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Research article

PHARMACOLOGICAL INHIBITION OF GSK3 ATTENUATES DNA DAMAGE-INDUCED APOPTOSIS *via* REDUCTION OF p53 MITOCHONDRIAL TRANSLOCATION AND Bax OLIGOMERIZATION IN NEUROBLASTOMA SH-SY5Y CELLS

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Abstract: Glycogen synthase kinase-3 (GSK3) and p53 play crucial roles in the mitochondrial apoptotic pathway and are known to interact in the nucleus. However, it is not known if GSK3 has a regulatory role in the mitochondrial translocation of p53 that participates in apoptotic signaling following DNA damage. In this study, we demonstrated that lithium and SB216763, which are pharmacological inhibitors of GSK3, attenuated p53 accumulation and caspase-3 activation, as shown by PARP cleavage induced by the DNA-damaging agents doxorubicin, etoposide and camptothecin. Furthermore, each of these agents induced translocation of p53 to the mitochondria and activated the mitochondrial pathway of apoptosis, as evidenced by the release of cytochrome C from the mitochondria. Both mitochondrial translocation of p53 and mitochondrial release of cytochrome C were attenuated by inhibition of GSK3, indicating that GSK3 promotes the DNA damage-induced mitochondrial translocation of p53 and the mitochondrial apoptosis pathway. Interestingly, the regulation of p53 mitochondrial translocation by GSK3 was only evident with wild-type p53, not with mutated

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Abbreviations used: ATCC – American Type Culture Collection; EDTA – ethylenediaminetetraacetic acid; EGTA – ethyleneglycoltetraacetic acid; FBS – fetal bovine serum; GSK3 – glycogen synthase kinase 3; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP – immunoprecipitation; MEM – minimum essential medium; PARP – poly ADP ribose polymerase; PMSF – phenylmethylsulfonylfluoride; PUMA – the p53 upregulated modulator of apoptosis; SDS –sodium dodecyl sulfate

p53. GSK3 inhibition also reduced the phosphorylation of wild-type p53 at serine 33, which is induced by doxorubicin, etoposide and camptothecin in the mitochondria. Moreover, inhibition of GSK3 reduced etoposide-induced association of p53 with Bcl2 and Bax oligomerization. These findings show that GSK3 promotes the mitochondrial translocation of p53, enabling its interaction with Bcl2 to allow Bax oligomerization and the subsequent release of cytochrome C. This leads to caspase activation in the mitochondrial pathway of intrinsic apoptotic signaling.

Key words: GSK3, p53, Mitochondrial translocation, Apoptosis, Bax oligomerization, Cytochrome C, Phosphorylation, Etoposide, Doxorubicin, Camptothecin

INTRODUCTION

GSK3 is a serine/threonine kinase that plays critical roles in the regulation of multiple signaling pathways, including the wnt/wingless, insulin and apoptotic signaling pathways. It is involved in the regulation of a variety of fundamental processes, including some related to cell structure, metabolism and survival [1, 2]. Moreover, it is believed to have a role in the etiology of many chronic diseases, including diabetes, Alzheimer's disease and multiple types of cancer [3].

GSK3 has been shown to promote apoptotic cell death induced by several types of damage that involve the intrinsic mitochondrial pathway. These include DNA damage [4], endoplasmic reticulum stress [5-7] and mitochondrial toxins [8, 9]. There are two isoforms, GSK3α and GSK3β. Overexpression of GSK3β is sufficient to cause apoptosis in a p53-dependent manner, and this is one of the key components in apoptosis signaling, particularly following DNA damage [10].

A direct interaction between GSK3 and p53 has been demonstrated in the nucleus. This interaction was found to increase GSK3 activity in the nucleus and promote the apoptotic action of p53 [11]. Moreover, GSK3 phosphorylates p53 at serines 33 [12], 315 and 376 [5], which are sites that increase the apoptotic action of p53 after phosphorylation. It is interesting to note that serine 376 of p53 is within the region that has been identified as necessary for the interaction of p53 and GSK3 [11].

These findings suggest that the interaction of GSK3 and p53 may be important for controlling p53-mediated apoptosis. It is well recognized that activation of the tumor suppressor protein p53, of which mutations are found in approximately 50% of human cancers [13], causes apoptosis. In part, p53 mediates apoptosis through transcriptional promotion of the expression of proapoptotic proteins such as Bax and PUMA [14]. Additionally, it has been demonstrated that p53 is capable of inducing apoptosis independently of its transcriptional activity via its translocation to the mitochondria [15-19]. Interestingly, GSK3 has been shown to promote the intrinsic pathway of apoptotic signaling in which mitochondria have a critical role [1, 3].

This study was designed to further investigate the role of GSK3 in the translocation of p53 to the mitochondria and p53 transcription-independent mitochondrial apoptotic processes. The results show that GSK3 controls p53 mitochondrial translocation and mitochondria-mediated apoptosis, in part by promoting p53 mitochondrial translocation and interaction with Bcl2, which allows Bax oligomerization and subsequently cytochrome C release and apoptosis.

