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ORIGINAL ARTICLE

Estradiol-17β protects against hypoxia-induced hepatocyte injury through ER-mediated upregulation of Bcl-2 as well as ER-independent antioxidant effects

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Although many previous studies have suggested that estrogen functions as a cytoprotective agent under oxidative stress conditions, the underlying mechanism by which this effect is exerted remains to be elucidated. This study assessed the effects of estradiol-17β (E_2) (10^{-8} M) on hypoxia-induced cell injury and its related signaling in primary cultured chicken hepatocytes. Hypoxic conditions were found to augment the level of DNA damage and to reduce cell viability and the level of [^{3}H]-thymidine incorporation, and these phenomena were prevented through treatment with E_2. Hypoxia also increased caspase-3 expression, but showed no evidence of an influence on the expression of Bcl-2. However, E_2 induced an increase in the level of Bcl-2 expression under hypoxic conditions and reduced the level of caspase-3 expression. The effects of E_2 on Bcl-2 and caspase expression were blocked by ICI 182780 (E_2 receptor (ER) antagonist, 10^{-7} M). In addition, hypoxia resulted in an increase in the intracellular reactive oxygen species (ROS) generated. These effects were blocked by E_2, but not by E_2-BSA and ICI 182780. Hypoxia also activated p38 mitogen-activated protein kinase (MAPK), c-JUN N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and nuclear factor-κB (NF-κB). These effects were blocked by E_2, but not by ICI 182780. The inhibition of p38 MAPK and JNK/SAPK blocked NF-κB activation. In conclusion, E_2 was found to protect against hypoxia-induced cell injury in chicken hepatocytes through ER-mediated upregulation of Bcl-2 expression and through reducing the activity of ROS-dependent p38 MAPK, JNK/SAPK and NF-κB.

Keywords: Hypoxia, estradiol-17β, chicken hepatocytes

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Introduction

Hypoxia generates reactive oxygen species (ROS, which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and nitric oxide (NO)), which induce tissue injury via oxidative stress [1]. Although some ROS function as secondary messengers in normal cellular signaling [2], excessive ROS generation is known to cause oxidative damage to DNA, lipids and proteins [3]. This results in hypoxic cell injury, which is mediated by ROS-induced intracellular signal molecules, including mitogen-activated protein kinases (MAPKs) [4] and nuclear factor-κB (NF-κB) [5, 6]. Antioxidant supplementation has been shown to inhibit the cellular damage that is induced by oxidative stress [4, 7, 8]. The liver is more vulnerable to oxidative stress under hypoxic conditions than other organs [9]. It has been previously demonstrated that the significant damage to cells that results from endogenous free radical attack contributes to the pathology of cancer [10] and neurodegenerative diseases [11]. The mechanism responsible for hypoxic liver injury has been the focus of a considerable amount of research, with the broader objective of preventing such injuries, and thereby improving the treatment [12, 13]. Studies using hepatocellular cell injury models have demonstrated that oxidative stress is mediated by ROS [14]. Natural estrogens showed evidence of significantly higher radical-scavenging antioxidant activity than vitamins C and E, with up to 2.5 times more activity

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[15, 16]. Considering the reported anti-apoptotic effects of estrogen on a variety of cell types [17-19], hepatoprotection mediated by estrogen may be the result of the prevention of hepatocyte apoptosis induced by hypoxia. However, the detailed molecular mechanisms that underlie the effects of estradiol-17β (E_2) in hepatocytes remain to be clearly elucidated.

This study used a chick liver model to assess the relevant estrogen responsive functions. In contrast to the oviduct, which requires estrogen for differentiation, the livers of chicks are differentiated and function fully in the absence of estrogen. This unique feature of the chick liver allows the examination of its functional regulation prior to, during and after estrogen administration [20]. Previous reports have suggested that there are certain differences in the liver-specific functions of hepatocytes between birds and mammals [21, 22]. It is possible that chicken hepatocytes may restore the expression of liver-specific functions in the later stages of culture, even in a simple monolayer culture aggregate. It was also previously reported that an aggregate of rat hepatocytes formed through the coating of dishes with synthetic substrates could maintain liver-specific functions for a longer period. Previous reports have suggested that the chicken hepatocyte system is a useful *in vitro* model for the examination of the functions of the liver, including albumin expression [23], P450 1A induction [23], tyrosine aminotransferase expression [24] and ascorbate recycling [25]. This study assessed the effects of E_2 on hypoxia and its associated mechanisms in primary cultured chicken hepatocytes.

