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Thirty-five percent oxygen pre-conditioning protects PC12 cells against death induced by hypoxia

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Abstract

The present study is designed to investigate the effect of pre-conditioning with 35% O_2 on PC12 cell death induced by hypoxia. This study investigated whether 35% O_2 pre-conditioning for 3 h, followed by 12 h recovery, can protect PC12 cells against death induced by subsequent exposure to hypoxia for 72 h. The result showed that pre-conditioning with 35% O_2 partly blocked the decrease in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction induced by hypoxia in PC12 cells. PC12 cells pre-conditioned with 35% O_2 could generate a small quantity of reactive oxygen species (ROS), which activated the extracellular signal-regulated kinase (ERK) signalling pathway, then the over-expression of the B-cell lymphoma/leukaemia-2 (Bcl-2) was induced, which subsequently protected PC12 cell against death resulting from hypoxia exposure. In conclusion, 35% O_2 pre-conditioning could protect PC12 cells against hypoxic insult.

Keywords: *ERK, ROS, PC12 cell, hypoxia, Bcl-2*

Abbreviations: *DMEM, Dulbecco's modified Eagle's medium; SiRNA, small interfering RNA; PYR, pyrogallol; CAT, catalase; H2O2, hydrogen peroxide; TEM, 4-hydroxyl-tempol; PD, PD98059; DCFH-DA, dichlorofluorescein diacetate; HEt, dihydroethidium; H7, 1-5-isoquinolinesulphonyl-2-methypoperazine; DMSO, dimethyl sulphoxide; WO, wortmannin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide; DCF, dichlorofluorescein; ROS, reactive oxygen species; IPC, ischemic pre-conditioning; PC12, pheochromocytoma; PMSF, phenylmethylsulphonylfluoride; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PKA, protein kinase A; PI3K, phosphoinositide-3-kinase; EGTA, ethylene glycol tetraacetic acid; CREB, cyclic AMP-responsive element binding protein; Bcl-2, B-cell lymphoma/leukaemia-2; NF-B, nuclear factor B; DEPC, diethyl-pyrocarbonate; AMV-RT, avian myeloblastosis virus reverse transcriptase.*

Introduction

High concentration oxygen is often used for treating patients with respiratory failure. However, high concentration oxygen may exacerbate the development of acute lung injury by excessive production of reactive oxygen species (ROS) [1–3]. Reactive oxygen species, the collective term for superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydrol radical ($^{\cdot}OH$)

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and others, have been traditionally regarded as cytotoxic, with the potential to cause damage to lipids, protein and DNA [4], and they are related to ageing, atherosclerosis and cancer in large quantities [4–6]. In contrast to this harmful condition, considerable evidence show that various stimuli including growth factors, hormones, casoactive factors, metal ions, etc. can stimulate the enzymatic generation of lower levels of ROS, acting as second messengers in response to these factors [6–9].

Previous studies have indicated that ischemic preconditioning (IPC) can protect tissues, such as brain, heart, etc. against injury [10,11]. IPC has emerged as a universal mechanism of cytoprotection in the living system. Now, the concept has been extended to pre-conditioning triggered by non-ischemic stress, such as generation of reactive oxygen radicals [12,13]. It has been demonstrated that preconditioning with oxidative stress may provide cytoprotection against injury [11–15].

Extracellular signal-regulated kinase (ERK), a prototype member of the mitogen-activated protein kinase (MAPK) family, is up-regulated by reactive oxygen species production in response to numerous stimuli [16,17]. The MAPK/ERK signal transduction pathway is important in protecting cells against various factors inducing cytotoxicity [18–20]. Moreover, ERK activation promotes Bcl-2 gene expression through a variety of transcription factors activation [21,22]. The Bcl-2 protein family has an important role in the regulation of programmed cell death [23,24]. It has been implicated that the over-expression of Bcl-2 plays a key role in cytoprotection of H_2O_2 pre-conditioning against apoptosis induced by H_2O_2 in PC12 cells [25].

This article is designed to investigate whether 35% oxygen pre-conditioning for 3 h can protect PC12 cells against the death induced by hypoxia (1% O_2) and whether 35% oxygen induced adaptive cytoprotection is related to the generation of ROS, ERK signal-transduction pathways and the over-expression of Bcl-2.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin liquid, poly-L-lysine, lipofectamine 2000 and small interfering RNA (SiRNA) were obtained from Invitrogen (Carlsbad, CA). Horse serum and foetal bovine serum were obtained from Hyclone (Rockville, MD). Pyrogallol (PYR), catalase (CAT), hydrogen peroxide (H_2O_2), 4-hydroxyl-tempol (TEM), PD98059 (PD), wortmannin (WO), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), dihydroethidium (HEt), 1-5-isoquinolinesulphonyl-2-methypoperazine (H7), anti-Bcl-2, anti-ERK1/2, anti-phospho ERK1/2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma (St. Louis, MO). GeneAmp RNA-PCR kit was from Promega (Promega, Madison, WI).