MATERIALS AND METHODS

Cell culture

SH-SY5Y human neuroblastoma cells (ATCC) were maintained in a 1:1 mixture of minimum essential medium (MEM) and Ham's F12 medium (Gibco) supplemented with 10% FBS (JR Scientific Inc.), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco), and cultured in 5% CO_2 at 37ºC. T47D cells (ATCC) were cultured in RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM D-glucose, 1 mM sodium pyruvate (Gibco) and 13.9 mM insulin (Sigma-Aldrich).

Subcellular fractionation

Subcellular fractionation was performed as described previously [20]. Briefly, cells were incubated for 10 min on ice with extraction buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.02% digitonin, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and a protease inhibitor cocktail. The cell extracts were then spun at 700 × g for 5 min at 4ºC yielding a pellet containing the nuclei and a supernatant containing the cytosol and mitochondria.

The pellet with the nuclear fraction was then washed with twice wash buffer containing 10 mM HEPES (pH 7.4), 10 mM NaCl, 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and a protease inhibitor cocktail, and lysed for 30 min at 4ºC with nuclear lysis buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 300 mM NaCl, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 50 mM NaF and a protease inhibitor cocktail. The nuclear extracts were clarified by centrifugation at 16,000 × g for 15 min at 4ºC. The supernatant was kept as the nuclear fraction.

The supernatant containing the cytosol and mitochondria was centrifuged at 16,000 × g for 30 min at 4ºC to separate the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The mitochondria were washed twice with wash buffer and lysed by incubation with the lysis buffer for 30 min on ice. The mitochondrial lysates were clarified by centrifugation at 16,000 × g for 15 min at 4ºC. The cytosol was subjected to ultracentrifugation at 110,000 × g for 30 min at 4ºC to collect the cytosolic fraction (supernatant).

Immunoblot analysis

Cells were lysed with lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet-P40, 0.1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF and a protease cocktail inhibitor (Calbiochem). Cell lysates were sonicated and then centrifuged at 20,000 × g for 15 min at 4ºC. The protein concentration was determined using Bradford reagent (Bio-Rad). Immunoblotting was performed as described previously [20].

The antibodies were: mouse anti-GSK3α/β, mouse anti-phosphotyrosine 279/216-GSK3α/β, mouse anti-p53, mouse anti-Bcl2 and rabbit anti-Bax (Millipore); mouse anti-PARP, p21 and β-tubulin antibodies (BD Pharmingen Bioscience); mouse anti-cytochrome C, goat anti-ATP synthase F1 subunit and mouse anti-histone H1 (Santa Cruz Biotechnology); rabbit anti-phospho-serine 21/9-GSK3α/β, phosphoserine 15, 33, and 37 p53 antibodies (Cell Signaling Technology); and mouse anti-β-actin (Sigma-Aldrich).

For immunoprecipitation, 60 μg of mitochondrial protein was incubated with 1 μg of p53 antibody for 2 h at 4ºC with gentle agitation followed by incubation with protein G-sepharose beads (GE Healthcare) for 2 h at 4ºC. The immune complexes were washed, mixed with Laemmli sample buffer (2% SDS) and boiled for 10 min. The eluted protein complexes were subjected to immunoblot analysis using anti-p53 and Bcl2 antibodies. To reprobe, the blots were stripped in stripping buffer consisting of 62.5 mM Tris (pH 6.8) and 2% SDS 100 mM β-mercaptoethanol, and incubated at 56ºC for 30 min.

All of the experiments were repeated three or more times. Protein bands were quantified using a densitometer (Imagescaner III, GE Healthcare), and normalized to their respective loading control on the same blot.

Chemical cross-linking

Mitochondrial protein chemical cross-linking was performed as described previously [20] using a bifunctional cross-linker, ethylene glycol bis(succinimidyl succinate) (EGS, 1 mM; Pierce). The cross-linked samples were resolved in 4-20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was probed with anti-Bax antibody. At least three independent experiments were completed for each treatment.

Statistical analysis

Statistical analyses were performed using Student's T-test. Differences were considered significant when $p < 0.05$.

RESULTS

GSK3 inhibition attenuates the increase in p53 levels and activation of caspase induced by DNA damage

To study p53-mediated apoptosis signaling, we used three chemotherapeutic agents that have mechanistically different modes of induction of DNA damage that induces increases in p53 levels. Doxorubicin is a DNA intercalating agent,

etoposide is a topoisomerase II inhibitor, and camptothecin is a topoisomerase I inhibitor [21].

Treating human neuroblastoma SH-SY5Y cells, which express wild-type p53, with doxorubicin, etoposide or camptothecin caused large increases in the level of p53 (Fig. 1). Activation of apoptosis was evident by the increases in the cleavage of PARP, which is a substrate of caspase 3, a key enzyme that is activated during apoptosis [6]. The levels of the anti-apoptotic protein Bcl2 and the pro-apoptotic protein Bax were not affected by doxorubicin, etoposide or camptothecin treatments (Fig. 1), suggesting that the apoptosis induced by these three inducers of DNA damage was not due to imbalances in these proteins.