Results

Effect of E_2 on hypoxia-induced hepatocyte apoptosis

The effect of hypoxia on the level of hypoxia-inducible factor-1α (HIF-1α) expression was first analyzed in order to verify whether the typical cell responses were induced by the hypoxic conditions. As is shown in Figure 1A, we noted a significant increase in HIF-1α under hypoxic conditions, which achieved a peak level at 12 h after the initiation of hypoxic incubation. This result was validated by immunofluorescence staining, which revealed an increase in the intranuclear expression levels of HIF-1α (Figure 1B). In order to determine whether hypoxia stimulates the generation of ROS in hepatocytes, the level of H_2O_2 production was assessed using CM-H_2DCFDA (DCF-DA, 10^{-5} M) as the fluorescent probe of the intracellular H_2O_2. As is shown in Figure 1C, in the cells exposed to hypoxic conditions, the level of H_2O_2 increased in a time-dependent manner (0-72 h). In an experiment designed to determine whether or not E_2 (10^{-8} M) influences hypoxia-induced DNA fragmentation, it was observed that hypoxia increased the level

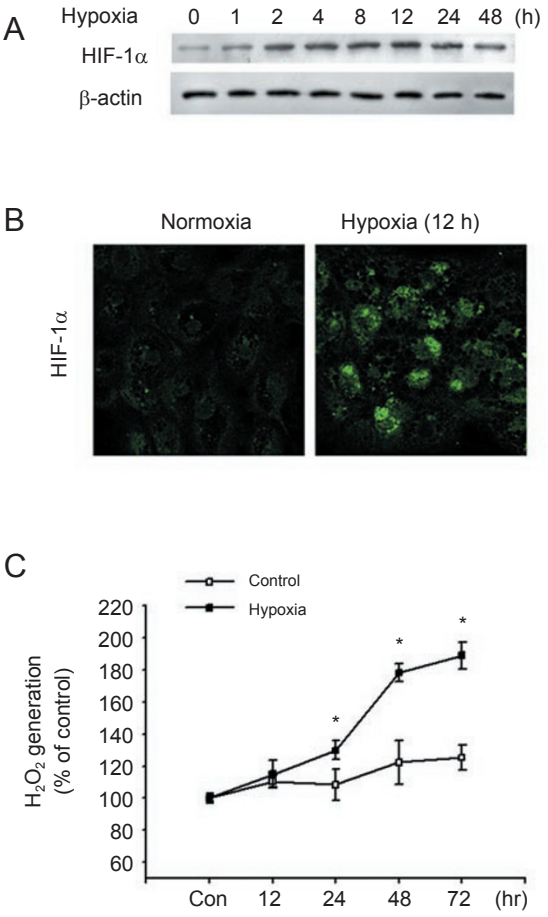

Figure 1 Time-dependent effect of hypoxia on hypoxia inducible factor (HIF)-1α. **(A)** Chicken hepatocytes were incubated under hypoxic conditions for various times (0-48 h), and the level of cellular HIF-1α expression was determined through western blotting. **(B)** The cells were incubated under normoxic or hypoxic conditions for 12 h and HIF-1α was detected using immunofluorescence staining. (**C**) The cells were incubated under hypoxic conditions for 0 to 72 h, and the cellular level of the hypoxia-induced H_2O_2 was measured using a luminometer after 10 μM DCF-DA staining for 30 min before counting. The values are expressed as the mean± SE of three independent experiments with triplicate dishes. **P* < 0.05 versus the control.

of DNA fragmentation on and after 48 h (Figure 2A), and that the E_2 treatment attenuated hypoxia-induced DNA fragmentation at 48 h (Figure 2B). In addition, the effects of E_2 on Bcl-2 and caspase-3 expression were assessed under hypoxic conditions. As is shown in Figure 3A, the level of caspase-3 expression increased under hypoxic conditions in a time-dependent manner. However, the levels of Bcl-2 expression remained unaffected. On the other hand, the level of caspase-3 expression was reduced by E_2 treatment and the level of Bcl-2 expression was increased (Figure 3B). As

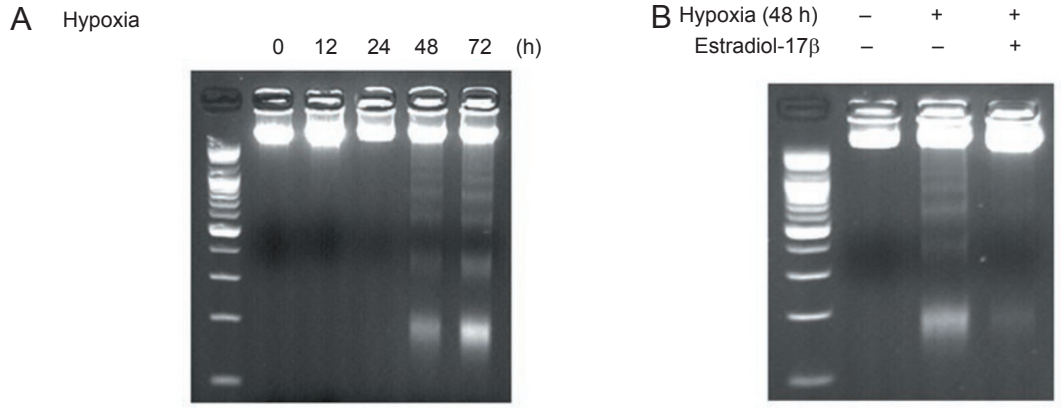

Figure 2 Effect of estradiol-17β (E_2) on DNA fragmentation. **(A)** The effect of hypoxia on DNA fragmentation. Chicken hepatocytes were incubated under hypoxic conditions for 0-72 h, and the total genomic DNA was then isolated from the cell pellets. The DNA was analyzed on 2% agarose gel and visualized with ethidium bromide. **(B)** The effect of E_2 (10^{-8} M) on the level of DNA fragmentation under hypoxic conditions for 48 h.

