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Impact of 2-bromopropane on mouse embryonic stem cells and related regulatory mechanisms

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2-Bromopropane (2-BP), a cleaning agent, is used as an alternative to ozone-depleting solvents. Previously, 2-BP was shown to have cytotoxic effects on mouse blastocysts and is associated with defects in their subsequent development, both *in vitro* and *in vivo*. In addition, it was found that 2-BP also has cytotoxic effects on oocyte maturation and subsequent pre- and post implantation development *in vitro* and *in vivo*, and significantly reduces the rate of oocyte maturation, fertilization, and embryonic development *in vitro*. This study shows that 2-BP (5 to 10 µM) induces apoptotic processes in mouse embryonic stem cells (ESC-B5), but exerts no effects at treatment dosages below 5 µM. In ESC-B5 cells, 2-BP directly increased the content of reactive oxygen species (ROS), significantly increased the cytoplasmic free calcium and nitric oxide (NO) levels, triggered a loss of mitochondrial membrane potential (MMP), activated caspases-9 and -3, and induced cell death. Pre-treatment with NO scavengers suppressed the apoptotic biochemical changes induced by 10 µM 2-BP and promoted the gene expression levels of p53 and p21, which are involved in apoptotic signaling. These results demonstrate for the first time that 2-BP triggers apoptosis in mouse embryonic stem cells via ROS, NO and the activation of mitochondria-dependent cell death signaling.

Key words: 2-Bromopropane, apoptosis, oxidative stress, calcium, nitric oxide.

INTRODUCTION

2-Bromopropane (2-BP), a cleaning agent, is used as an alternative to ozone-depleting solvents. In 1995, 2-BP was shown to have caused a series of reproductive and hematopoietic disorders in female and male workers commonly exposed to the solvent in an electronics factory located in South Korea (Kim et al., 1996; Park et al., 1997). Moreover, earlier reports found a high incidence of oligozoospermia in male workers after long-term exposure to 2-BP (Kim et al., 1996; Li et al., 2001; Park et al., 1997). Several animal studies have further confirmed the potential of 2-BP to affect the reproductive, hematopoietic, central nervous and immune systems (Ichihara et al., 1997; Kim et al., 2004; Omura et al., 1999; Son et al., 1999; Wu et al., 2002; Yu et al., 1999a; Yu et al., 1999b; Zhao et al., 2002). In cytotoxicity experiments, mouse embryos treated with 2-BP formed micronuclei and shows a decrease in embryonic cell numbers (Ishikawa et al., 2001). Moreover, 2-BP was recently identified as a potent DNA damaging agent (Wu et al., 2002; Zhao et al., 2002). These results collectively suggest that 2-BP induces various toxicities via DNA damage. A reproductive toxicity investigation further demonstrates that exposure to 2-BP induced testicular or ovarian dysfunction, causes injury to early types of spermatogenic cells or primordial follicles and oocytes in rats (Omura et al., 1999; Yu et al., 1999b).

In an experiment investigating the effects of 2-BP on pre- and postnatal development, exposure of pregnant or lactating female rats to 2-BP results in a decreased delivery rate, increase in pre- and postnatal death,

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decreased body weight, and a higher incidence of reproductive organ dysfunction (Kang et al., 2002). To date, however, no clinical study, epidemiological study or case report has demonstrated a direct relationship between reproductive problems and the exposure of pregnant workers to 2-BP. Specifically, there is no evidence to suggest that the solvent negatively affects embryonic development or infant growth in humans. However, it is very important to explore the health risks associated with the exposure of female workers, especially those who are pregnant, to 2-BP.

Importantly, the solvent is very volatile and can permeate human skin. The major exposure route is *via* inhalation in the workplace or factory (Kim et al., 1996; Park et al., 1997). Moreover, recent study by our group shows that 2-BP induces cellular apoptosis in both the inner cell mass (ICM) and trophectoderm cells (TE) of mouse blastocysts, leading to decreased implantation, reduced embryonic development, and a loss of embryonic viability (Chan, 2010a). Notably, it was further discovered that 2-BP significantly reduced the rates of oocyte maturation, fertilization, and *in vitro* embryonic development (Chan, 2010b). These results clearly indicate that 2-BP may be a serious risk factor that affects both the pre- and post-implantation stages of embryonic development. However, the precise regulatory mechanisms underlying the potentially adverse effects of 2-BP on early embryonic development require further investigation.

Numerous chemical and physical treatments capable of inducing apoptosis stimulate oxidative stress via the generation of reactive oxygen species (ROS) in cells (Khan et al., 2013; Rhee et al., 2013), suggesting that there is a close relationship between oxidative stress and apoptosis. Nitric oxide (NO) is an important second messenger involved in a variety of cellular responses and biological functions, including tumor development, metastasis and apoptosis (Kruzliak et al., 2013; Sun et al., 2013). Recent studies have demonstrated that NO is predominantly produced in mitochondria through the actions of a Ca^{2+}-sensitive mitochondrial NO synthase (NOS) (Lu et al., 2006; Nazarewicz et al., 2007). NOS-mediated NO production may regulate oxygen consumption and mitochondrial membrane potential through cytochrome c oxidase.

The NO molecule is subsequently reactivated with superoxide to produce peroxynitrite, leading to further modification of its target substrates and induction of oxidative stress (Brookes, 2004; Dennis and Bennett, 2003; Ghafourifar and Cadenas, 2005; Wang et al., 2013). Oxidative stress and changes in intracellular Ca^{2+} act as upstream regulators of mitochondrial NOS activity (Dedkova et al., 2004; Elfering et al., 2002), and we recently showed that changes in intracellular Ca^{2+} were significantly involved in ROS generation-triggered cell apoptosis ESC-B5 cells(Chan et al., 2013).

Embryonic stem cells (ESCs) are pluripotent, early embryo-derived cells. When cultured in the presence of anti-differentiation agents, such as embryonic fibroblasts or leukemia inhibitory factor (LIF), ESCs proliferate while maintaining their capacity to differentiate into any cell type in the body (Evans and Kaufman, 1981). When the anti-differentiation agent is withdrawn, ESCs spontaneously differentiate and develop in a manner that recapitulates early embryogenesis (Keller, 1995). Following differentiation in suspension culture, ESCs typically form three-dimensional aggregates known as embryoid bodies (EBs), which consist of ectodermal, mesodermal and endodermal tissues resembling the egg-cylinder stage of embryos. In view of these properties, EB formation is a useful *in vitro* system for analyzing early embryonic development and differentiation processes.

To elucidate the precise regulatory mechanisms of 2-BP-triggered cytotoxicity in mouse ESCs, the effects of 2-BP on ESC-B5 cells is examined, and herein propose a model for 2-BP-induced cell injury signaling in mouse ESCs. Our results reveal for the first time that 2-BP treatment triggers apoptosis in mouse ESCs via ROS- and NO-related mitochondria-dependent apoptotic signaling, and causes hazardous effects on embryonic development.

MATERIALS AND METHODS

Chemicals and reagents

2-Bromopropane, 3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, 2',7'-dichlorofluorescin diacetate (DCF-DA), dihydrorhodamine 123 (DHR 123), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), N-acetyl cysteine (NAC), 2',7'-dichlorofluorescein diacetate (DCF-DA), propidium iodide, Hoechst 33342 and ethyleneglycol-bis(β-aminoethylether)tetraacetic acid (EGTA) were purchased from Sigma (St. Louis, MO). CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) was purchased from Boehringer Mannheim (Mannheim, Germany), while bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL, U.S.A.).