To test whether GSK3 is involved in the regulation of p53 induction and apoptosis, we used lithium and SB216763, which are two mechanistically different pharmacological inhibitors of GSK3. The activity is of GSK3 mainly regulated by phosphorylation. N-terminal serine phosphorylation (serine 21 for GSK3α and serine 9 for GSK3β) inhibits its activity, and tyrosine phosphorylation (tyrosine 279 for GSK3α and tyrosine 216 for GSK3β) promotes it [1]. Lithium-mediated GSK3 inhibition caused increases in the serine phosphorylation of both GSK3 isoforms (Fig. 1), as reported previously [22, 23]. SB216763 directly inhibits GSK3 by competition at the ATP-binding site [24], which resulted in decreases in both the serine and tyrosine phosphorylation of GSK3α/β (Fig. 1).

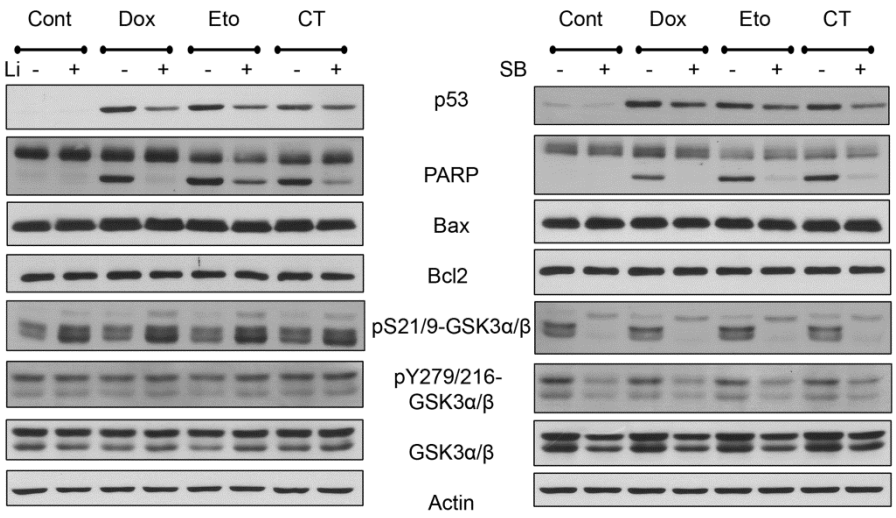

Fig. 1. GSK3 inhibition attenuates the DNA damage-induced increase in p53 levels and caspase activation. SH-SY5Y cells were treated with 2 μM doxorubicin (Dox), 10 μM etoposide (Eto) or 5 μM camptothecin (CT) for 5 h with or without a 30 min pretreatment with the GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM). The levels of p53, PARP, pS21/9-GSK3α/β, pY279/216-GSK3α/β, GSK3α/β, Bcl2 and Bax were analyzed by immunoblot analysis and β-actin was used as a loading control. Caspase activation was determined by measuring PARP proteolysis.

Pretreatment with lithium or SB216763 reduced the increase in p53 levels and cleavage of PARP induced by treatment with doxorubicin, etoposide and camptothecin (Fig. 1), indicating that GSK3 contributes to the increased p53 expression and caspase activation caused by each of these DNA-damaging agents. However, lithium and SB216763 treatment did not alter the levels of Bcl2 or Bax (Fig. 1), indicating that the apoptotic protection provided by GSK3 inhibition is unlikely to be through modulation of the levels of Bcl2 or Bax.

Inhibition of GSK3 reduces p53 translocation to the mitochondria and apoptosis

Since translocation of p53 to the mitochondria can induce apoptosis [25], we investigated the contribution of GSK3 to p53 mitochondrial translocation and its activation of mitochondria-mediated apoptosis. SH-SY5Y cells were treated with 2 μM doxorubicin, 10 μM etoposide or 5 μM camptothecin for 5 h, and then the mitochondrial, cytosolic and nuclear fractions were prepared. The purity of the mitochondrial, cytosolic and nuclear preparations was verified by immunoblotting for markers for each fraction: ATP synthase F1 subunit, β-tubulin and histone H1, respectively (Fig. 2A).

Treatment with doxorubicin, etoposide or camptothecin caused significant increases in the levels of p53 in the mitochondria (Fig. 2B) and cytochrome C release into the cytosol, although cytochrome C release after 5 h camptothecin treatment was mild (Fig. 3), which indicates activation of the mitochondrial apoptosis pathway. Inhibition of GSK3 by lithium or SB216763 significantly attenuated p53 translocation to the mitochondria following doxorubicin, etoposide or camptothecin treatment (Fig. 2B).