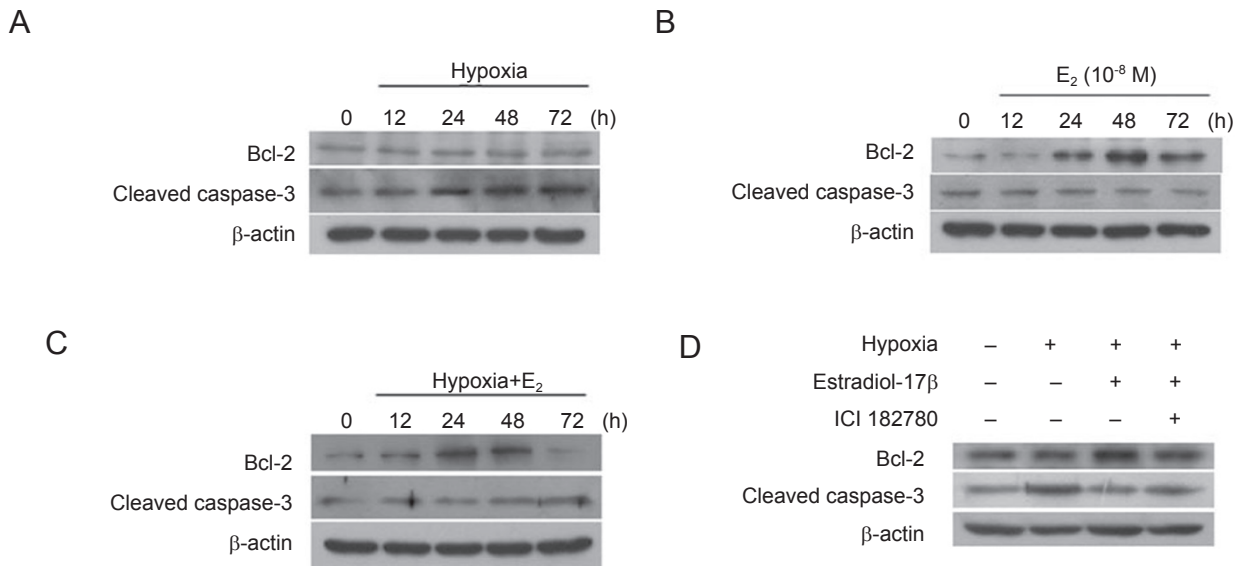

Figure 3 Effect of estradiol-17β (E_2) on Bcl-2 and caspase-3 expression on hypoxia. **(A-C)** Chicken hepatocytes were incubated for various times (0-72 h) under hypoxic conditions, under treatment with E_2 or under hypoxic conditions with pretreatment with E_2, and the levels of Bcl-2 and caspase-3 expression were determined through western blotting. **(D)** Cells were pretreated with ICI 182780 (E_2 receptor antagonist, 10^{-7} M) for 30 min prior to 48 h of incubation under hypoxic conditions with E_2. The levels of Bcl-2 and cleaved caspase-3 were then measured through western blotting. The example shown is a representative of three independent experiments.

is shown in Figure 3C, E_2 treatment increased the Bcl-2 expression level under hypoxic conditions, and the level of hypoxia-induced caspase-3 expression was inhibited by E_2 treatment. These effects of E_2 were inhibited by ICI 182780 (E_2 receptor antagonist, 10^{-7} M).

Effect of E_2 on hypoxia-induced hepatocyte H_2O_2 production and cell injury

Hypoxia-induced H_2O_2 generation was attenuated by either E_2 or diethylstilbesterol (DES, 10^{-8} M), but was unaffected by ICI 182780 or E_2-BSA (10^{-8} M). These results were confirmed through the detection of DCF-sensitive intracellular H_2O_2 using confocal microscopy. H_2O_2 was used as a positive control for hypoxia-induced H_2O_2 generation (Figure 4A and 4B). [^{3}H]-thymidine incorporation was conducted in an effort to determine the effects of hypoxia

on cell proliferation. We noted no significant differences in the levels of [^{3}H]-thymidine incorporation under normoxic conditions. However, after ≥ 48 h under hypoxic conditions, the level of [^{3}H]-thymidine incorporation was reduced, and the effects of hypoxia were attenuated by treatment with E_2 (Figure 4C). A trypan blue exclusion assay was conducted in an effort to determine the effects of hypoxia on cell viability. Cell viability was reduced by 48 h of exposure to the hypoxic conditions compared with what was observed under normoxic conditions, and the hypoxia-induced reduction in cell viability was attenuated as a result of treatment with E_2 and vitamin C (antioxidant, 10^{-3} M). H_2O_2 was used as a positive control (Figure 4D).