Cell culture

PC12 cells, a rat cell line derived from pheochromoytoma cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal bovine serum, 10% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2 and 95% air. PC12 cells were pre-conditioned with exposure to 35% O_2 for 180 min followed by 12 h recovery and subsequent exposure to hypoxia (1% O_2) for 72 h. Prior to exposing to 35% O_2 and 1% O_2, the medium was replaced with serum-free medium. Cells were pre-incubated with related drugs for 60 min before exposing to 35% O_2.

MTT assay

Cells grown in 150 mm plates were washed twice with phosphate-buffered saline (PBS) and resuspended in DMEM. Viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide) method. Briefly, cells were plated on poly-L-lysine pre-coated 96-well plates and grown in complete medium until they reached 30–50% confluence (usually 12–24 h). Then medium was replaced with serum-free medium and cells were placed under normoxic (21% O_2) or hyperoxic condition (35% O_2) for 3 h, followed by 0 h, 12 h or 24 h recovery and subsequent exposure to hypoxia (1% O_2) for 72 h. After treatments, MTT diluted in PBS was added to the culture medium to a final concentration of 1.5 mg/ml and all samples were incubated at 37°C for 4 h under normoxic conditions. Finally, medium was removed and precipitated MTT salts were solubilized in DMSO (100 μl). Product formation was monitored by reading absorbance at 550 nm on the Microplate Reader (model 550, Bio-Rad Laboratories).

Detection of ROS

Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (HEt). DCFH-DA is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant (H_2O_2). Superoxide anion is able to oxidize HEt to yield ethidium and then the fluorescence intensity was analysed by flow cytometry. PC12 cells were plated in 6-well plates and grown in complete medium until they reached

~80% confluence. Thereafter, culture medium was replaced with serum-free medium and cells were pre-conditioned with 3 h exposure to 35% O_2 and 21% O_2, respectively. Following treatment, cells were washed with cold PBS, suspended in PBS at 5×0^5 cells/ml and incubated with 5 μM DCFH-DA and 10 μM HEt (40 min, 37°C in darkness). The relative fluorescence intensity was monitored using flow cytometry (Becton-Dickinson, USA).

Transfection and infection

PC12 cells were plated in 6-well plates and grown in complete medium until they reached 30–50% confluence. Thereafter, culture medium was replaced with serum-free medium without antibiotics. Twenty-four hours later, PC12 cells were transfected using lipofectamine 2000, as recommended by manufacturer's instructions. Briefly, 5 μl SiRNA and 10 μl of lipofectamine 2000 were incubated in 50 μl optima-medium, respectively, at room temperature for 5 min. Then the diluted SiRNA and lipofectamine were incubated for 20 min for complex formation. The complexes were added to wells. One day after infection, the cells were treated with the indicted design.

RT-PCR analysis

Total cellular RNA was extracted from 1×10^6 PC12 cells with Trizol (Promega, Madison, WI) according to the manufacturer's protocol. The RNA pellet was washed in 70% cold ethanol, air-dried and redissolved in 20 μl of diethyl-pyrocarbonate (DEPC)-treated water. The reverse transcription reaction was performed in a final volume of 30 μl reaction mixture including 1.5 mM $MgSO_4$, 1 μM each primer (sense and antisense), 250 μM dNTP, 3 U avian myeloblastosis virus reverse transcriptase (AMV-RT), 3 U Tfl DNA polymerase and 2 μg of total RNA. The reverse transcription was allowed to proceed at 45°C for 45 min, then AMV-RT was inactivated in 94°C for 2 min.

PCR was performed in a DNA thermal cycler (Hybaid, Middlesex, UK) for 40 cycles in the following conditions: 30 s at 94°C for melting, 45 s at 60°C for annealing, 2 min at 68°C for extension and 7 min at 68°C for final extension. The following primers synthesized by Promega (Promega, Madison, WI) were used: ERK 5′-TTTTGGACCTGGTCT GCTCT-3′and 5′- TCATAGCCCTTCCATTCCA G-3′; β-actin 5′-AAGTACCCCATTGAACACGG-3′and 5′- AACACAGCCTGGATGGCTAC-3′. The PCR products were electrophoresed on 1.5% agarose and visualized by ethidium bromide stain and UV illumination. The band intensities were analysed on a digital imaging system (UVI Tech, Cambridge, UK).

Western Blot analysis

Cells were plated in 6-well plates and grown in complete medium until they reached ~80% confluence. Thereafter, culture medium was replaced with serum-free medium and cells were pre-conditioned with 3 h exposure to 35% O_2 and 21% O_2 followed by 0, 12, 24 h recovery, respectively, and subsequent exposure to hypoxia (1% O_2) for 72 h. After treatment, PC12 cells were rinsed with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM Hepes, 1 mM dithiothreitol, 20 mM EGTA, 10% glycerol, 50 mM β-glycerophosphate, 10 mM NaF, 1% tritonX-100, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM Na_3VO_4, 2 μM aprotinin, 100 μM leupeptin, 2 μM pepstatin). Cell debris was removed by centrifugation at $14\ 000 \times g$ for 10 min. The supernatant was utilized for the western blot analysis. Protein concentration was determined using the BIO-Rad Protein Assay Dye Reagent. Equal amounts of sample (30 μg) were seperated by SDS-polyacrylamide gel electrophoresis using 12.5% polyacrylamide gels. The resolved proteins were then electroblotted onto immobilon polyvinylidene difluoride membranes (milipore, Bedford, MA). Membranes were blocked with 5% bovine serum albumin and incubated with the indicted primary antibody (anti-ERK1/2, anti-phospho ERK1/2, anti-Bcl-2) for 1 h at room temperature. After being washed and incubated for 1 h with the second antibody, respectively, blots were then washed and immunoreactive bands were detected by enhanced chemiluminescence and recorded using Kodak XAR-5 film and then analysed by using Multi Gauge software version 3.0 (Fujifilm Science Systems, Tokyo, Japan).