Cell culture and 2-BP treatment

Mouse embryonic stem cells (ESC-B5) were cultured in DMEM supplemented with 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were plated on 60 mm culture dishes, and 2-BP treatments were performed the following day. For 2-BP treatment, cells were incubated in a medium containing various concentrations of 2-BP at 37°C in a CO_2 incubator for 24 h. Cells were then washed twice with ice-cold PBS and lysed on ice for 10 min in 400 µl lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 µM sodium pyrophosphate and 1 mM sodium orthovanadate). Cell lysates were sonicated on ice for 3 × 10 s followed by centrifugation at 15,000 × *g* for 20 min at 4°C. The supernatants were used as cell extracts.

MTT assay

Cell survival was monitored using the MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) test. Briefly, cells were treated with the indicated concentrations of 2-BP for 24 h, and then treated with 100 µL of 0.45 g/L MTT solution. The cells were incubated at 37°C for 60 min to allow color development, and then 100 µL of 20% sodium dodecyl sulphate (SDS) in DMF:H_2O (1:1) solution was added to each well to stop the reaction. The plates were incubated overnight at 37°C for solubilization of formazan products, and spectrophotometric data were measured using an enzyme linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm.

Assessment of necrosis and apoptosis

Oligonucleosomal DNA fragmentation (a hallmark of apoptosis) was measured using the cell death detection ELISA^{plus} kit (Roche Molecular Biochemicals, Mannheim, Germany). Cells (1×10^5) were treated with or without the indicated concentrations of 2-BP at 37°C for 24 h, the procedures were performed according to the manufacturer's protocol, and spectrophotometric data were obtained using an ELISA reader at 405 nm. In addition, cells were incubated with propidium iodide (1 µg/ml) and Hoechst 33342 (2 µg/ml) at room temperature for 10 min, and fluorescent microscopy was used to identify the percentage of propidium iodide-impermeable cells having condensed/fragmented nuclei (apoptotic) and the percentage of propidium iodide-permeable cells (necrotic). In each experiment, 8-10 independent fields (~500-800 nuclei in total) were counted per condition. The activity of lactate dehydrogenase (LDH) present in the culture medium was evaluated as an additional index of necrosis, as previously described (Behl et al., 1994; Chan and Chang, 2006; Wu and Chan, 2007). Briefly, cells (5×10^4) were cultured in 96-well microtiter plates (100 µL medium/well), LDH activity was assayed using, and the absorption values at 490 nm were determined with an ELISA reader, according to the manufacturer's instructions (Promega, Madison, WI). Blanks consisted of test substances added to cell-free medium.

ROS assay

ROS were measured in arbitrary units using 2',7'-dichlorofluorescein diacetate (DCF-DA) or dihydrorhodamine 123 (DHR 123) dye. Cells (1.0×10^6) were incubated in 50 µL PBS containing 20 µM DCF-DA or DHR123 for 1 h at 37°C, and relative ROS units were determined using a fluorescence ELISA reader (excitation 485 nm, emission 530 nm). An aliquot of the cell suspension was lysed, the protein concentration was determined, and the results were expressed as arbitrary absorbance units/mg protein.

Detection of intracellular calcium concentration ($[Ca^{2+}]i$)

The $[Ca^{2+}]i$ was detected with Fluo-3 AM fluorescence dye, using a modification of the previously reported method (Aoshima et al., 1997; Lu et al., 2006). Briefly, cells were co-treated with 2-BP, harvested and washed, and then loaded with 6 µM Fluo-3 AM in standard medium (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 20 mM Hepes, pH of 7.4). After 30 min, the cells were washed 3 times with PBS and then resuspended in standard medium or Ca^{2+}-free standard medium. The fluorescence intensity of Fluo-3 was determined using a fluorescence spectrophotometer (Hitachi, F-2000; excitation at 490 nm, emission at 526 nm).

Detection of intracellular NO content

The DAF-2DA fluorescence dye was used to detect intracellular NO, according to a modification of the previously reported method (Lu et al., 2006; Nakatsubo et al., 1998). Briefly, treated or control cells were collected and washed, and then incubated with 3 µM DAF-2DA. After 60 min, the cells were washed three times with PBS and the fluorescence intensity was measured by a fluorescence spectrophotometer (Hitachi, F-2000; excitation at 485 nm, emission at 515 nm).

Caspase activity assays

Caspase-9 activity was assayed using the Colorimetric Caspase-9 Assay Kit (Calbiochem, CA). Caspase-3 activity was measured using the Z-DEVD-AFC fluorogenic substrate, as previously described (Chan et al., 2003; Hsieh et al., 2003).

Real-time RT-PCR assay

Total RNA was extracted with the TRIzol reagent (Life Technologies) and purified with an RNeasy Mini kit (Qiagen) according to the manufacturers' protocols. Real-time PCR was carried out with an ABI 7000 Prism Sequence Detection System (Applied Biosystems). The β-actin mRNA levels were quantified as an endogenous control, and used for normalization. The primers used for PCR were as follows: p53, 5′-CCC ATC CTC ACC ATC ATC AC-3′ and 5′-GTC AGT GGG GAA CAA GAA GTG-3′; p21, 5′-GCC GAA GTC AGT TCC TTG TGG A-3′ and 5′-GTG GGC GGA TTA GGG CTT-3′.

siRNA knockdown

Lipofectamine was used to transfect mononuclear cells with 150 nM of siRNA for targeting against p53 (5'-GACUCCAGUGGUAAUCUACTT-3'; sip53) or a scrambled control duplex (5'-GCGCGCUUUGUAGGAUUCG-3'; siScr). Twenty-four hours post-transfection, fresh culture medium was added, and the cells were treated with or without 10 µM 2-BP for another 24 h.

Embryoid body formation

Embryoid bodies were generated as previously described (Chan, 2006). Briefly, ESC-B5 cells were dissociated with trypsin-EDTA (0.25%) and cultured in leukemia inhibitory factor (LIF)-free stem cell medium to induce differentiation. Cell suspension liquid cultures ($<10^4$ cells/ml) were dispensed to 10 cm petri dishes (10 ml per dish). EB formation was initiated in hanging drop cultures prepared with 10-µL droplets, each containing an appropriate number (~100) of ESC-B5 cells. The ESC-B5 cells were allowed to aggregate in the hanging drops for two days, and were then transferred to liquid suspension culture.

Statistics

Data were analyzed using one-way ANOVA, and differences were evaluated using a two tailed Student's t-test and analysis of variance. $P < 0.05$ was considered significant.