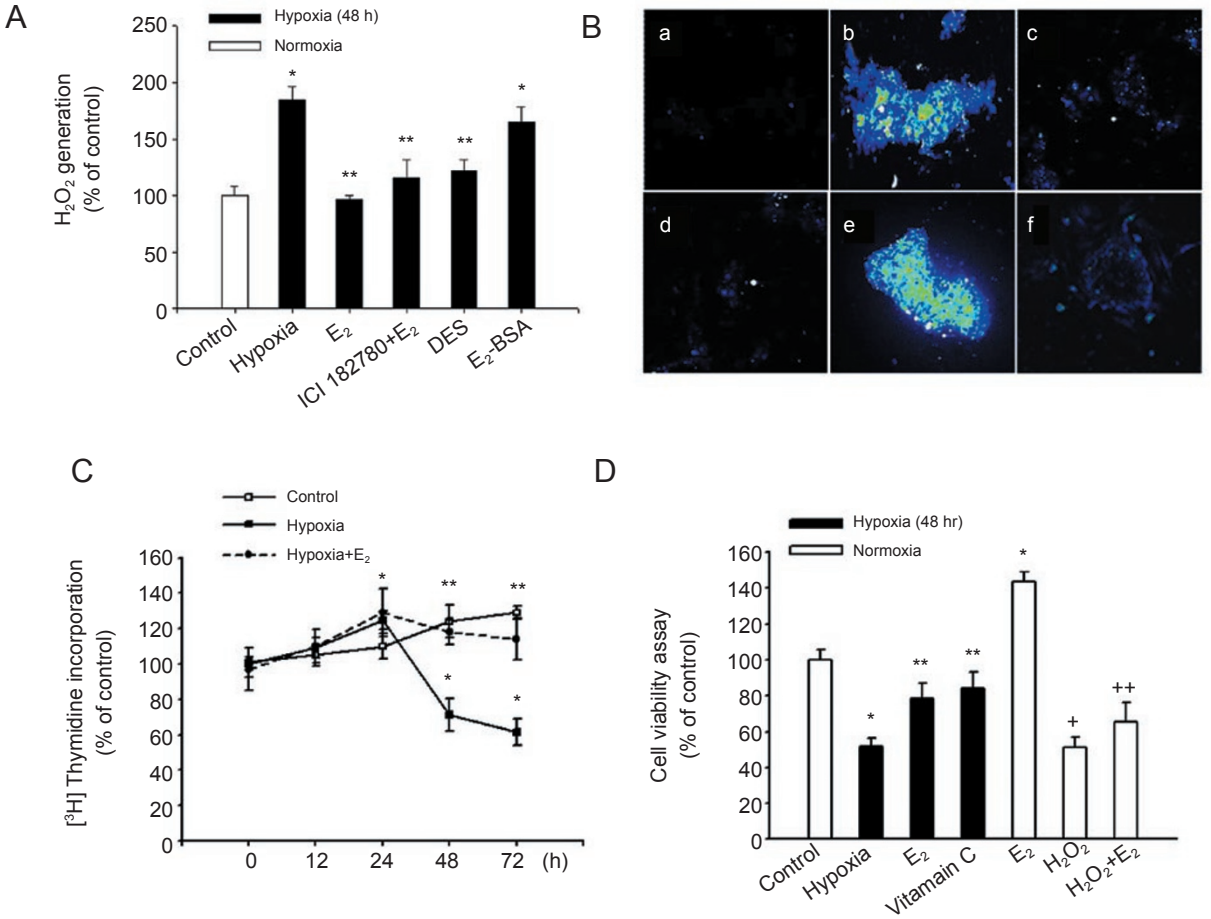

Figure 4 Effect of E_2 on the generation of hypoxia-induced reactive oxygen species (ROS) and cell injury. **(A)** Cellular H_2O_2 levels were measured after 48 h of incubation of the chicken hepatocytes under hypoxic conditions. The chicken hepatocytes were pretreated with E_2, with E_2 and ICI 182780 (E_2 receptor antagonist, 10^{-7} M), with diethylstilbesterol (DES, 10^{-8} M), and with E_2-BSA (10^{-8} M) for 30 min prior to hypoxic incubation, and the cellular levels of hypoxia-induced H_2O_2 were measured. The values were expressed as the means ± SE from three independent experiments with triplicate dishes. *P < 0.05 versus control, **P < 0.05 versus hypoxia alone. **(B)** Dichlorofluorescein (DCF)-sensitive cellular ROS was assessed using confocal microscopy. The chicken hepatocytes were incubated under normoxic conditions (a), hypoxic conditions (b), hypoxic conditions with E_2 (10^{-8} M) (c), hypoxic conditions with E_2 after pretreatment with ICI 182780 (d), normoxic conditions with H_2O_2 (10^{-4} M) (e), and normoxic conditions with H_2O_2 after 48 h of pretreatment with E_2 (f). The example shown is a representative of three independent experiments. **(C)** Effect of hypoxia on the level of [^{3}H]-thymidine incorporation. The chicken hepatocytes were incubated under normoxic or hypoxic conditions, or hypoxic conditions with E_2, for 0-72 h, and then pulsed with 1 μCi of [^{3}H]-thymidine. **(D)** Chicken hepatocytes were pretreated with E_2 (10^{-8} M) or vitamin C (antioxidant, 10^{-3} M) for 30 min before 48 h of incubation under normoxic or hypoxic conditions. Under normoxic conditions, E_2, H_2O_2 (10^{-4} M), and H_2O_2 with E_2 were used as positive controls and compared with the hypoxia groups. The trypan blue exclusion test was conducted as described in Materials and Methods. The values are expressed as the mean ± SE from three independent experiments with triplicate dishes. *P < 0.05 versus control, **P < 0.05 versus hypoxia alone, ^+P < 0.05 versus control, ^{++}P < 0.05 versus H_2O_2 alone.