Statistical analysis

Data were presented as mean ± SD. Statistical analysis of the data was performed by one-way analyses of variance (ANOVA). A p-value less than 0.05 was considered statistically significant.

Results

Effects of pre-conditioning with 35% O_2 on hypoxia-induced PC12 cells

To explore the possible cytoprotective effects of pre-conditioning by different oxygen concentration prior to exposing to hypoxia (1%), oxygen pre-conditioning was, respectively, performed in PC12 cells cultures by an incubation with different concentrations (75%, 50%, 35%) of O_2 for 3 h followed by 12 h of recovery as described above. As is shown in Figure 1, hypoxia (1%) could cause considerable PC12 cells death. However, the effect was inhibited by pre-conditioning with 35% O_2 for 3 h followed by 12 h recovery ($p < 0.01$) and no effect was found by 0 h or 24 h recovery, while pre-conditioning by 50% and 75% O_2

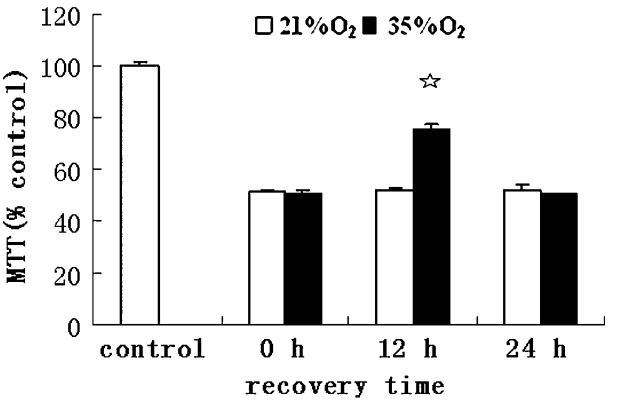

Figure 1. Preconditioning with 35% O_2 protected PC12 cells against death induced by hypoxia. After the medium was replaced with serum free medium, PC12 cells were placed under normoxic (21% O_2) or hyperoxic condition (35% O_2) for 3 h, followed by different time recovery and subsequent exposure to hypoxia (1% O_2) for 72 h. The data are shown as mean ± SD. from at least three independent experiments, each of which performed in duplicate or triplicate. * $p < 0.01$ vs 21% O_2 group.

just showed the opposite results in MTT assay (data not show). The above indicted that 35% O_2 pre-conditioning for 3 h followed by 12 h recovery could protect PC12 cells against death induced by hypoxia, so PC12 cell pre-conditioned with 35% O_2 for 3 h followed by 12 h recovery then exposed to 72 h hypoxia was selected as the model of this experiment.

ROS production

The mean fluorescence intensity of dichlorofluorescein and ethidium were used to assess the production of ROS induced by 35% O_2 pre-conditioning by flow cytometric analysis [26–28].

To identify the production and role of ROS in the 35% O_2 pre-conditioning, 20 μM pyrogallol, a donor to release $O_2^{\cdot-}$ and 30 μM H_2O_2 were added to the medium for 1 h before cells were pre-conditioned with 21% O_2 [26–28]. In contrast, 300 u/ml catalase, a scavenger of H_2O_2, and 10 mM tempol, a scavenger