Quantitative analyses were performed using ATP synthase in the same blot as a loading control. The results revealed that inhibition of GSK3 with lithium or SB216763 significantly reduced DNA damage-induced p53 translocation to the mitochondria (Fig. 2C). It should be noted that although GSK3 inhibition caused a small reduction in the DNA damage-induced p53 mitochondrial translocation, the observation was highly reproducible. Treatment with the GSK3 inhibitors did not alter the levels of p53 in the cytosol and nucleus (Fig. 2B). These results indicate that active GSK3 contributes to p53 mitochondrial translocation after DNA damage. Additionally, GSK3 inhibition reduced cytochrome C release (Fig. 3). These results indicate that GSK3 promotes both p53 mitochondrial translocation and the mitochondrial apoptosis pathway after DNA damage.

GSK3 regulates mitochondrial translocation of wild-type p53 but not mutant p53

It has been reported that some transcriptionally incompetent mutants of p53 are capable of inducing apoptosis [26, 27] and are present in the mitochondria after the induction of apoptosis [17, 28]. Therefore, we investigated whether GSK3 controls the mitochondrial translocation of mutant p53. Breast cancer T47D cells expressing L194F mutant p53 [29] were used. Unlike wild-type p53, which is expressed at very low levels in unstressed cells (e.g., SH-SY5Y cells), mutant p53 is expressed at high levels in untreated T47D (Fig. 4A). Additionally, we

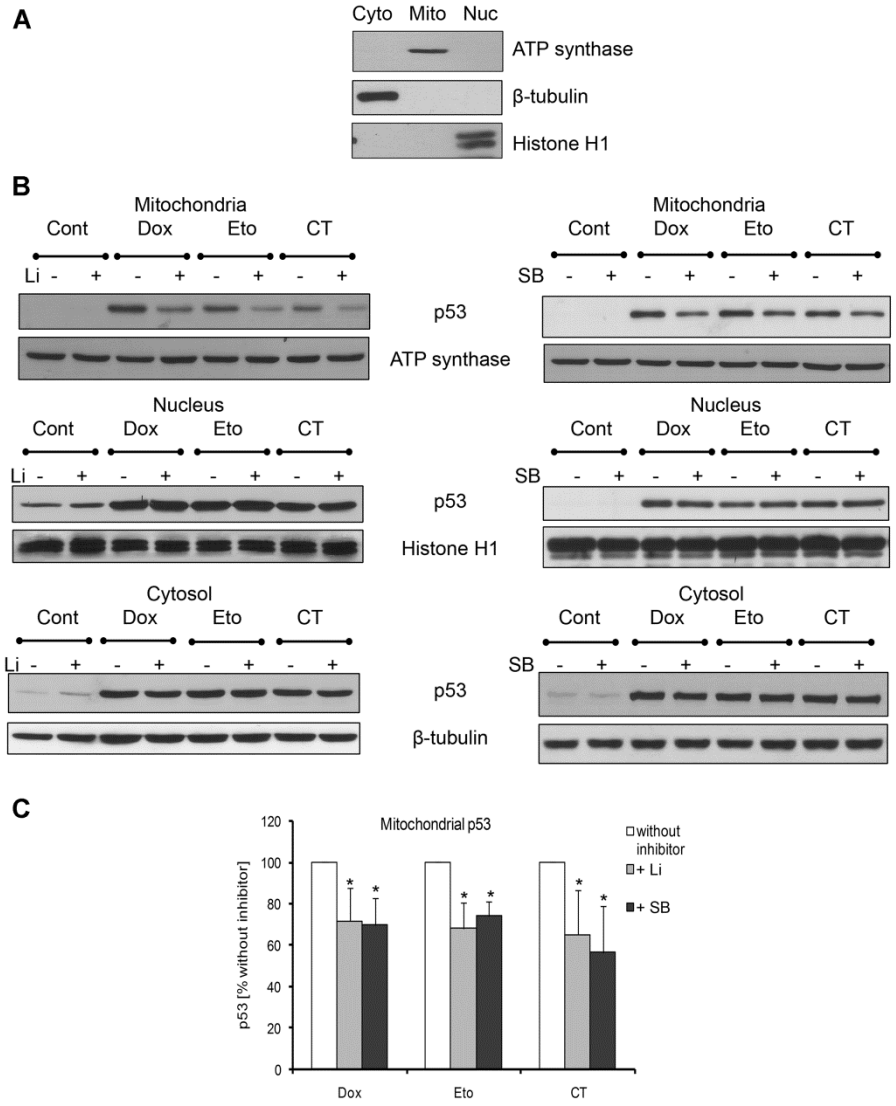

Fig. 2. Inhibition of GSK3 reduced p53 mitochondrial translocation. The mitochondrial, nuclear and cytosolic fractions were prepared from cells treated with doxorubicin (Dox), etoposide (Eto) or camptothecin (CT) for 5 h with or without a 30 min pretreatment with the GSK3 inhibitors, lithium (Li) or SB216763 (SB). A – Mitochondria (Mito), nuclear (Nuc) and cytosolic (Cyto) fractions were immunoblotted with a protein marker for each fraction (the F1 subunit ATP synthase for mitochondria, β-tubulin for the cytosol and histone H1 for the nucleus) to verify the purity of each fraction. B – The levels of mitochondrial, cytosolic and nuclear p53 were detected by immunoblotting. C – The quantitative data of mitochondrial p53 were normalized to the level of ATP synthase on the same blot, and were expressed as percent individual chemical treatment without GSK3 inhibition (mean ± SD; n = 3). * $p < 0.05$.