Effect of E_2 on hypoxia-induced activation of MAPKs and NF-κB

The signaling molecules associated with hypoxia-induced cell injury, p38 MAPK, c-JUN N-terminal kinase/stress-activated protein kinase (JNK/SAPK and NF-κB, were evaluated. The maximum level of p38 MAPK and JNK/SAPK activation was observed at 4 and 2 h, respectively, after the initiation of hypoxic incubation (Figure 5A). As is shown in Figure 5B, SB 203580 (p38 MAPK inhibitor, 10^{-6} M) and SP 600125 (JNK/SAPK inhibitor, 10^{-6} M) partially protected the cells against hypoxia-induced injury. In addition, the maximal increase in the level of phospho NF-κB was observed at between 2 and 4 h of hypoxic incubation, after which phosphorylation gradually decreased to basal levels (Figure 5C). The inhibitory effect of hypoxia on cell viability was partially blocked by Bay 11-7082 (NF-κB inhibitor, 2×10^{-5} M) (Figure 5D).

As is shown in Figure 6A, the hypoxia-induced activation of p38 MAPK and JNK/SAPK was blocked by E_2, but the effect of E_2 was not inhibited by ICI 182780. In addition, treatment with E_2 attenuated the hypoxia-induced activation of NF-κB, but ICI 182780 did not block the effect of E_2 (Figure 6B). The relationship between MAPKs and NF-κB was subsequently assessed. The hypoxia-induced phosphorylation of NF-κB was inhibited by pretreatment with SB 203580 and SP 600125 (Figure 6C).

Discussion

The results of this study show that E_2 exerts a protec-

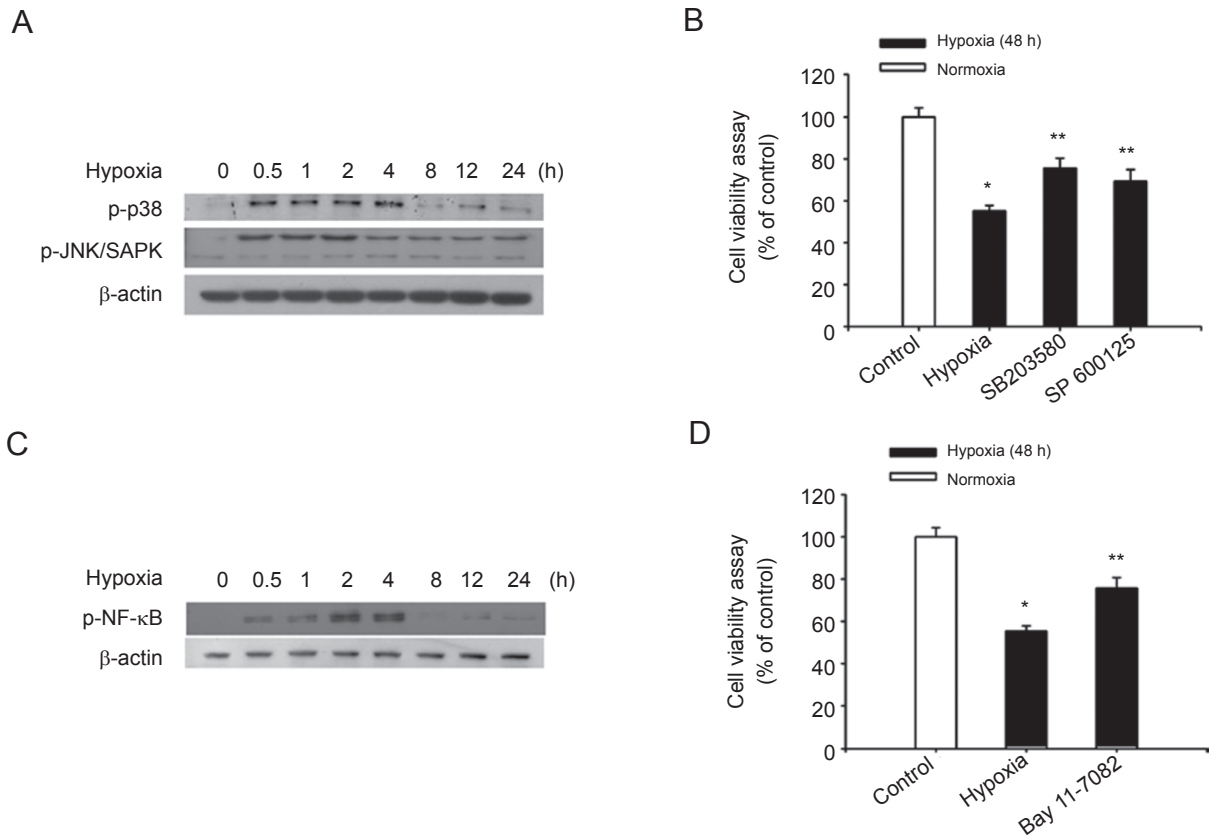

Figure 5 Involvement of the mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) in hypoxia-induced cell injury. **(A)** Chicken hepatocytes were incubated under hypoxic conditions from 0 to 24 h. The phosphorylated p38 MAPK and JNK/SAPK were then detected through western blotting. **(B)** Chicken hepatocytes were pretreated with SB 203580 (p38 MAPK inhibitor, 10^{-6} M) and SP 600125 (JNK/SAPK inhibitor, 10^{-6} M) for 30 min prior to 48 h of incubation under hypoxic conditions. The trypan blue exclusion test was conducted as described in Materials and Methods. **(C)** Chicken hepatocytes were incubated under hypoxic conditions from 0 to 24 h. The phosphorylated NF-κB was then detected through western blotting. **(D)** Chicken hepatocytes were pretreated for 30 min with Bay 11-7082 (NF-κB inhibitor, 2×10^{-5} M) prior to 48 h of incubation under hypoxic conditions. The trypan blue exclusion test was conducted as described in Materials and Methods. The values are expressed as the mean ± SE from three independent experiments with four dishes. *P < 0.05 versus control, **P < 0.05 versus hypoxia alone.