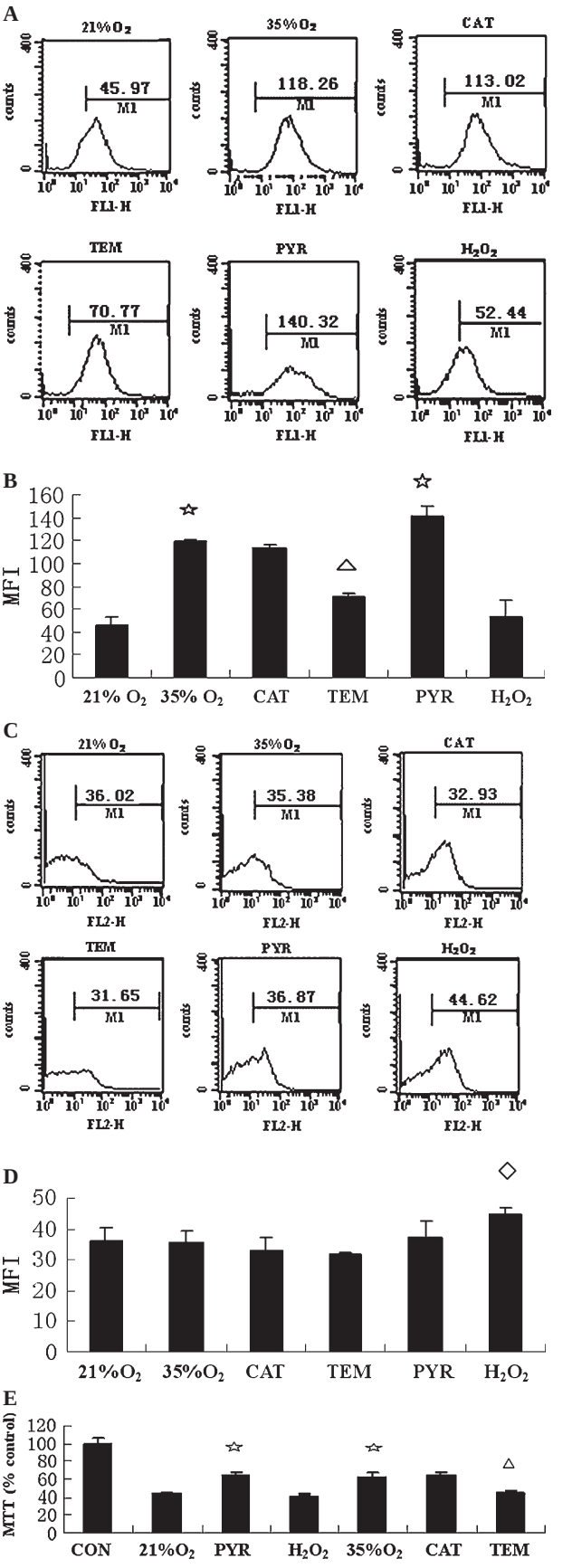

Figure 2. Super oxygen anion produced by 35% O_2 pre-conditioning protected PC12 cells against hypoxia-induced death. ROS were detected at the end of 3 h pre-conditioning. MTT was examined at the end of 72 h hypoxia exposure. (A and B) Production of $O_2^{\cdot-}$. (C and D) Production of H_2O_2. (E) Super oxygen anion produced by 35% O_2 pre-conditioning protected PC12 cells against hypoxia-induced cytotoxicity. MFI: Mean fluorescence intensity. CAT: 1 h pre-treatment with 300 u/ml catalase before pre-conditioning with 35% O_2. TEM: 1 h pre-treatment with 10 mM 4-hydroxyl-tempol before pre-conditioning with 35% O_2. PYR: 1 h pre-treatment with 20 μM pyrogallol before pre-conditioning with 21% O_2. H_2O_2: 1 h pre-treatment with 30 μM H_2O_2 before pre-conditioning with 21% O_2. The data were shown as mean ± SD, from at least three independent experiments, each of which performed in duplicate or triplicate. * $p < 0.01$ vs 21% O_2 group, $^{\Delta}$ $p < 0.05$ vs 35% O_2 group, $^{\diamond}$ $p < 0.05$ vs 21% O_2 group.

of $O_2^{\cdot-}$, were added to the medium for 1 h before cells were pre-conditioned with 35% O_2 [26–28].

4-hydroxyl-tempol at a concentration of 10 mM was routinely used to clear $O_2^{\cdot-}$ in the medium [26–28]. Catalase, a scavenger of H_2O_2, may clear H_2O_2 at a concentration of 300 u/ml [29–32]. According to our preliminary experiment, pyrogallol at a concentration of 20 μM was routinely used to realease $O_2^{\cdot-}$ and H_2O_2 at a concentration of 30 μM was used to provide H_2O_2 in the medium but did not cause obvious damage to PC12 cell (data not show).

PC12 cells in 35% O_2 and pyrogallol group produced more $O_2^{\cdot-}$ than that in the 21% O_2 group and the $O_2^{\cdot-}$ in tempol group decreased more significantly than that in the 35% O_2 group (Figure 2A and B). The production of H_2O_2 by 35% O_2 pre-conditioning did not increased significantly compared with that of 21% O_2 pre-conditioning (Figure 2 C and D).

The cell viability increased significantly in the pyrogallol and 35% O_2 group ($p < 0.05$) and reduced significantly in the tempol group (Figure 2E). From the above, it could be concluded that PC12 cells preconditioned with 35% O_2 could generate $O_2^{\cdot-}$, which played a vital role in protecting PC12 cell against cytotoxicity induced by hypoxia.

ERK expression

To determine which signalling pathway was involved in the 35% O_2 pre-treatment, the effect of three signalling pathway inhibitors, PD98059, 1–5-isoquinolinesulphonyl-2-methypoperazine (H7) and wortmannin, were tested. PD98059, H7 and wortmannin were widely used as inhibitors of mitogen-activated protein kinase (MAPK) pathway, protein kinase A/C (PKA/PKC) pathway and phosphoinositide-3-kinase (PI3k/Akt) pathway, the effective concentrations for inhibiting these pathway in cultured cells were 50 μM, 100 μM and 0.25 μM, respectively [33–39].

The results showed that 35% O_2 pre-treatment significantly enhanced the cell viability and this effect was obviously attenuated by PD98059, not by H7 and wortmannin (Figure 3A).