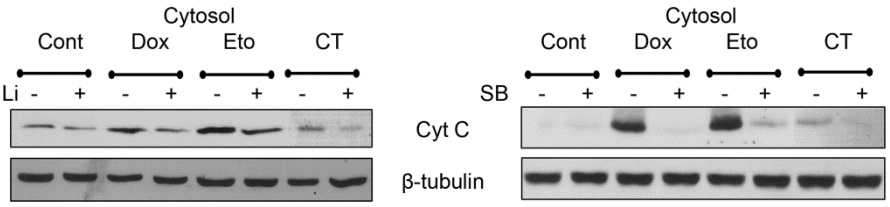

Fig. 3. Inhibition of GSK3 reduced cytochrome C release from the mitochondria. SH-SY5Y cells were treated with 2 μM doxorubicin (Dox), 10 μM etoposide (Eto) or 5 μM camptothecin (CT) for 5 h with or without a 30 min pretreatment with the GSK3 inhibitors, lithium (Li, 20 mM) or SB216763 (SB, 20 μM). Cytochrome C (Cyt C) released into the cytosol was measured by immunoblotting.

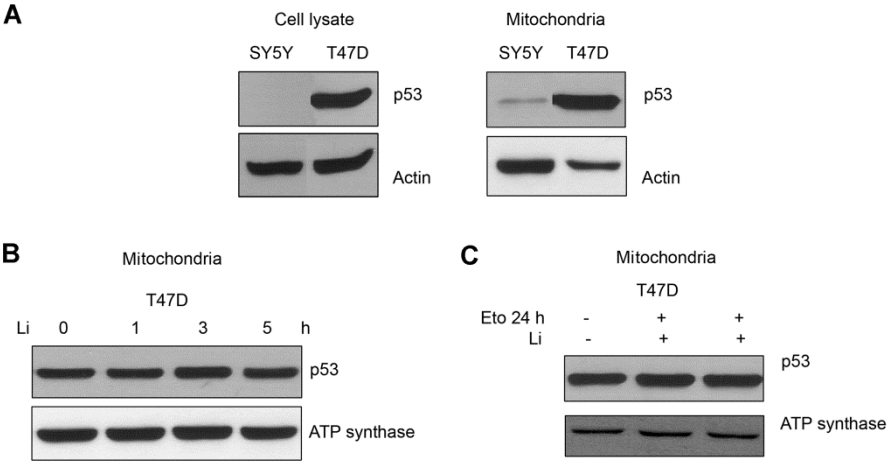

Fig. 4. GSK3 regulates the mitochondrial translocation of wild-type but not mutant p53. A – Levels of mitochondrial p53 in SH-SY5Y (wild-type p53) and T47D (L194F p53) cells. B – T47D cells were treated with 20 mM lithium (Li) for 1, 3 or 5 h and the levels of mitochondrial p53 were measured by immunoblot analysis. C – T47D cells were treated with 10 μM etoposide (Eto) with or without 20 mM lithium for 24 h and the levels of mitochondrial p53 were analyzed by immunoblotting.

found higher levels of mutant p53 than wild-type p53 are in the mitochondria of untreated cells (Fig. 4A). Inhibition of GSK3 with lithium did not affect the basal levels of mutant p53 in the mitochondria in T47D cells (Fig. 4B). Moreover, treatment with etoposide did not significantly affect the mitochondrial levels of mutant p53 and GSK3 inhibition did not alter mitochondrial mutant p53 levels following treatment with etoposide (Fig. 4C). These results indicate that GSK3 only regulates the mitochondrial translocation of wild-type p53, but not mutant p53, or that GSK3 only regulates the increase in mitochondrial p53 that follows the activation of apoptotic signaling, which was absent in T47D cells.

Inhibition of GSK3 reduces p53 phosphorylation at serine 33 in the mitochondria

p53 is known to be phosphorylated at multiple sites in response to DNA damage, and some of these phosphorylation events contribute to its apoptotic action [30]. To examine whether GSK3 inhibition affected p53 phosphorylation following DNA damage, the level of p53 phosphorylation was detected by immunoblot analysis using phospho-specific p53 antibodies. After doxorubicin, etoposide and camptothecin treatment, phosphorylation of p53 at serines 15, 33 and 37 increased substantially in the mitochondria (Fig. 5). Inhibition of GSK3 by lithium caused a reduction in p53 phosphorylation at serine 33 in the mitochondrial fractions, while its phosphorylation at serines 15 and 37 was not affected (Fig. 5).