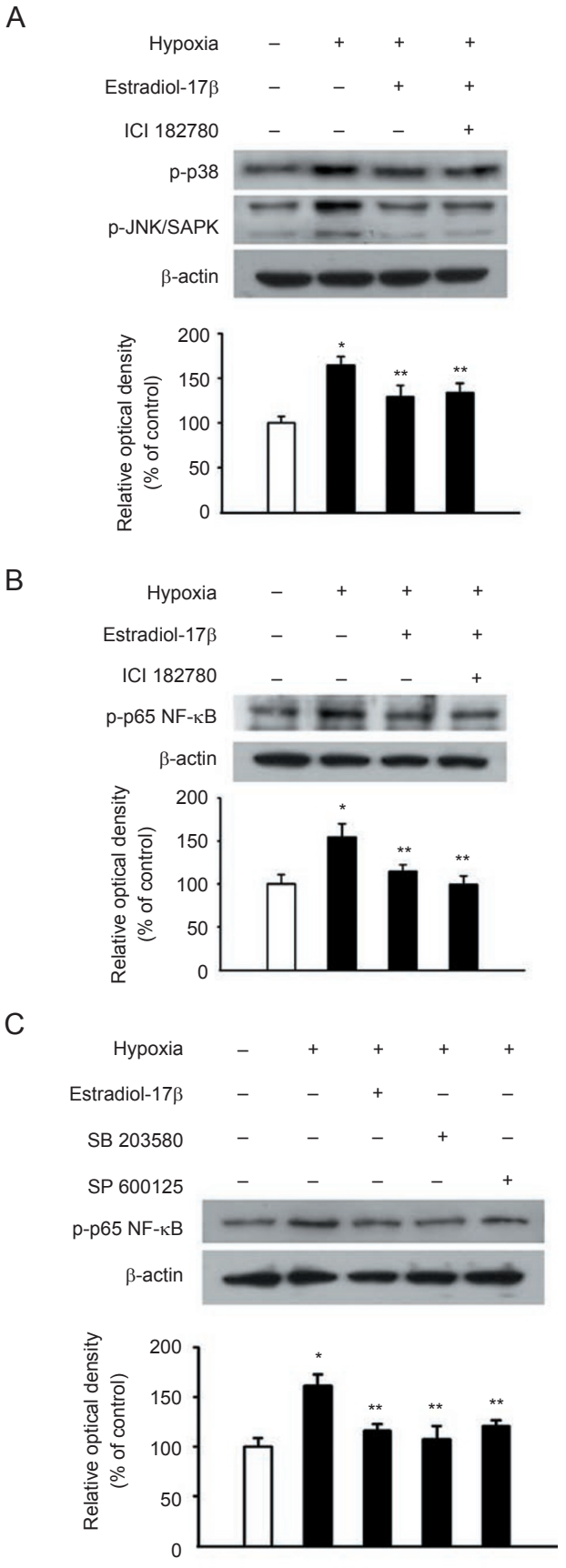

Figure 6 Effect of E_2 on the hypoxia-induced activation of MAPKs and NF-κB. **(A, B)** Chicken hepatocytes were pretreated with E_2 and E_2 with ICI 182780 for 30 min prior to hypoxic incubation, and the levels of p38 MAPK, JNK/SAPK and NF-κB were then measured. **(C)** Chicken hepatocytes were treated with E_2, SB 203580 and SP 600125 for 30 min prior to incubation under hypoxic conditions, and the levels of NF-κB were then assessed. The levels of MAPKs and NF-κB activation were determined by western blotting. The example shown is representative of three experiments. The values are expressed as the means ± SE from three independent experiments. **P* < 0.05 versus control, ***P* < 0.05 versus hypoxia alone.