In order to explore whether 35% O_2 pre-conditioning modulated the expression of activated ERK1/2 or not in PC12 cells, the levels of expression of total and phosphorylated ERK1/2 were measured by Western blot analysis. After PC12 cells pre-conditioned with 35% O_2 for 3 h, recovered for 12 h and exposed to hypoxia for 72 h, the levels of activated ERK1/2 expression obviously increased compared with that of 21% O_2 pre-conditioning and this effect was obviously attenuated by PD98059, not by H7 and wortmannin. However, PC12 cells pre-conditioned with 35% O_2 for 3 h and recovered for 0 or 24 h did not remarkably enhance the levels of activated ERK1/2 expression (Figure 3B and C).

The activated ERK1/2 over-expression induced by 35% O_2 was obviously reduced by treatment with 10 mM 4-hydroxyl-tempol (a scavenger of $O_2^{\cdot-}$). Pre-conditioning with 21% O_2 and 20 μM pyrogallol (a donor to release $O_2^{\cdot-}$), could also induce the over-expression of phosphorylated ERK1/2. Contrastively, pre-treatment with 35% O_2 and 300 u/ml catalase (a scavenger of H_2O_2) or pre-conditioning with 21% O_2 and 30 μM H_2O_2 did not change the ERK1/2 expression obviously compared with that of 35% O_2 or 21% O_2 pre-conditioning, respectively (Figure 3C).

The above results (Figure 3C) indicted that MAPK signal transduction pathway was involved in the cytoprotective effect of 35% O_2 pre-conditioning and it was $O_2^{\cdot-}$ not H_2O_2 resulting from 35% O_2 pre-conditioning that played an important role during this process.

Effects of ERK on hypoxia-induced PC12 cells proved by ERK SiRNA

To identify the role of the ERK1/2 in the 35% O_2 pre-conditioning, silencing of gene expression by small interfering RNAs (SiRNAs) was performed. RT-PCR analysis showed that ERK mRNA expression was remarkably inhibited by SiRNA. The intensity of ERK mRNA in PC12 cells transfected with SiRNA (SP group) was significant lower than that of normal PC12 cells (NP group) (Figure 4A). Western blot analysis showed that the expression of total and activated ERK1/2 protein in SP group was strongly suppressed; the intensity of total and activated ERK1/2 were lower than that of NP group ($p < 0.01$); and there were no difference in the expression of total and phosphorylated ERK1/2 protein between the 21% O_2 and 35% O_2 group in PC12 cells transfected with the SiRNA. However, in normal PC12 cells, the amount of activated ERK1/2 protein expressed in the 35% O_2 group was obviously higher than that of 21% O_2 group (Figure 4B).

ERK SiRNA reduced PC12 cell viability. The viability of PC12 cells transfected with ERK SiRNA was remarkably more reduced than that of normal PC12 cells ($p < 0.05$) and there was no significant reduction of viability between the 21% O_2 and 35% O_2 group in the SiRNA transfected PC12 cells. However, in normal PC12 cells, the viability in the 21% O_2 group was obviously more reduced than that of 35% O_2 group (Figure 4C).

The production of ROS in the SP group was just like that of in the NP group, PC12 cells exposed to 35% O_2 produced more $O_2^{\cdot-}$ rather than H_2O_2 and the production of H_2O_2 by 35% O_2 pre-conditioning did not increase significantly compared with control (21% O_2 pre-conditioning) and there were no difference in the production of $O_2^{\cdot-}$ and H_2O_2 between the SP group and NP group (Figure 4 D–G).