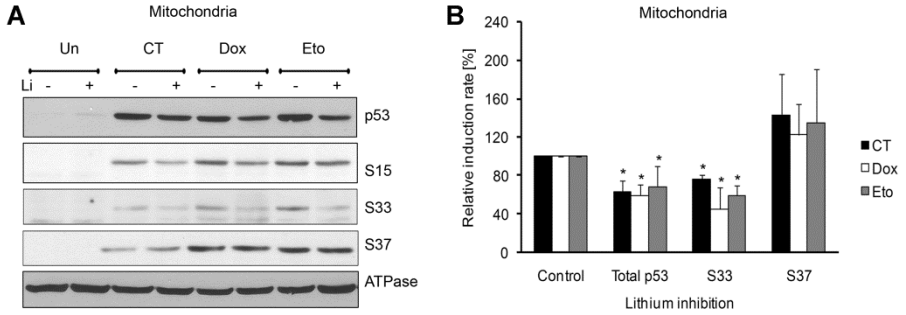

Fig. 5. Inhibition of GSK3 reduces DNA damage-mediated p53 phosphorylation at serine 33 in the mitochondria. A – The mitochondrial fractions prepared from cells treated with doxorubicin (Dox), etoposide (Eto) or camptothecin (CT) for 5 h with or without a 30 min pretreatment with the GSK3 inhibitor lithium (Li) were subjected to immunoblot analysis and probed with phospho-serine 15, 33 and 37 p53 antibodies. B – The quantitative data for the total and phosphorylated p53 in the mitochondria were normalized to the level of ATP synthase on the same blot, and were expressed as percent of individual treatment without GSK3 inhibition (mean ± SD; n = 3). * $p < 0.05$.

Inhibition of GSK3 reduces the interaction of p53 with Bcl2 and Bax oligomerization

It has been shown that p53 directly induces mitochondrial apoptosis through its interaction with Bcl2, a key protein regulating the permeability of the mitochondrial outer membrane [31]. Therefore, we examined p53 and Bcl2 interaction in the mitochondria using co-immunoprecipitation. The level of p53 in the mitochondria increased after etoposide or doxorubicin treatment, whereas the level of Bcl2 remained unaltered (Fig. 6A). Co-immunoprecipitation of p53 and Bcl2 was observed in the etoposide- and doxorubicin-treated cells, and this p53 and Bcl2 interaction was drastically reduced by treatment with the GSK3 inhibitor lithium (Fig. 6A). The interaction of p53 and other proteins in the Bcl2 family, Bcl-xl and Bax, was also examined but there was none of the interaction of p53 and Bcl-xl or p53 and Bax observed under our experimental conditions (data not shown).

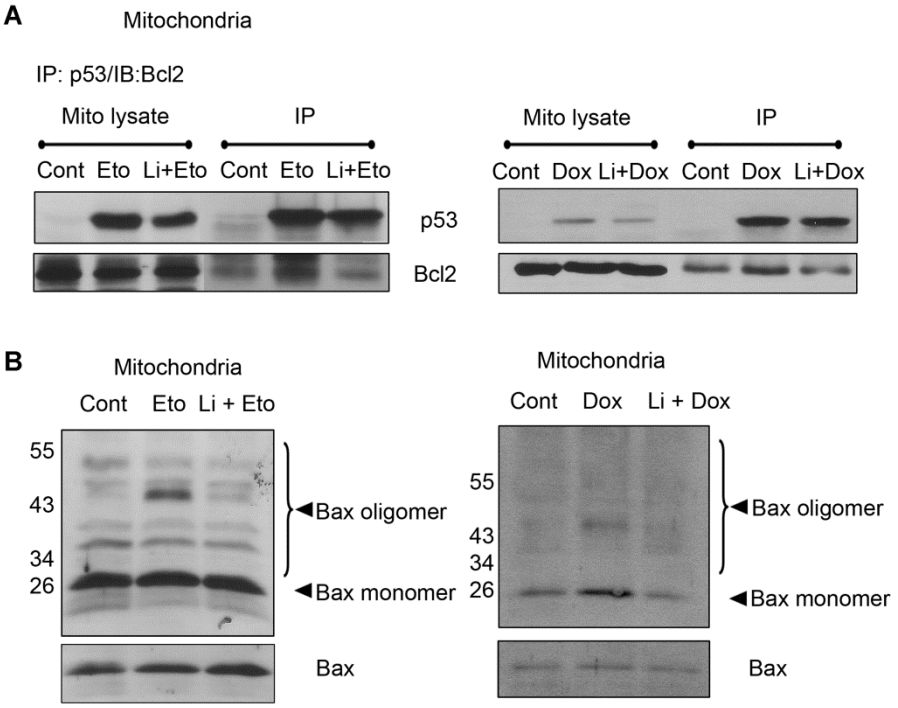

Fig. 6. Inhibition of GSK3 reduced the interaction of p53 with Bcl2 and Bax oligomerization associated with the mitochondria. SH-SY5Y cells were treated with 10 μM etoposide (Eto) or 2 μM doxorubicin (Dox) for 5 h with or without a 30 min pre-incubation with the GSK3 inhibitor lithium (Li, 20 mM). A – Mitochondrial p53 was immunoprecipitated. The interaction of p53 and Bcl2 was detected by co-immunoprecipitation. B – Mitochondria were lysed with 2% CHAPS lysis buffer followed by chemical cross-linking with EGS. Cross-linked proteins were resolved in 4-20% SDS-PAGE, and immunoblotted for Bax. The total amount of mitochondrial Bax in the samples is shown in the bottom panel.