tive effect against hypoxia-induced hepatocyte injury by increasing the level of Bcl-2 expression, as well as by scavenging intracellular ROS or by blocking the ROS-dependent activation of MAPKs and NF-κB. The physiological concentrations of serum estradiol are within a nanomolar range, and are dependent on sex and menstrual/menopausal status [26]. In this study, it was determined that E_2 protects against hypoxia-induced injury at low concentrations (10^{-9} M). On the basis of our previous results, however, we utilized 10^{-8} M E_2 to elucidate the action mechanism of E_2 in a more stable and reproducible situation [16]. In this study, DNA damage and a cell viability assay were used to support the finding that E_2 blocks the apoptotic cell death that is induced by hypoxia in chicken hepatocytes. The protection against hypoxic injury that is offered by E_2 may occur through activating the intracellular estrogen receptors and through regulating the transcription of the genes that encode for the molecules associated with the balance between apoptosis and cell survival [27]. The results described herein indicate that E_2 increases Bcl-2 levels and attenuates caspase-3 activity through the E_2 receptors under hypoxic conditions. This suggests that the Bcl-2 gene is positively regulated by E_2, and that Bcl-2 expression represents one of the most important ways in which E_2 protects hepatocytes against hypoxia. One of the mechanisms that has been proposed to explain this role of Bcl-2 is that Bcl-2 functions as an apparent antioxidant, which appears to be important due to the abundance of evidence suggesting that ROS perform an important function in the regulation of apoptosis. Indeed, Bcl-2 is an anti-apoptotic factor [28] that exerts its effects though heterodimerization with Bax. Bcl-2 is cleaved by caspase-3 and is converted to pro-apoptotic proteins that are similar to Bax. These events in turn lead to the activation of caspase-3, and ultimately result in cell death [29]. Despite the varying conditions under which cellular injury can occur, caspase-3 activation is a universal event, and is considered for this reason to be an apoptotic marker. The reduction in caspase-3 activity in the presence of E_2 also supports the notion that E_2 induces

the suppression of apoptosis. The results of this study also demonstrate that treatment with E_2 provides a significant amount of protection from the apoptosis-signaling cascade (including caspase) that is induced by hypoxia. Consistent with these results, it has been reported that estrogen attenuates the ischemia-induced activation of caspase-3 and reduces the number of TUNEL-positive cells and DNA fragmentation [30, 31]. Therefore, these results provide evidence for the close relationship that exists between the suppression of hepatocyte injury and the upregulation of Bcl-2. For these reasons, it has been suggested that E_2 may trigger the upregulation of Bcl-2, a physiological reaction that is relevant to the protection of cells against oxidative stress-induced hepatocyte injury.

In this study, it was shown that the levels of intracellular ROS are increased through hypoxic incubation, and are reduced by treatment with E_2, as was demonstrated through DCF fluorescence imaging using confocal microscopy. The results obtained using chicken hepatocytes are consistent with those from previous studies, which report that estrogens exert anti-oxidant effects in hepatocytes and rat hippocampal neurons [32, 33]. It was also previously reported that E_2 can function as a potent antioxidant and can inhibit lipid peroxidation [34, 35]. The antioxidant effects of estrogen may originate from its biochemical properties, rather than from its genomic action, because the protective effects of estrogens are not affected by the estrogen receptor antagonist. E_2 itself is a phenolic antioxidant that harbors an OH group in its molecular structure (an A ring), such as vitamin E [32]. This aspect was observed when the cells were treated with DES, but not with the membrane E_2 receptor agonist, E_2-BSA. According to the results of a recent study, direct antioxidant effects can be observed in the absence of functional estrogen receptors, as has been shown in a variety of neuronal cell lines (for example, HT22 clonal hippocampal cells) [36]. MAPKs, which respond to hypoxia [37], exert a primary effect in the mediation of the stress-induced expression of certain genes. These results demonstrate the rapid activation of p38 MAPK and JNK/SAPK through phosphorylation in hepatocytes that were incubated under hypoxic conditions. Hypoxia-induced hepatocyte injury was attenuated by E_2 or MAPK inhibitors, thereby suggesting that the p38 MAPK and JNK/SAPK signal pathways function in hypoxia-induced cellular injury. p38 MAPK and JNK/SAPK phosphorylation culminates in the activation of the downstream transcription factors, including NF-κB, which control a broad variety of intracellular signaling processes. These signal molecules perform an important function in the regulation of cellular responses to hypoxia [5, 38]. However, in this study, it was demonstrated that hypoxia-induced cell injury was attenuated partially by inhibition of p38 MAPK, JNK/SAPK and NF-κB. This raises the possibility of an antioxidant effect of E_2. Therefore, more study will be required to elucidate the molecular mechanisms that underlie the antioxidative functions of E_2. In conclusion, E_2 protects against hypoxia-induced cell injury through ER-mediated upregulation of Bcl-2 expression, as well as ER-independent reduction in ROS levels or the activity of p38 MAPK, JNK/SAPK and NF-κB in primary cultured chicken hepatocytes.

Materials and Methods

Materials

Two-week-old White Leghorn male chickens were obtained from the Dae Han Experimental Animal Co. Ltd. (Chungju, Korea). All animal management procedures were conducted in accordance with the standard operation protocols established by Seoul National University. The Institutional Review Board at Chonnam National University approved the research proposal, as well as all relevant experimental procedures, including animal care. The experimental samples were appropriately managed, and quality control protocols were applied to all laboratory facilities and equipment used in this study. The class IV collagenase and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY, USA). The fetal bovine serum was acquired from Biowhittaker (Walkersville, MD, USA). The 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate and acetyl ester (CM-H2DCFDA) were purchased from Eugene (Oregon, USA). The E_2, DES, E_2-BSA, hydrogen peroxide solution, SB 203580 and SP 600125 were acquired from Sigma Chemical Company (St Louis, MO, USA). The ICI 182780, BAY 11-7082 was purchased from NEN (Boston, MA). The phospho-p38 MAPK, phospho-SAPK/JNK, and phospho-NF-κB antibodies were obtained from New England Biolabs (Hertfordshire, UK). The HIF-1α, Bcl-2 and caspase-3 antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-rabbit IgG was obtained from Jackson Immunoresearch (West Grove, PA, USA). The liquiscint was purchased from National Diagnostics (Parsippany, NY, USA). All other reagents were of the highest purity commercially available.