RESULTS

Cytotoxic effects of 2-BP on ESC-B5 cells

To assess the potential cytotoxicity of 2-BP, the MTT

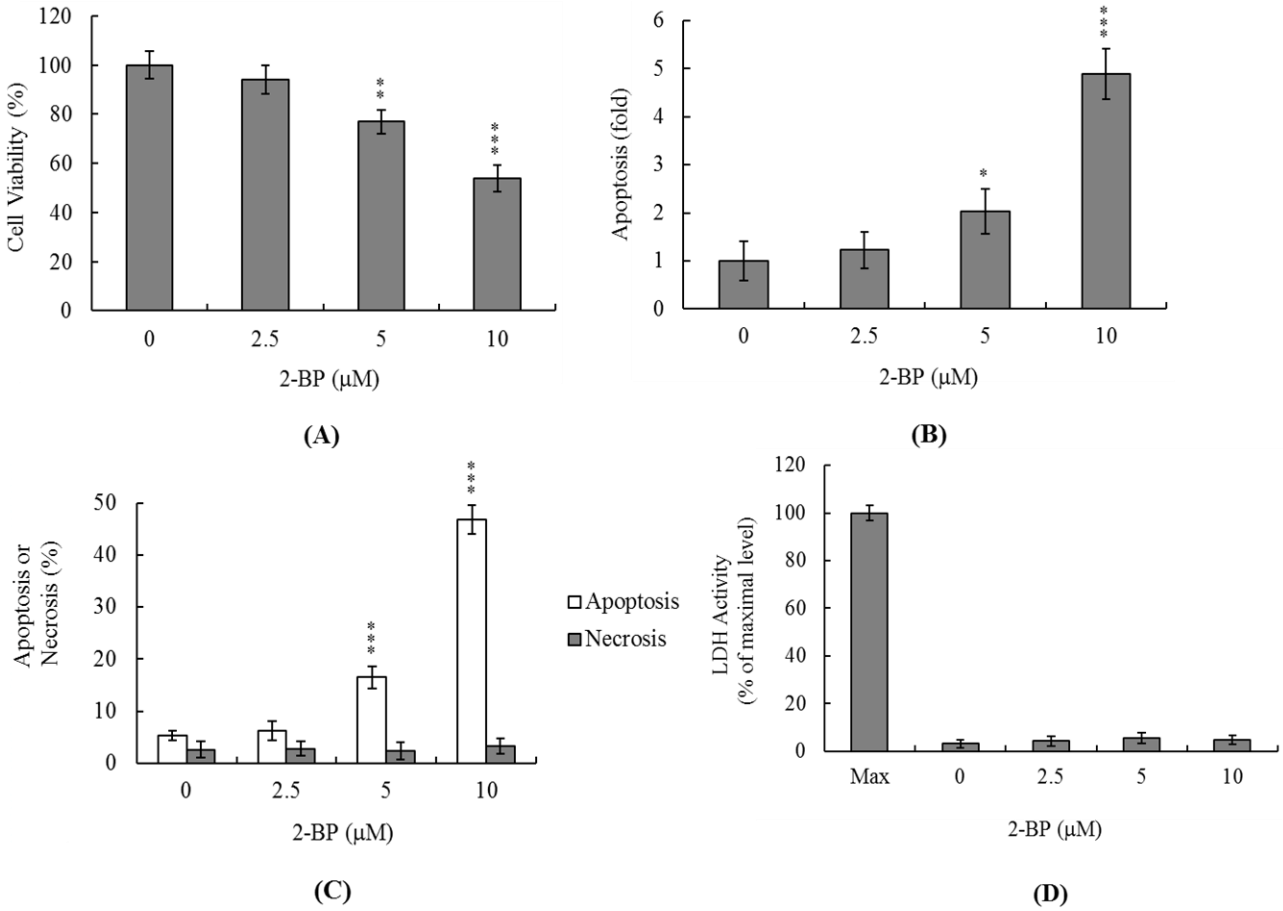

Figure 1. Effects of 2-BP on ESC-B5 cells: ESC-B5 cells were incubated with 0–10 μM 2-BP for 24 h. **(A)** Cell viability was determined using the MTT assay. **(B and C)** Apoptosis was detected with a cell death detection ELISA kit (B), followed by staining with propidium iodide and Hoechst 33342 (C). **(D)** Necrosis was further assessed in terms of LDH activity in the culture medium, and the data are expressed as a percentage of the maximal level (Max) of LDH activity determined after total cell lysis. Values are presented as means ± SEM of eight determinations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the untreated control group.

assay was used to examine the viability of ESC-B5 cells incubated in medium containing 0 to 10 μM 2-BP for 24 h. Results revealed that the viability of treated ESC-B5 cells was dose-dependently decreased by approximately 23 to 46% at concentrations higher than 5 μM 2-BP (Figure 1A). We next used a TUNEL ELISA kit to examine apoptosis, in an effort to determine the mode of 2-BP-induced cell death. Notably, 10 μM 2-BP induced a 4.9-fold increase in TUNEL positivity, compared with untreated cells (Figure 1B). The percentages of apoptotic and necrotic cells were further evaluated by staining with propidium iodide and Hoechst 33342. As shown in Figure 1C, the proportion of apoptotic cells was significantly increased following treatment with 5 to 10 μM 2-BP. However, no necrotic cells were identified in the 2-BP-treated group (Figures 1C and 1D). Our findings indicate that 2-BP induces apoptosis, but not necrosis, at concentrations greater than 5 μM in ESC-B5 cells.

ROS levels increase in ESC-B5 cells treated with 2-BP

In view of our previous finding that numerous chemical stimuli trigger apoptosis via ROS generation (Chan, 2007; Huang et al., 2007), DCF-DA and DHR 123 were used to examine ROS formation in ESC-B5 cells treated with 2-BP. The results revealed that 2-BP (5–10 μM) stimulated ROS generation about 2.8 to 5.3-fold, compared with the untreated control group (Figure 2A). Notably, pretreatment with N-acetyl cysteine (NAC), a commonly used ROS scavenger, effectively prevented ROS generation in the presence of 5 to 10 μM 2-BP (Figure 2B). These results clearly indicate that 2-BP triggers apoptosis of ESC-B5 cells via ROS generation.

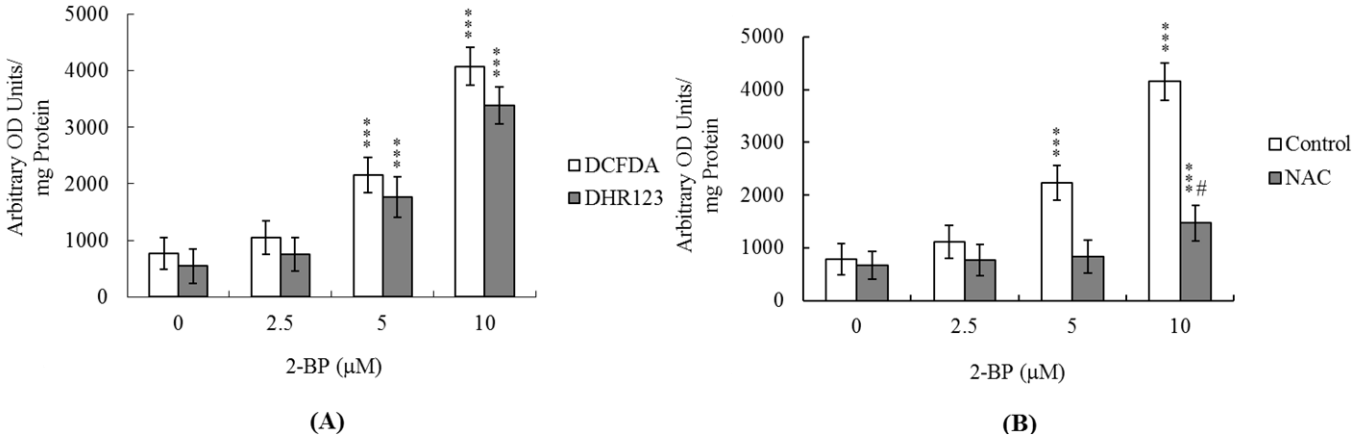

Figure 2. 2-BP promotes oxidative stress in ESC-B5 cells: **(A)** ESC-B5 cells were treated with 0 to 10 μM 2-BP for 24 h and ROS generation was assayed using DCF-DA (20 μM) or dihydrorhodamine 123 (DHR 123; 20 μM). **(B)** ESC-B5 cells were pre-incubated with N-acetyl cysteine (NAC; 300 μM) for 30 min, followed by treatment with or without 2-BP, as indicated. ROS generation was assayed using DCF-DA. Data are representative of eight independent experiments. ***, $P < 0.001$ versus the untreated control group; #, $P < 0.001$ versus the 2-BP-treated group.