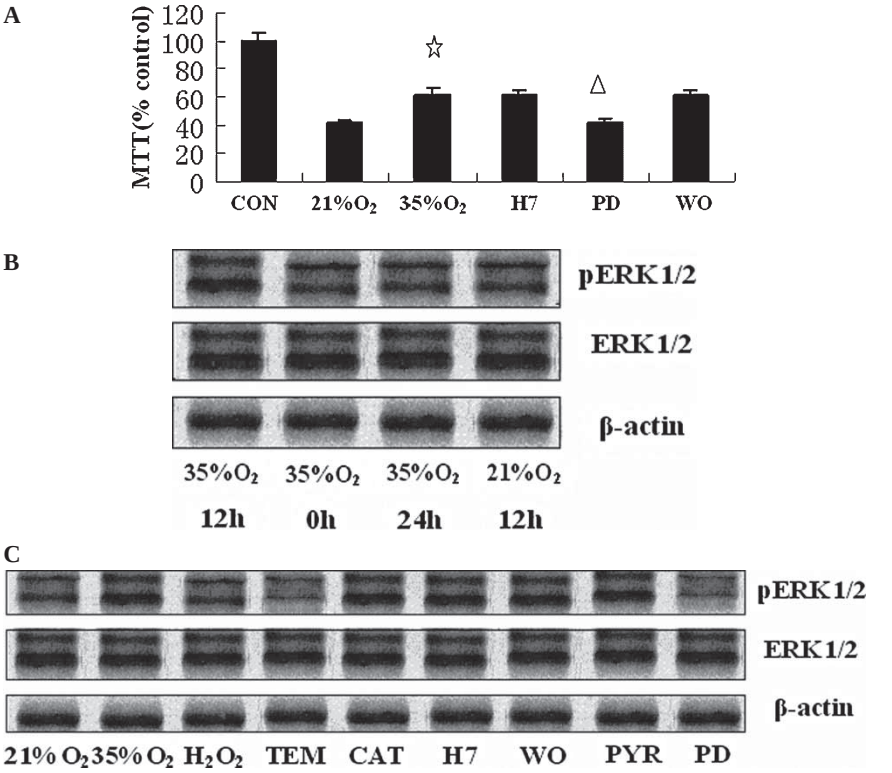

Figure 3. MAPK signal transduction pathway was involved in the cytoprotective process of 35% O_2 pre-conditioning. The levels of total ERK1/2 and pERK1/2 was determined by western blot at the end of 72 h hypoxia exposure after 3 h pre-conditioning and 12 h recovery. (A) Effect of different signal transduction pathway inhibitors on the cell viability. (B) Expression of phosphorylated ERK1/2 induced by 35% O_2 pre-conditioning. (C) Effect of ROS and different signalling pathway inhibitors on the expression of ERK1/2. CAT: 1 h pre-treatment with 300 u/ml catalase before pre-conditioning with 35% O_2. TEM: 1 h pre-treatment with 10 mM 4-hydroxyl-tempol before pre-conditioning with 35% O_2. PYR: 1 h pre-treatment with 20 μM pyrogallol before pre-conditioning with 21% O_2. H_2O_2: 1 h pre-treatment with 30 μM H_2O_2 before pre-conditioning with 21% O_2. H7: 1 h pre-treatment with 100 μM 1-5-isoquinolinesulphonyl-2-methypoperazine (H7) before pre-conditioning with 35% O_2. PD: 1 h pre-treatment with 50 μM PD98059 before pre-conditioning with 35% O_2. WO: 1 h pre-treatment with 0.25 μM wortmannin before pre-conditioning with 35% O_2. The data shown are mean ± SD. from at least three independent experiments, each of which performed in duplicate or triplicate. Photographs shown are representative of at least three experiment. * $p <$ 0.05 vs 21% O_2 group, $^{\Delta}$ $p < 0.05$ vs 35% O_2 group.

In summary, we could conclude that ERK1/2 protein played an important role in the 35% O_2 pre-conditioning and the ERK signalling pathway was involved in the protective effect of 35% O_2 pre-conditioning.

Bcl-2 expression

In order to explore whether the over-expression of Bcl-2 was involved in the 35% O_2 pre-conditioning in PC12 cells, the levels of Bcl-2 expression were measured by Western blot analysis. The levels of Bcl-2 and phosphorylated ERK expression increased obviously in the 35% O_2 group compared with that in other groups and decreased significantly in the PD group and SiRNA group compared with that in the 21% O_2 or 35% O_2 group ($p < 0.01$). The expression of total ERK did not change significantly among the 21% O_2, 35% O_2 and PD groups but decreased more significantly in the SiRNA group than in any of the other groups ($p < 0.01$).

Figure 5 indicted that ERK signal pathway was necessary to the over-expression of the Bcl-2 protein in PC12 cells pre-conditioned with 35%O_2 and Bcl-2 played an important role in this process.

Discussion

Hyperoxia-inducible $O_2^{\cdot-}$ generation has been associated with increased mitochondrial production and a role for NAD(P)H oxidases. NAD(P)H oxidases catalyse the univalent reduction of molecular oxygen to $O_2^{\cdot-}$ at the expense of NADPH/NADH as the electron donor [2,40].

Our result indicated that high concentration of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), caused by 75% O_2 or 50% O_2 pre-conditioning, showed a cytotoxic effect (data not show). Excessive production of ROS can cause damage to lipids, protein and DNA, mediate cell death in lung epithelium [41] and are associated with ageing, atherosclerosis and cancer [4–6]. In contrast