We further investigated whether GSK3 inhibition affected Bax oligomerization, which can lead to mitochondrial outer membrane permeabilization and subsequent cytochrome C release. To detect Bax oligomerization, after treatment, the mitochondrial proteins were cross-linked by treatment with EGS, followed by western blot analysis. The results show that etoposide and doxorubicin treatment increased the immunoreactivity of a slow-migrating band above the 21 kD Bax monomer band (Fig. 6B) indicating increased Bax oligomerization associated with the mitochondria following etoposide treatment. Inhibition of GSK3 with lithium treatment attenuated etoposide- and doxorubicin-induced Bax oligomerization in the mitochondria. The level of Bax in the mitochondria was not altered by co-treatment with lithium and etoposide or doxorubicin (Fig. 6B bottom panel), indicating that the reduction in the level of Bax oligomerization after GSK3 inhibition was not due to a decrease in Bax in the mitochondria.

These results indicate that GSK3 regulates mitochondrial apoptotic signaling in part by facilitating p53 translocation to the mitochondria, where it binds and neutralizes the anti-apoptotic protein Bcl2, allowing Bax oligomerization, which increases mitochondrial permeabilization and cytochrome C release.

DISCUSSION

Considering the recently identified direct action of p53 in the mitochondria in inducing apoptosis [31, 32] and the direct interaction between p53 and GSK3 involved in activating the mitochondrial pathway of apoptosis [4, 11], we tested whether GSK3 plays a role in regulating mitochondrial p53. Our findings show that after DNA damage, GSK3 promotes p53 translocation to the mitochondria, Bax oligomerization associated with the mitochondria, and mitochondria-mediated apoptotic signaling. The results indicate that GSK3 facilitates the mitochondrial pathway of apoptosis in part through promotion of p53 translocation to the mitochondria, where it binds and sequesters the anti-apoptotic protein Bcl2, which allows Bax oligomerization, which in turn leads to the release of cytochrome C from the mitochondria to promote caspase activation.

The apoptotic activity of p53 has been demonstrated to include both transcription-dependent and -independent actions. The transcription-dependent apoptotic action of p53 is mediated by transactivation of pro-apoptotic genes including Bax and PUMA [14], and transrepression of anti-apoptotic genes, such as Bcl2 [14]. The transcription-independent apoptotic action of p53 was identified by overexpression of transcriptionally deficient p53 mutants that were capable of triggering apoptosis [27]. It has also been shown that expression of mitochondria-targeted p53 caused apoptosis in p53-null cells [16, 17, 25], indicating that transcription-independent p53-mediated apoptosis occurred via its action in the mitochondria.

We recently reported that inhibition of GSK3 reduced p53 translocation to the mitochondria and mitochondrial apoptotic cell death following arsenite treatment, suggesting a regulatory role for GSK3 in the translocation of p53 to the mitochondria and the mitochondrial apoptotic pathway [20]. This study extended these findings by demonstrating that inhibition of GSK3 by two structurally unrelated, selective GSK3 inhibitors, lithium and SB216763, attenuated p53 translocation to the mitochondria in SH-SY5Y cells when induced by three different DNA-damaging agents that induce apoptosis by activating p53, namely doxorubicin, etoposide and camptothecin.

Since mutant p53 has been reported to reside in the mitochondria, we also examined the GSK3-mediated regulation of mitochondrial translocation of mutant p53. We observed that in T47D cells expressing mutant p53 (p53L194F), basal mitochondrial p53 levels were relatively high, which is consistent with previous reports [17, 28]. Unlike in the case of wild-type p53, inhibition of GSK3 did not affect the mitochondrial level of mutant p53 with or without an

apoptotic stimulus, suggesting that GSK3 only regulates the translocation of wild-type p53 to the mitochondria.