Changes in intracellular Ca^{2+} concentrations and NO levels are involved in 2-BP-induced cell apoptosis

Changes in $[Ca^{2+}]i$ in 2-BP-treated ESC-B5 cells were detected using the fluorescent dye, Fluo-3AM. Treatment with 5 to 10 μM 2-BP elicited an increase in $[Ca^{2+}]i$ (Figure 3A), and cells cultured in Ca^{2+}-containing or Ca^{2+}-free culture media displayed a ~3-fold increase in $[Ca^{2+}]i$ following treatment with 10 μM 2-BP (Figure 3A). These findings indicate that the 2-BP-induced increase in $[Ca^{2+}]I$ is primarily due to the release of internal Ca^{2+}, likely from the endoplasmic reticulum, mitochondria, nucleus and/or calcium-binding proteins (Figure 3A).

PTIO, an inhibitor of NOS and a scavenger of NO, and L-NMMA, an inhibitor of NO synthase (NOS), had little effect on the $[Ca^{2+}]i$ increase induced by 2-BP, but pretreatment with NAC significantly suppressed this increase (Figure 3B). Thus, it seems that the 2-BP-induced increase in $[Ca^{2+}]i$ is regulated by ROS but not NO. We further used the NO-sensitive dye, DAF-2DA, to measure intracellular NO generation during 2-BP-induced apoptosis. The results revealed that intracellular NO levels were increased in ESC-B5 cells treated with 10 μM 2-BP (Figure 3C), but this increase was prevented by pretreatment of cells with the NOS inhibitor, L-NMMA (Figure 3C), or with 500 μM EGTA (a Ca^{2+} chelator) (Figure 3C).

Based on these findings, it was proposed that intracellular Ca^{2+} levels play an important role in the NOS activation and NO increase observed in 2-BP-treated ESC-B5 cells. Furthermore, our results clearly indicate that 2-BP triggers apoptosis of ESC-B5 cells via ROS generation and further demonstrate that NAC rescues the viability of 2-BP-treated cells.

PTIO inhibits mitochondrial membrane potential (MMP) changes and caspase activation during 2-BP-induced cell apoptosis

Next, we analyzed changes in MMP, a major apoptotic event during mitochondria-mediated apoptosis. Uptake of DiOC6(3) and TMRE into the mitochondria of ESC-B5 cells was observed following treatment with 10 μM 2-BP, indicating that there was a significant loss of MMP (Figure 4A). In addition, we monitored the activations of caspase-9 and -3, which are involved in the mitochondria-mediated apoptotic pathways, and found that the treatment of ESC-B5 cells with 10 μM 2-BP stimulated caspase-9 (Figure 4B) and -3 (Figure 4C) activities. Importantly, the 2-BP-induced MMP loss and caspase activation were significantly inhibited when cells were pretreated with 20 μM PTIO prior to with the application of 10 μM 2-BP (Figures 4A to 4C). These results indicate that the NO level may act as an upstream regulator of MMP changes and caspase-9 and -3 activation during the 2-BP-induced apoptosis of ESC-B5 cells.

Changes in mRNA levels following 2-BP treatment of ESC-B5 cells

Data from real-time RT-PCR analyses revealed significant upregulations of p53 and p21 mRNA levels in ESC-B5 cells treated with 10 μM 2-BP. These responses were blocked by pretreatment with NAC or PTIO (Figures 5A and B).

Treatment of ESC-B5 cells with p53 siRNA blocks 2-BP-induced apoptosis

To further determine the roles of p53 and p21 in

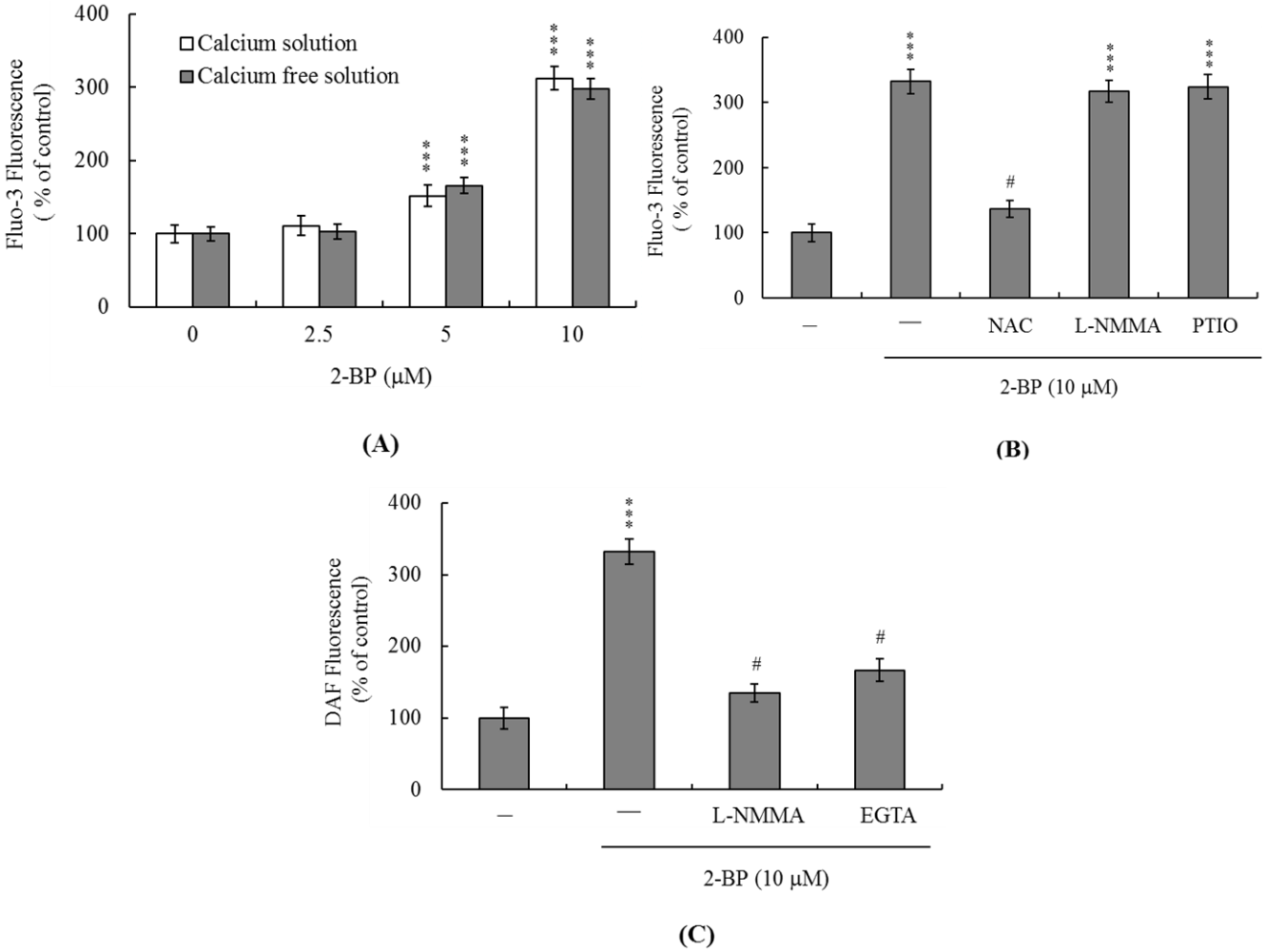

Figure 3. 2-BP stimulates changes in the intracellular calcium and NO contents of ESC-B5 cells: **(A)** ESC-B5 cells were incubated with 0 to 10 μM 2-BP for 24 h. Intracellular Fluo-3 fluorescence intensity was measured in the presence/absence of extracellular Ca^{2+}. **(B)** Changes in intracellular Ca^{2+} level upon treatment with 10 μM 2-BP, and the effects of ROS and NO inhibitors (NAC, 300 μM; L-NMMA, 400 μM; PTIO, 20 μM) were measured. **(C)** ESC-B5 cells were pretreated with L-NMMA (400 μM) or EGTA (500 μM) for 30 min, followed by incubation with or without 10 μM 2-BP. Intracellular NO generation was measured using the fluorescent dye, DAF-2DA. Data are presented as a percentage of the control group. ***, P < 0.001 versus the untreated control group; #, *P* < 0.001 versus the 2-BP-treated group.