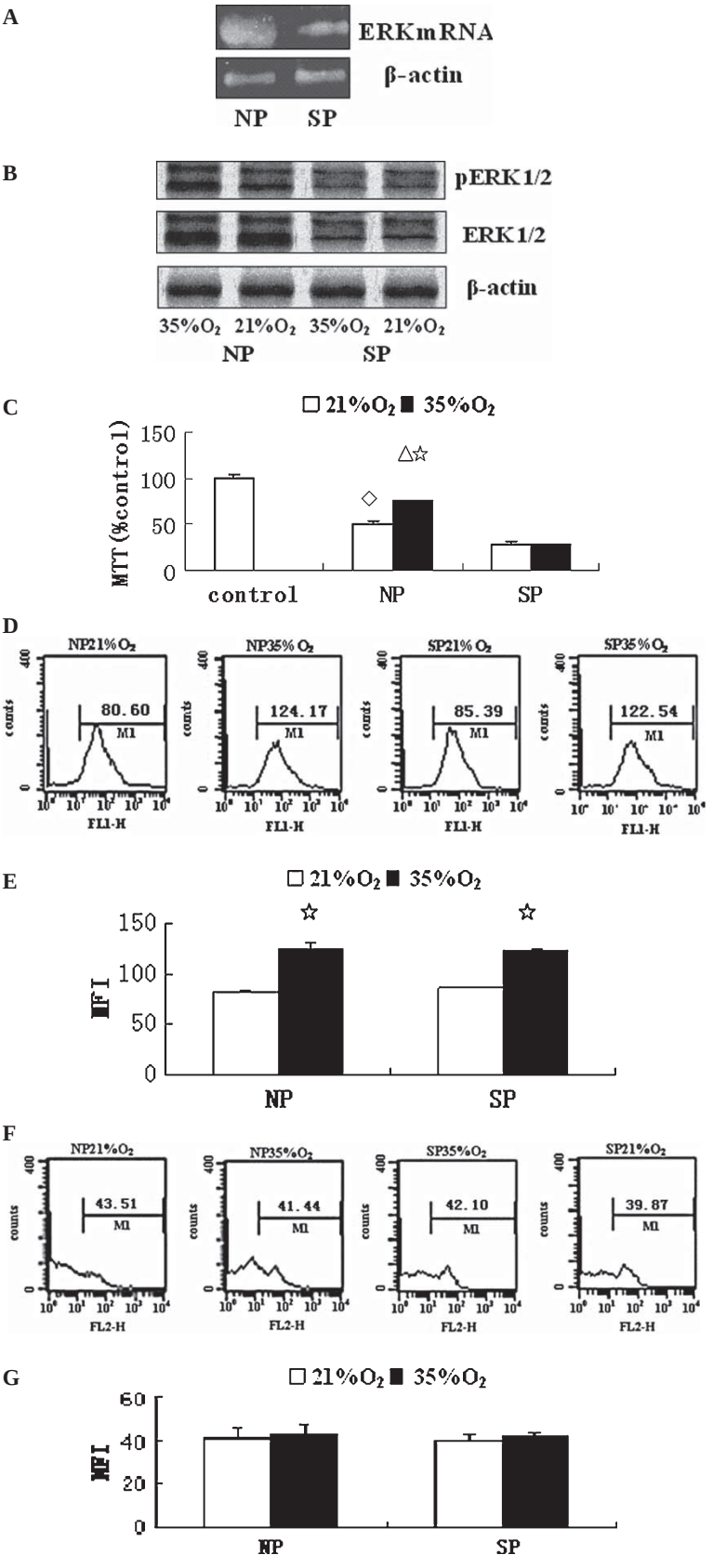

Figure 4 (*Continued*)

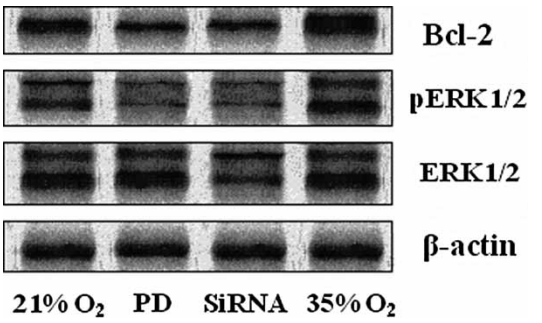

Figure 5. The Bcl-2 and ERK1/2 expression in PC12 cell pre-conditioned with 35% O_2. PD: 1 h pre-treatment with 50 μM PD98059 before pre-conditioning with 35% O_2. SiRNA: Transfection with the ERK SiRNA before pre-conditioning with 35% O_2. The levels of Bcl-2, phosphorylated ERK and total ERK were determined by western blot at the end of 72 h hypoxia exposure after 3 h 35% O_2 pre-conditioning and 12 h recovery. Photographs shown are representative of at least three experiments.

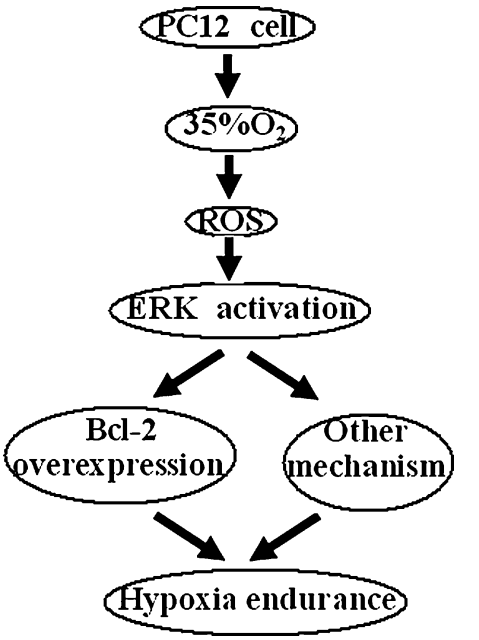

Figure 6. A model of the protective role of 35% O_2 pre-conditioning. PC12 cells pre-conditioned with 35% O_2 for 3 h could generate lower levels of ROS, which acted as the second messenger to activate the ERK signalling transduction pathway, through which the over-expression of Bcl-2 was induced and Bcl-2 played a vital role in protecting PC12 cells against the death induced by subsequently 72 h hypoxic exposure.

to this harmful condition, lower levels of ROS may modulate the redox condition of the cell and act as second messengers in response to the outside stimulation including growth factors, hormones, casoactive factors, metal ions, etc. [6–9]. PC12 cells pre-conditioned with 35% O_2 could generate a small quantity of ROS, which was essential to protect PC12 cells against death induced by hypoxia. This study just accords with the reports that oxidative pre-conditioning can protect cells against apoptosis induced by H_2O_2 [25,42].