Although recent studies have demonstrated the importance of mitochondrial p53 in the mitochondrial pathway of apoptosis, little is known about the mechanisms that regulate its translocation. These findings add GSK3 to known regulators of p53 mitochondrial translocation, which also include the previously identified mono-ubiquitination [33] and Tid1 (mtHSP40) [28]. The apoptosis-promoting action of GSK3 has been demonstrated previously, showing that GSK3 promotes the intrinsic mitochondrial pathway of apoptosis but not the extrinsic pathway of apoptosis [1, 3]. The finding that GSK3 promotes p53 translocation to the mitochondria, which has been shown to induce mitochondrial apoptosis, led us to examine the contribution of GSK3 to the actions of p53 in mitochondria. In the mitochondria, p53 has previously been reported to form complexes with Bcl2 and Bcl-xl, anti-apoptotic members of the Bcl2 family that play critical roles in controlling mitochondrial outer membrane permeabilization (MOMP). This interaction of p53 with Bcl2 or Bcl-xl induced MOMP, leading to cytochrome C release and subsequently apoptosis [17]. Our study also found that p53 interacts with Bcl2 following etoposide treatment, and inhibition of GSK3 reduced this interaction. However, we were unable to detect the interaction of p53 with Bcl-xl in these cells. Additionally, we found that inhibition of GSK3 reduced etoposide-induced Bax oligomerization, which leads to MOMP [34]. Bax oligomerization results from disruption of Bcl2-Bax complexes that inhibit MOMP following the sequestration of Bcl2 by p53 [32, 35]. p53 has been shown to form a pore complex with Bax, suggesting the potential for a direct interaction with Bax in triggering MOMP [32]. However, we did not detect p53 complexed with oligomerized Bax under our experimental conditions, which is consistent with a previous report [17]. Interestingly, although Bax phosphorylation by GSK3 has been shown to increase its translocation to the mitochondria [36], GSK3-mediated Bax phosphorylation is unlikely to be critical for Bax mitochondrial translocation under our experimental conditions as the level of Bax in the mitochondria was not affected by GSK3 inhibition. It must be noted that Tan *et al.* reported a similar finding that inhibition of GSK3 reduced DNA damage-induced p53 accumulation in colorectal cancer cells. However, they found that GSK3 inhibition promoted p53 apoptosis upon DNA damage [37]. This discrepancy may be due to different background protein expression, especially of those proteins regulated by GSK3, such as β-catenin and adenomatous polyposis coli (APC) which are frequently aberrant in colorectal cancer cells [38].

Phosphorylation modification plays an important role in p53 stabilization and activation [30], but its contribution to mitochondrial translocation of p53 remains unclear. Nemajerova *et al.* reported that mitochondrial translocation of p53 was independent of its phosphorylation state [39]. However, other studies have suggested that p53 translocation to the mitochondria could be promoted by its phosphorylation at serines 15 and 33 [40, 41]. It is interesting to note that

GSK3 was reported to phosphorylate p53 at serine 33, and that pre-phosphorylation at serine 37 was required for GSK3-mediated p53 phosphorylation [12]. Our finding that inhibition of GSK3 reduced DNA damage-mediated p53 phosphorylation at serine 33 in the mitochondria may implicate the phosphorylation of p53 by GSK3 in the regulation of p53 mitochondrial localization. Together with previously reported results showing that apoptotic stimulation by camptothecin activated GSK3 in the mitochondria [42], we speculate that upon apoptotic induction by DNA damage, p53 phosphorylation by activated GSK3 may sequester p53 in the mitochondria, and the reduction in p53 phosphorylation due to GSK3 inhibition allows p53 to shuttle out of the mitochondria. Thus, the reduction in the level of mitochondrial translocation of p53 after DNA damage by inhibition of GSK3 may be at least in part linked to p53 phosphorylation at serine 33. However, the role of GSK3-mediated p53 phosphorylation for regulating its mitochondrial translocation will require further analysis.

It is clearly evident that mitochondrial p53 can induce apoptosis independent of its transcriptional activity [27, 28] and that induction of apoptosis is one of the major mechanistic targets for chemotherapeutic agents, especially those that cause DNA damage. As an extension of our findings that GSK3 promotes DNA damage-induced p53 mitochondrial translocation and the mitochondrial pathway of apoptosis, enhanced GSK3 activity may be beneficial for chemotherapy of cancer cells harboring wild-type p53. Moreover, mitochondrial p53 was experimentally demonstrated to have tumor suppressor activity *in vivo* as the expression of mitochondrial-targeted p53 suppressed tumor growth in p53-null lymphoma xenograft mice [43]. Thus, augmentation of p53 levels in the mitochondria by GSK3 activation may improve p53 tumor suppressor action. It is interesting to note that generally, under normal physiological conditions, GSK3 activity is suppressed in response to a number of cellular signaling pathways, including growth factor-mediated Akt activation and insulin signaling [2], so deactivation of those signals would increase GSK3 activity and could lead to a promotion of p53 tumor suppression. However, it remains to be seen whether increased GSK3 activity is sufficient to enhance p53 mitochondrial translocation under unstressed condition.

In summary, this study found that inhibition of GSK3 reduced wild-type p53 mitochondrial translocation but not that of mutant p53 and that the mitochondrial interaction of wild-type p53 with Bcl2 and Bax oligomerization was concomitant with a reduction in cytochrome C release. These results indicate that GSK3 promotes mitochondrial apoptosis partly through facilitating p53 translocation to the mitochondria, where it binds and neutralizes Bcl2, thus allowing Bax oligomerization and the subsequent release of cytochrome C in the mitochondrial apoptotic signaling pathway.

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Conflict of interest. The authors declare no conflict of interest.

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