Effects of 2-BP on the development and differentiation of ESC-B5 cells *in vitro*

To further determine the effects of 2-BP on early embryonic development in a stem cell assay model, we incubated cells in the presence or absence of 2-BP and assessed their ability to form embryoid bodies *in vitro*. Embryoid body formation was not affected by 2-BP at concentrations of less than 5 μM, but the application of 10 μM 2-BP significantly decreased embryoid body formation (Figure 7). These findings show that low doses of 2-BP (< 5 μM) have no injury effects on ESC-B5 cells, whereas higher concentrations (5 to 10 μM) trigger apoptosis and injury to development of mouse ESCs *in vitro.*

DISCUSSION

Oxidative stress stimulates several cell responses, including apoptosis (Khan et al., 2013; Rhee et al., 2013). A previous study demonstrated a significant decrease in superoxide dismutase (SOD) and an increase in glutathione peroxidase (GSH-PX) activity in Leydig cells treated with 0.10 and 1.00 mM 2-BP (Wu et al., 2002), suggesting that 2-BP impaired the antioxidant defenses in Leydig cells. The same study further demonstrated that 2-BP also induces DNA damage and enhances lipid peroxidation in cultured Leydig cells (Wu et al., 2002). Here, it was demonstrated that 2-BP directly induced oxidative stress in ESC-B5 cells (Figure 2), and that pretreatment with antioxidants effectively prevented ROS-

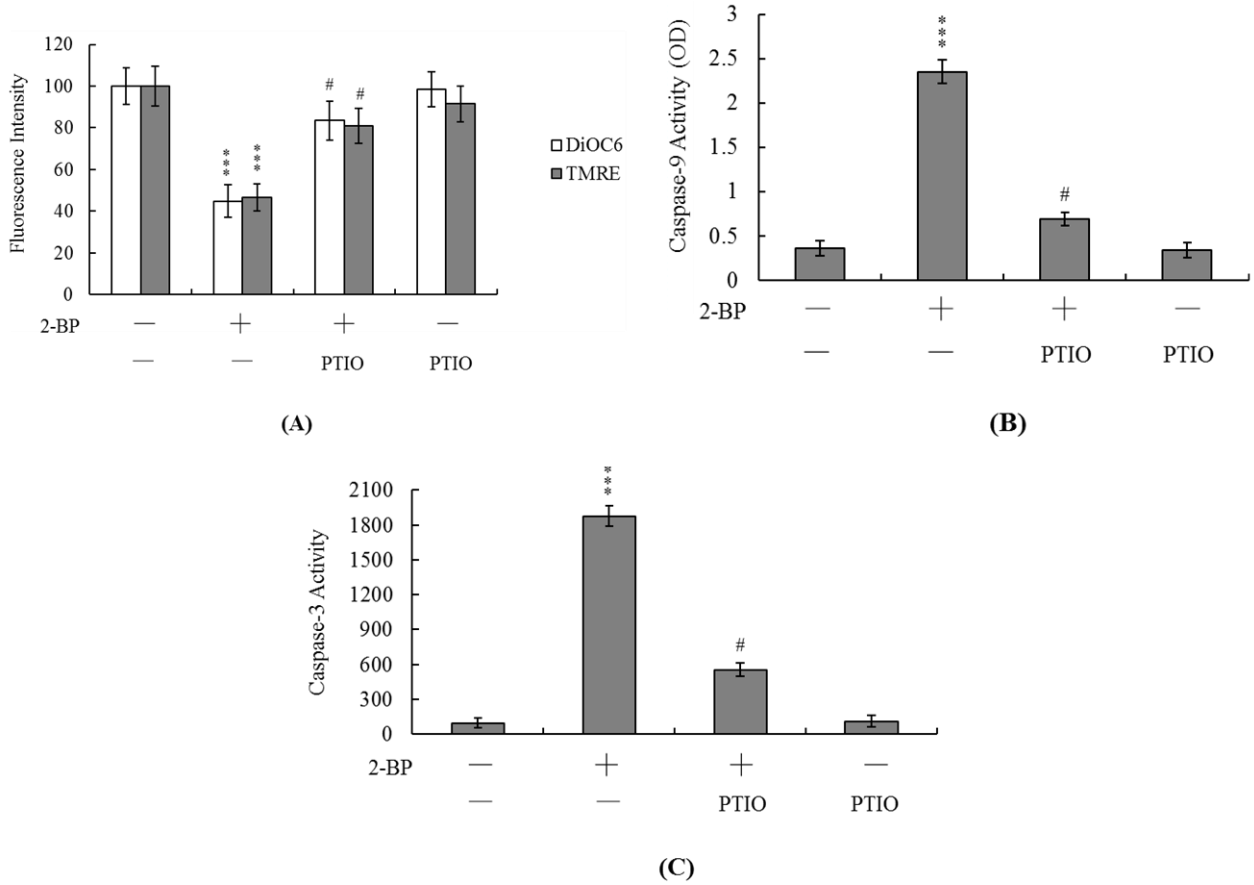

Figure 4. MMP alterations and activation of caspase-9 and -3 following 2-BP treatment of ESC-B5 cells. ESC-B5 cells were pretreated with PTIO (20 μM) for 1 h, and treated with or without 10 μM 2-BP for a further 24 h. **(A)** MMP changes were analyzed using 40 nM DiOC6(3) or 1 μM TMRE. **(B)** Caspase-9 activity was assayed using a Colorimetric Caspase-9 Assay kit. **(C)** Cell extracts (60 μg) were analyzed for caspase-3 activity using Z-DEVD-AFC as the substrate. ***, $P < 0.001$ versus the untreated control group; #, $P < 0.001$ versus the 2-BP-treated group.

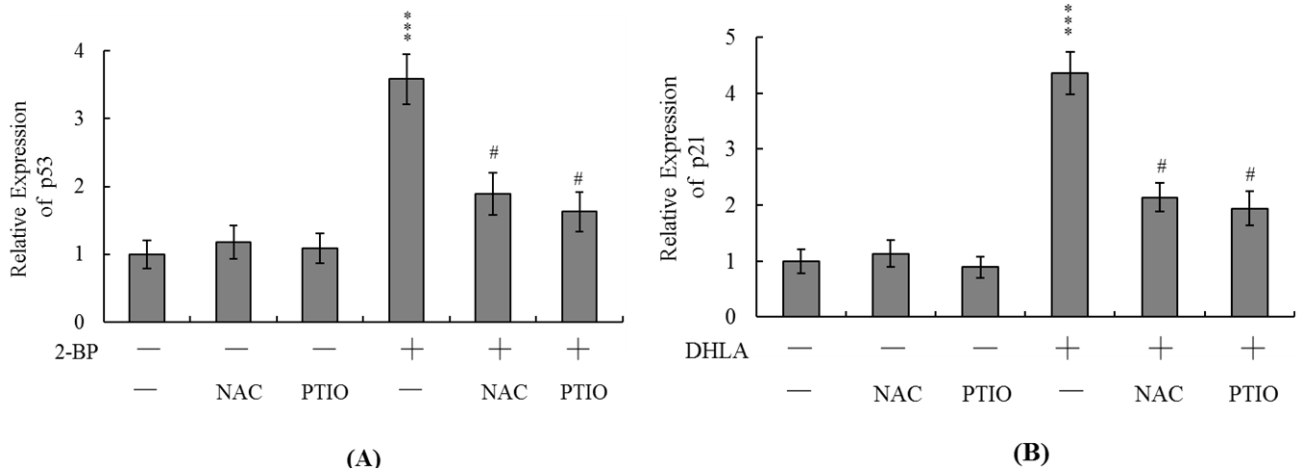

Figure 5. Effects of NAC and PTIO on p53 and p21 mRNA levels: ESC-B5 cells were pre-incubated with or without NAC (300 μM) and PTIO (20 μM) for 1 h, followed by treatment with 10 μM 2-BP for another 24 h. The mRNA levels of p53 **(A)** and p21 **(B)** were analyzed using real-time PCR. Values are representative of eight determinations. ***, $P < 0.001$ versus the untreated control group; #, $P < 0.001$ versus the 2-BP-treated group.