The study demonstrated that PC12 cells exposed to 35% O_2 produced more $O_2^{\cdot-}$ than H_2O_2 and the superoxide anion appeared to be a key trigger for ERK1/2 activation in 35% O_2 induced cell protection. Pyrogallol, a donor to realease $O_2^{\cdot-}$, could cause the same result of 35% O_2 pre-conditioning and the pre-treatment with tempol, a scavenger of $O_2^{\cdot-}$, virtually inhibited both generation of ROS and ERK1/2 activation. Inhibiting ERK1/2 activation with PD98059 or suppressing the expression of ERK1/2 protein by small interfering RNAs (SiRNAs) did not significantly change the production of ROS. This indicated that ROS function upstream of ERK signalling pathway in the induction of the cell protection.

Reactive oxygen species are important components of normal cell signalling. A small amount of ROS carry out redox signalling and mediate specific signalling pathways by modulating gene expression through regulation of several transcription factors, such as activator protein 1 (AP-1) and nuclear factor B (NF-B) [16]. ROS-dependent redox cycling is critical for the establishment of the protein–protein interactions that regulate the activity of important proteins in signal transduction including the extracellular signal-regulated kinase. ERK can be phosphorylated and activated in response to oxidant-induced alterations of the redox state [16,17].

Thirty-five percent oxygen pre-conditioning enhanced the expression of phosphorylated ERK1/2 in PC12 cells. PD98059, a specific inhibitor of the MAPK pathway, suppressed the 35% O_2 pre-conditioning induced rescue of cell viability of hypoxic PC12 cells. This protective effect was absent in PC12 cells treated with ERK SiRNA. The results indicated that ERK1/2 lied in a signal transduction pathway that mediated the protective effect of 35% O_2 pre-conditioning and ERK1/2 was crucial for the prevention of hypoxia-induced death in PC12 cells.

Figure 4. The ERK1/2 mRNA expression, ERK1/2 protein expression, viability and ROS production of PC12 cells induced by ERK SiRNA. (A) Inhibitory effect of the expression of ERK1/2 mRNA by SiRNA. (B) Inhibitory effect of the expression of ERK1/2 protein by SiRNA. (C) ERK SiRNA reduced PC12 cell viability. (D and E) The production of $O_2^{\cdot-}$ in the SP and NP group. (F and G) The production of H_2O_2 in the SP and NP group. SP: PC12 cells transfected with SiRNA. NP: Normal PC12 cells. MFI: Mean fluorescence intensity. The data were shown as mean ± SD, from at least three independent experiments, with each performed in duplicate or triplicate. Photographs shown are representative of at least three experiments. * $p < 0.05$ vs 21% O_2 group, $^{\Delta}$ $p < 0.05$ vs SP group, $^{\diamond}$ $p < 0.05$ vs SP group.

In line with these findings, others have shown that MAPK plays an important role in protecting cells against various factors inducing cytotoxicity [18–20]. In cultured skin cells, MAPK/ERK signal transduction pathway is associated with cellular proliferation, differentiation and cytoprotection [43]. Previously others have suggested that ERK are potent anti-apoptotic kinases [44,45]. ERK activation could suppress apoptotic processes through the regulation of anti-apoptotic genes or existing proteins through phosphorylation [46]. After activation, ERK translocates into the nucleus where it activates a variety of transcription factors including transcription factor NF-κB and cyclic AMP-responsive element binding protein (CREB), which in turn promotes Bcl-2 gene expression [21,22].

The results showed that the levels of Bcl-2 expression obviously increased in the PC12 cells pre-treated with 35% O_2 compared with control, while the Bcl-2 values decreased significantly in the PD group and the ERK SiRNA group compared with the 35% O_2 group, which indicated that ERK was upstream of Bcl-2 in the 35% O_2 pre-conditioning. The reduced ERK expression resulting by PD and SiRNA could induce the less expression of Bcl-2.

The Bcl-2 protein family has an important role in the regulation of programmed cell death induced by diverse death stimuli. Members of this family can either promote or inhibit apoptosis [22–25,42,47]. Numerous studies have shown that the Bcl-2 protein can regulate apoptosis by blocking the mitochondrial permeability transition pore (PTP) opening; Bcl-2 modulates the release of mitochondrial apoptogenic factors like cytochrome *c* or apoptosis-inducing factor that activate caspases and other proteases; and Bcl-2 acts as an anti-oxidant that can exert a buffering effect on the production of mitochondrial reactive oxygen species [22,23,25,42,47].

In conclusion, PC12 cells treated with 35% O_2 preconditioning could generate a small quantity of ROS, which acted as the second messenger to activate the ERK signalling transduction pathway. The ERK pathway was essential to the over-expresssion of Bcl-2, which played a vital role in protecting PC12 cells against the death induced by hypoxia (Figure 6). Further study is required to completely elucidate the mechanism of the protective role of 35% O_2 preconditioning in PC12 cell.

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