Primary culture of chicken hepatocytes

The chicken liver cells were prepared and maintained in a monolayer culture, as described elsewhere [23]. In brief, the chicken hepatocytes were isolated by perfusing the liver with 0.05% collagenase from a chicken which had been starved for 3 h. Hepatocytes with better than 90% viability, as verified via a trypan blue exclusion test, were used for subsequent plating. The hepatocytes were plated (5.0×10^5 cells/60 mm collagen-coated dish) with an incubation medium (Basal Medium Eagle supplemented with essential amino acids) containing 75 U/ml of penicillin, 75 U/ml of streptomycin and 5% calf serum, and were incubated for 4 h at 37 °C in an atmosphere containing 5% CO_2. The medium was then replaced with fresh incubation medium, and the hepatocytes were incubated for an additional 20 h in order to achieve a monolayer culture.

Hypoxic stimulation

The chicken hepatocytes were cultured in 35 or 60 mm culture dishes and washed twice in phosphate-buffered saline (PBS). The medium was then exchanged with fresh William's medium without

supplements. The experiments were conducted in an incubator at 37 °C under either normoxic conditions by maintaining the cells in an atmosphere containing 92.3% air and 5.5% CO_2 or hypoxic conditions by incubating the cells for 30 min in a modular incubator chamber gassed with 2.2% O_2, 5.5% CO_2 and 92.3% N_2 (Billups-Rotheberg Inc, CA, USA) at a flow rate of 20 l/min. The chamber was purged with gas, sealed and placed in a conventional incubator at 37 °C.

[^{3}H]-thymidine incorporation

The [^{3}H]-thymidine incorporation experiment was conducted using the methodology established by Brett *et al*. [39]. In brief, the chicken hepatocytes were serum-starved for 24 h before being subjected to hypoxic conditions. Immediately before the beginning of the study, the medium was exchanged with Williams' medium without serum. The cells were incubated under normoxic or hypoxic conditions. The cells were then pulsed with 1 μCi of [^{3}H]-thymidine for 4 h at 37 °C under normoxic or hypoxic conditions. All values are expressed as the mean (± the SE) of triplicate experiments, and were converted from absolute counts to a percentage of the control.

DNA fragmentation assay

The semi-confluent chicken hepatocytes in 60 mm dishes were harvested after incubation in a hypoxia chamber. The cells were suspended in lysis buffer (10 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EDTA, pH 8.0, 0.5% Triton-X 100). The cell lysates were treated with 200 μg/ml of proteinase K for 6 h at 60 °C. The DNA was extracted with phenol/chloroform and precipitated with isopropyl alcohol. The DNA samples were digested at 37 °C for 1 h with 10 μg/ml of TE-RNAse and electrophoresed on 2% agarose gel. The gel was stained with ethidium bromide and then photographed under ultraviolet (UV) light.

Measurements of intracellular H_2O_2 levels

CM-H_2DCFDA (DCF-DA), which functions as a H_2O_2-sensitive fluorophore, was used to detect the intracellular H_2O_2. The cells were plated on gelatin-coated dishes and incubated under hypoxic conditions. The cells were then moved into the dark with 10 μM DCF-DA for 30 min at room temperature under normoxic or hypoxic conditions. The cells were imaged using laser confocal microscopy (400 ×; Fluoview 300, Olympus) with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The intracellular H_2O_2 level was quantified by treating the cells with DCF-DA, then rinsing them twice in ice-cold PBS and then scraping them. A 100 μl cell suspension was loaded into a 96-well plate and assessed using a luminometer (Victor3, Perkin-Elmer) and a fluorescent plate reader at an excitation and emission wavelength of 485 and 535 nm, respectively.

Trypan blue exclusion assay

The cells were grown in 35 mm dishes until they achieved confluence. The monolayers were then washed twice in PBS. The cells were detached from the culture dishes using a 0.05% trypsin/0.5 mmol/l EDTA solution, and the proteolytic activity was then inhibited using a soybean trypsin inhibitor (0.05 mg/ml). A 0.4% (w/v) trypan blue solution (500 μl) was subsequently added to the cell suspension. The cells were counted with a hemocytometer under an optical microscope while maintaining a separate count of the blue cells. The cells failing to exclude the dye were considered to be non-viable. The data were expressed as the percentage of viable cells.

Western blot analysis

The cell homogenates (30 μg of protein) were separated using 10% SDS-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride (PVDF) transfer membranes. The blots were then washed in tris-buffered solution containing Tween-20 (TBST, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20), blocked with 5% skim milk powder in TBST for 1 h, and incubated for 12 h with the appropriate primary antibody at the dilutions recommended by the supplier (1:1 000). The membranes were then washed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (1:10 000). The bands were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, England, UK) in accordance with the manufacturer's protocols.

Statistical analysis

The results are expressed as the means ± the standard error (SE). The difference between the two mean values was analysed using an ANOVA. A P value of < 0.05 was considered to be significant.

Acknowledgments

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