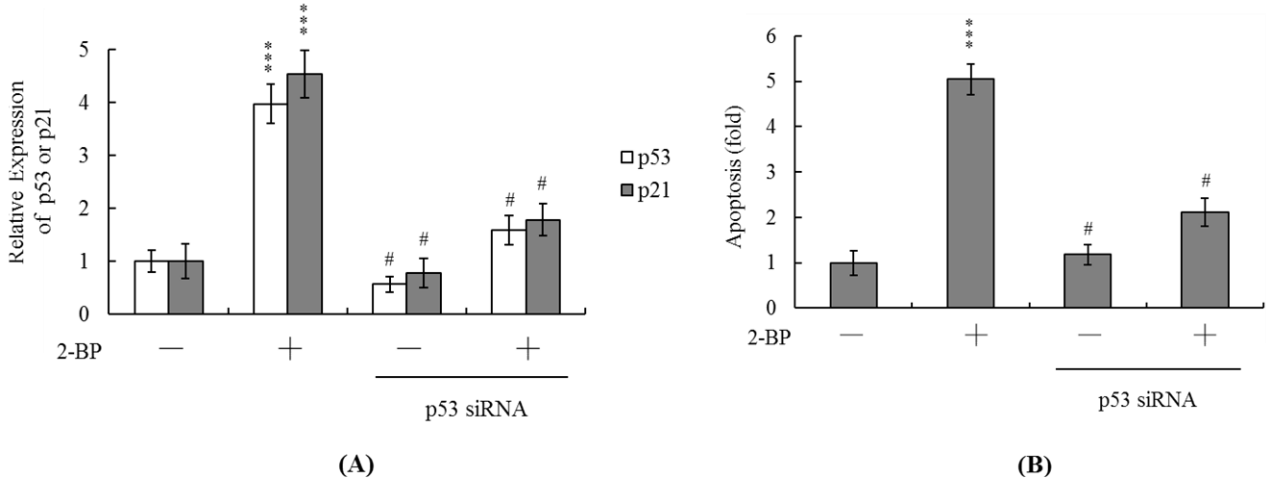

Figure 6. Knockdown of p53 protects ESC-B5 cells against 2-BP-induced apoptosis: ESC-B5 cells were transfected with siRNA targeting p53, incubated for 24 h, and treated with 10 μM 2-BP for a further 24 h. **(A)** The mRNA levels of p53 and p21 were analyzed using real-time PCR. **(B)** Apoptosis was measured as described in Figure 1. ***, $P < 0.001$ versus the untreated control group; #, $P < 0.001$ versus the 2-BP-treated group.

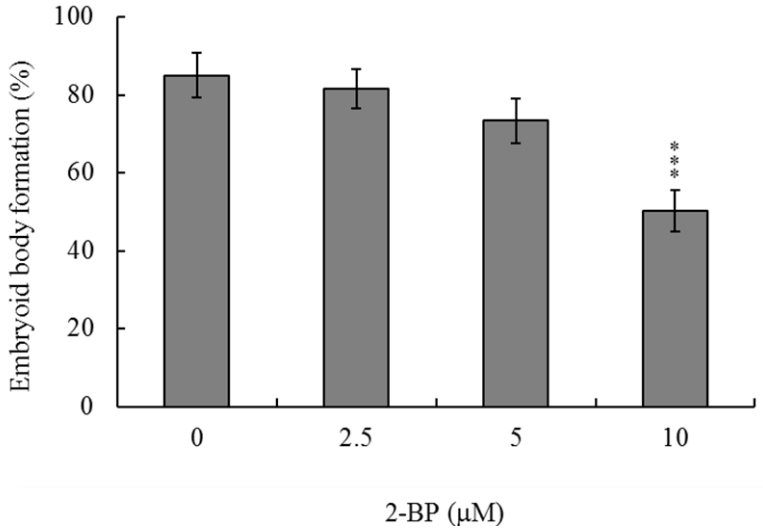

Figure 7. Effects of 2-BP on differentiation of ESC-B5 cells: ESC-B5 cells were incubated with or without the indicated concentrations of 2-BP for 24 h. Cells were dissociated with trypsin-EDTA, and cultured in medium without LIF to induce differentiation. Embryoid bodies were formed with the hanging drop method, as described in the Materials and Methods section. Values are presented as means ± SEM. ***, $P < 0.001$ versus the 2-BP-free group.

mediated 2-BP-induced apoptotic biochemical changes (Figures 2 and 3). ROS is an important upstream regulator of the apoptotic cascade in various mammalian cells (Webster, 2012). These results, coupled with our findings, strongly indicate that oxidative injury plays a pivotal role in 2-BP-induced apoptosis. However, the mechanisms underlying 2-BP-triggered ROS generation remain to be determined.

The intracellular calcium level plays an important role in regulating cell death (Dinicola et al., 2013). A previous study by our group showed that increases in intracellular calcium levels were involved in cell apoptosis triggered by specific chemical compounds (Chan, 2008; Hsieh and Chan, 2009). Furthermore, intracellular ROS is known to act as an upstream regulator of calcium levels and the induction of apoptosis (Chan, 2008; Hsieh and Chan,

2009). This study was meant to determine whether 2-BP induces apoptosis via an increase in intracellular calcium. Notably, an increase in $[Ca^{2+}]i$ following 2-BP treatment was observed, which shows that this could be largely attributed to the release of internal Ca^{2+} from storage organelles (Figures 3A and B). This increase was significantly blocked by NAC (Figure 3B), indicating that ROS generation is responsible for increasing the intracellular calcium concentration in 2-BP-treated ESC-B5 cells.

NO, which is an endogenous product of NADPH, O_2 and L-arginine catalysis by NOS, is involved in apoptosis triggered by several types of stimuli (Chan, 2008; Hsieh and Chan, 2009; Lu et al., 2006; Nazarewicz et al., 2007). The regulatory actions of NO on the mitochondrial apoptotic signaling pathways have been well documented. Additionally, previous studies have shown that tamoxifen and co-treatment with methylglyoxal and high glucose increase the intramitochondrial Ca^{2+} concentration, stimulating mitochondrial NO synthase activity and NO production in rat livers, human breast cancer MCF-7 cells, and human mononuclear cells (Chan, 2008; Hsieh and Chan, 2009; Nazarewicz et al., 2007). In the present study, we observed NO generation following 2-BP treatment of ESC-B5 cells, with ~3.3-fold higher intracellular NO levels in treated cells versus untreated controls (Figure 3C). Pretreatment with EGTA substantially prevented this increase in intracellular NO (Figure 3C), indicating that NO production in 2-BP-treated ESC-B5 cells is dependent on the intracellular calcium concentration. The regulatory role of NO in apoptosis is complex, and NO-mediated apoptotic effects are modulated via different mechanisms in distinct cell types (Chan, 2008; Hsieh and Chan, 2009; Li and Wogan, 2005; Rao, 2004). For instance, NOS substrates or NO donors inhibit apoptosis induced by photodynamic treatment in CCRF-CEM cells (Gomes et al., 2002), and an NOS inhibitor was shown to suppress the high glucose and methylglyoxal-stimulated mitochondria-dependent apoptotic pathway (Hsieh and Chan, 2009).

Importantly, it was discovered that PTIO attenuated the loss of mitochondrial membrane potential (MMP) and suppressed caspase activation in the system (Figure 4), suggesting that NO is an important mediator of apoptosis in 2-BP-treated ESC-B5 cells.

NO-mediated apoptotic processes are associated with p53 gene activation, which is essential for regulation of the cell cycle and/or apoptotic signaling occurring through $p21^{Waf1/Cip1}$ or Bax (Li et al., 2004; Okada and Mak, 2004). Moreover, a previous study by our group revealed apoptotic signaling pathway-induced increases in p53 and p21 expression levels in human mononuclear cells following co-treatment with high glucose and methyl-glyoxal (Hsieh and Chan, 2009). Here, we observed up regulation of both p53 and p21 mRNA following treatment with 2-BP, and found that this increase was blocked by pretreatment with PTIO and NAC (Figure 5). The p53 pro-tein, an expression product of the *p53* tumor suppressor gene, prevents the proliferation of cells with damaged DNA. More specifically, damaged DNA triggers the activation of ATM kinase, which catalyzes the phosphorylation of p53 proteins. Accumulation of phosphorylated p53 proteins in DNA-damaged cells activates two types of events: cell cycle arrest and apoptosis. The p53 protein also acts a transcription factor that can activate specific genes, including *p21*. The p21 protein, a member of the Cdk inhibitor family, blocks activation of the Cdk-cyclin complex, leading to cell cycle arrest. The p53 protein additionally activates genes encoding proteins that trigger cell apoptotic processes through binding and inactivating the apoptosis inhibitor, Bcl2.

Finally, p53 plays an important role in the self-renewal of ESCs and is involved in cell differentiation (Lin et al., 2005; Meletis et al., 2006). A previous study reported higher p53 protein levels in embryonic cells compared to differentiated cells (Sabapathy et al., 1997). Moreover, a recent report showed that p53 largely localizes to the cytoplasm of ESCs (Aladjem et al., 1998; Solozobova et al., 2009), but shows a mainly nuclear or perinuclear localization in many other cell lines (Shaulsky et al., 1990). These results highlight the important regulatory role of p53 in the differentiation of mouse ESCs. Here, we showed that 2-BP induces the expression of p53 (Fig. 5A), and siRNA-mediated knockdown of p53 mRNA prevented the 2-BP-induced the upregulation of *p21* mRNA expression and suppressed subsequent apoptosis (Figure 6A and B). Our data collectively indicate that p53 and p21 are activated by 2-BP treatment and play a critical regulatory role during 2-BP-induced apoptosis of ESC-B5 cells, but are not involved in regulating the differentiation potential of these cells. We hypothesize that in 2-BP-treated ESC-B5 cells, ROS generation triggers DNA damage, thereby activating p53 and downstream apoptotic signaling cascades.

These results collectively demonstrate that 2-BP has no injury effects on ESC-B5 cells at doses lower than 5 μM, but induces apoptosis at concentrations greater than 5 μM. Moreover, 2-BP directly triggers ROS generation, which in turn induces the release of calcium from intracellular storage organelles, increasing intracellular Ca^{2+} concentrations and stimulating NO production. Pretreatment with NAC or PTIO blocks the 2-BP-induced upregulation of critical genes and rescues cell viability. Based on these data, we propose that both intracellular ROS and NO play critical roles in the 2-BP-induced apoptosis of ESC-B5 cells.

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REFERENCES

Aladjem MI, Spike BT, Rodewald LW, Hope TJ, Klemm M, Jaenisch R, and Wahl GM (1998). ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Curr. Biol. 8:145-155.

Aoshima H, Satoh T, Sakai N, Yamada M, Enokido Y, Ikeuchi T, and Hatanaka H (1997). Generation of free radicals during lipid hydroperoxide-triggered apoptosis in PC12h cells. Biochim. Biophys. Acta. 1345:35-42.

Behl C, Davis JB, Lesley R, and Schubert D (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817-827.

Brookes PS (2004). Mitochondrial nitric oxide synthase. Mitochondrion 3:187-204.

Chan WH (2006). Ginkgolide B induces apoptosis and developmental injury in mouse embryonic stem cells and blastocysts. Hum. Reprod. 21:2985-2995.

Chan WH (2007). Citrinin induces apoptosis via a mitochondria-dependent pathway and inhibition of survival signals in embryonic stem cells, and causes developmental injury in blastocysts. Biochem. J. 404:317-326.

Chan WH (2008). Citrinin induces apoptosis in mouse embryonic stem cells. IUBMB Life. 60:171-179.

Chan WH (2010a). Cytotoxic effects of 2-bromopropane on embryonic development in mouse blastocysts. Int. J. Mol. Sci. 11:731-744.

Chan WH (2010b). Hazardous apoptotic effects of 2-bromopropane on maturation of mouse oocytes, fertilization, and fetal development. Int. J. Mol. Sci. 11:4361-4380.

Chan WH, Chang YJ (2006). Dosage effects of resveratrol on ethanol-induced cell death in the human K562 cell line. Toxicol. Lett. 161:1-9.

Chan WH, Houng WL, Lin CA, Lee CH, Li PW, Hsieh JT, Shen JL, Yeh HI, Chang WH (2013). Impact of dihydrolipoic acid on mouse embryonic stem cells and related regulatory mechanisms. Environ. Toxicol. 28:87-97.

Chan WH, Wu CC, Yu JS (2003). Curcumin inhibits UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermoid carcinoma A431 cells. J. Cell. Biochem. 90:327-338.

Dedkova EN, Ji X, Lipsius SL, Blatter LA (2004). Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells. Am. J. Physiol. Cell. Physiol. 286:C406-415.

Dennis J, Bennett JP, Jr. (2003). Interactions among nitric oxide and Bcl-family proteins after MPP+ exposure of SH-SY5Y neural cells I: MPP+ increases mitochondrial NO and Bax protein. J. Neurosci. Res. 72:76-88.

Dinicola S, Mariggio MA, Morabito C, Guarnieri S, Cucina A, Pasqualato A, D'Anselmi F, Proietti S, Coluccia P, Bizzarri M (2013). Grape seed extract triggers apoptosis in Caco-2 human colon cancer cells through reactive oxygen species and calcium increase: extracellular signal-regulated kinase involvement. Br. J. Nutr. 25:1-13.

Elfering SL, Sarkela TM, Giulivi C (2002). Biochemistry of mitochondrial nitric-oxide synthase. J. Biol. Chem. 277:38079-38086.

Evans MJ, Kaufman MH (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Ghafourifar P, Cadenas E (2005). Mitochondrial nitric oxide synthase. Trends Pharmacol. Sci. 26:190-195.

Gomes ER, Almeida RD, Carvalho AP, Duarte CB (2002). Nitric oxide modulates tumor cell death induced by photodynamic therapy through a cGMP-dependent mechanism. Photochem. Photobiol. 76:423-430.

Hsieh MS, Chan WH (2009). Impact of Methylglyoxal and High Glucose Co-treatment on Human Mononuclear Cells. Int. J. Mol. Sci. 10:1445-1464.

Hsieh YJ, Wu CC, Chang CJ, Yu JS (2003). Subcellular localization of Photofrin determines the death phenotype of human epidermoid carcinoma A431 cells triggered by photodynamic therapy: when plasma membranes are the main targets. J. Cell. Physiol. 194:363-375.

Huang LH, Shiao NH, Hsuuw YD, Chan WH (2007). Protective effects of resveratrol on ethanol-induced apoptosis in embryonic stem cells and disruption of embryonic development in mouse blastocysts. Toxicology 242:109-122.

Ichihara G, Asaeda N, Kumazawa T, Tagawa T, Kamijima M, Yu X, Kondo H, Nakajima T, Kitoh J, Yu IJ, Moon YH, Hisanaga N, and Takeuchi Y (1997). Testicular and haematopoietic toxicity of 2-bromopropane, a substitute for ozone layer-depleting chlorofluorocarbons. J. Occup. Health 39:57-63.

Ishikawa H, Tian Y, Yamauchi T (2001). Induction of micronuclei formation in preimplantation mouse embryos after maternal treatment with 2-bromopropane. Reprod. Toxicol. 15:81-85.

Kang KS, Li GX, Che JH, Lee YS (2002). Impairment of male rat reproductive function in F1 offspring from dams exposed to 2-bromopropane during gestation and lactation. Reprod. Toxicol. 16:151-159.

Keller GM (1995). In vitro differentiation of embryonic stem cells. Curr. Opin. Cell. Biol. 7:862-869.

Khan M, Li T, Ahmad Khan MK, Rasul A, Nawaz F, Sun M, Zheng Y, and Ma T (2013). Alantolactone induces apoptosis in HepG2 cells through GSH depletion, inhibition of STAT3 Activation, and mitochondrial dysfunction. BioMed. Res. Int. 2013:719858.

Kim JC, Kim SH, Shin DH, Ahn TH, Kim HC, Kim YB, Jiang CZ, Han J, Chung MK (2004). Effects of prenatal exposure to the environmental pollutant 2-bromopropane on embryo-fetal development in rats. Toxicology 196:77-86.

Kim Y, Jung K, Hwang T, Jung G, Kim H, Park J, Kim J, Park J, Park D, Park S, Choi K, Moon Y (1996). Hematopoietic and reproductive hazards of Korean electronic workers exposed to solvents containing 2-bromopropane. Scand. J. Work Environ. Health 22:387-391.

Kruzliak P, Kovacova G, Pechanova O (2013). Therapeutic potential of nitric oxide donors in the prevention and treatment of angiogenesis-inhibitor-induced hypertension. Angiogenesis 16:289-295.

Li CQ, Wogan GN (2005). Nitric oxide as a modulator of apoptosis. Cancer Lett. 226:1-15.

Li CQ, Robles AI, Hanigan CL, Hofseth LJ, Trudel LJ, Harris CC, Wogan GN (2004). Apoptotic signaling pathways induced by nitric oxide in human lymphoblastoid cells expressing wild-type or mutant p53. Cancer Res. 64:3022-3029.

Li GX, Kang KS, Lee YS (2001). 2-Bromopropane induced germ cell apoptosis during spermatogenesis in male rat. J. Vet. Med. Sci. 63:373-382.

Lin T, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E, Xu Y (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat. Cell. Biol. 7:165-171.

Lu Z, Tao Y, Zhou Z, Zhang J, Li C, Ou L, Zhao B (2006). Mitochondrial reactive oxygen species and nitric oxide-mediated cancer cell apoptosis in 2-butylamino-2-demethoxyhypocrellin B photodynamic treatment. Free Radic. Biol. Med. 41:1590-1605.

Meletis K, Wirta V, Hede SM, Nister M, Lundeberg J, and Frisen J (2006). p53 suppresses the self-renewal of adult neural stem cells. Development 133:363-369.

Nakatsubo N, Kojima H, Kikuchi K, Nagoshi H, Hirata Y, Maeda D, Imai Y, Irimura T, Nagano T (1998). Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. FEBS Lett. 427:263-266.

Nazarewicz RR, Zenebe WJ, Parihar A, Larson SK, Alidema E, Choi J, and Ghafourifar P (2007). Tamoxifen induces oxidative stress and mitochondrial apoptosis via stimulating mitochondrial nitric oxide synthase. Cancer Res. 67:1282-1290.

Okada H, Mak TW (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. Nat. Rev. Cancer. 4:592-603.

Omura M, Romero Y, Zhao M, Inoue N (1999). Histopathological evidence that spermatogonia are the target cells of 2-bromopropane. Toxicol. Lett. 104:19-26.

Park JS, Kim YH, Park DW, Choi KS, Park SH, and Moon YH (1997). An outbreak of hematopoietic and reproductive disorders due to solvents containing 2-bromopropane in an electronic factory, South Korea: Epidemiological survey. J. Occup. Health 39:138-143.

Rao CV (2004). Nitric oxide signaling in colon cancer chemoprevention.

Mutat Res. 555:107-119.
Rhee JS, Yu IT, Kim BM, Jeong CB, Lee KW, Kim MJ, Lee SJ, Park GS, Lee JS (2013). Copper induces apoptotic cell death through reactive oxygen species-triggered oxidative stress in the intertidal copepod *Tigriopus japonicus*. Aquat. Toxicol. 132-133C:182-189.
Sabapathy K, Klemm M, Jaenisch R, Wagner EF (1997). Regulation of ES cell differentiation by functional and conformational modulation of p53. EMBO J. 16:6217-6229.
Shaulsky G, Goldfinger N, Ben-Ze'ev A, Rotter V (1990). Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. Mol. Cell. Biol. 10:6565-6577.
Solozobova V, Rolletschek A, Blattner C (2009). Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. BMC Cell. Biol. 10:46.
Son HY, Kim YB, Kang BH, Cho SW, Ha CS, Roh JK (1999). Effects of 2-bromopropane on spermatogenesis in the Sprague-Dawley rat. Reprod. Toxicol. 13:179-187.
Sun GG, Hu WN, Zhang J, Li CL, Yang CR (2013). Effect of Nitric Oxide on Esophageal Cancer Cell Line TE-1. Chinese medical sciences journal = Chung-kuo i hsueh k'o hsueh tsa chih / Chinese Academy of Medical Sciences. 28:44-49.
Wang Y, Lin A, Loake GJ, Chu C (2013). H2 O2 -induced Leaf Cell Death and the Crosstalk of Reactive Nitric/Oxygen Species(F.). J. Integr. Plant Biol. 55:202-208.
Webster KA (2012). Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. Future Cardiol. 8:863-884.
Wu HJ, Chan WH (2007). Genistein protects methylglyoxal-induced oxidative DNA damage and cell injury in human mononuclear cells. Toxicol. In Vitro. 21:335-342.
Wu X, Faqi AS, Yang J, Pang BP, Ding X, Jiang X, Chahoud I (2002). 2-Bromopropane induces DNA damage, impairs functional antioxidant cellular defenses, and enhances the lipid peroxidation process in primary cultures of rat Leydig cells. Reprod. Toxicol. 16:379-384.
Yu X, Ichihara G, Kitoh J, Xie Z, Shibata E, Kamijima M, Asaeda N, Hisanaga N, Takeuchi Y (1999a). Effect of inhalation exposure to 2-bromopropane on the nervous system in rats. Toxicology 135:87-93.
Yu X, Kamijima M, Ichihara G, Li W, Kitoh J, Xie Z, Shibata E, Hisanaga N, and Takeuchi Y (1999b). 2-Bromopropane causes ovarian dysfunction by damaging primordial follicles and their oocytes in female rats. Toxicol. Appl. Pharmacol. 159:185-193.
Zhao LX, Kim EK, Lim HT, Moon YS, Kim NH, Kim TH, Choi H, Chae W, Jeong TC, Lee ES (2002). Synthesis, characterization and *in vtro* identification of N7-guanine adduct of 2-bromopropane. Arch. Pharm. Res. 25:39-44.