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IGF-1 prevents oxidative stress induced-apoptosis in induced pluripotent stem cells which is mediated by microRNA-1

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ABSTRACT

Oxidative stress contributes to tissue injury and cell death during the development of various diseases. The present study aims at investigating whether oxidative stress triggered by the exposure to hydrogen peroxide (H_2O_2) can induce apoptosis of induced pluripotent stem cells (iPS cells) in a mechanism mediated by insulin-like growth factor (IGF-1) and microRNA-1 (miR-1). iPS cells treated with H_2O_2 showed increases in miR-1 expression, mitochondria dysfunction, cytochrome-c release and apoptosis, Addition of IGF-1 into the iPS cell cultures reduced the H_2O_2 cytotoxicity. Prediction algorithms showed that 3′-untranslated regions of IGF-1 gene as a target of miR-1. Moreover, miR-1 mimic, but not miR-1 mimic negative control, diminished the protective effect of IGF-1 on H_2O_2-induced mitochondrial dysfunction, cytochrome-c release and apoptosis in iPS cells. In conclusion, IGF-1 inhibits H_2O_2-induced mitochondrial dysfunction, cytochrome-c release and apoptosis. IGF-1′s effect is, at least partially, regulated by miR-1 in iPS cells.

1. Introduction

Recent success in reprogramming somatic cells into induced pluripotent stem cells (iPS cells) with a cluster of nuclear transcription factors, such as Oct4, Sox2, Klf4, and c-Myc, opens up a new era in regenerative medicine [1,2]. However, the generation and application of iPS cells have several obstacles, including the difficulty of iPS cell long-term survival in the microenvironment with heightened oxidative stress and inflammation [3]. Although in primary cultures, iPS cells have low levels of reactive oxygen species (ROS), increased ROS levels occur during passage that causes iPS cell damage [4]. In addition, transplanted iPS cells may be challenged for their survival in disease conditions, as they may be stressed out by high levels of ROS, which are considered a key factor to trigger cell damage and apoptosis.

Mitochondria play a key role in regulation of apoptosis under a variety of pathological conditions, including oxidative stress [5–7]. The mitochondrial function is highly sensitive to apoptotic stimulation. In mammalian cells, mitochondrial oxidation can be monitored by staining with a fluorochome, such as Deep Red, which fluoresces in direct proportion to mitochondrial function [8]. Decreased mitochondrial function occurs in cells undergoing apoptosis induced by oxidative stress agents, such as hydrogen peroxide (H_2O_2) [9]. Injured mitochondria can release cytochrome-c into the cytoplasm when cells are treated with proapoptotic stimuli [10,11]. On entry into the cytosol, cytochrome-c activates apoptosome that contains the caspase-activating protein Apaf-1, and subsequently the caspase cascade [12]. It has been shown that H_2O_2 exposure can induce apoptosis of endothelial cells [13]. It is interesting to test the sensitivity of iPS cells to H_2O_2-induced apoptosis, and the underlying mechanisms.

Insulin-like growth factor (IGF-1) is an anti-apoptotic factor that regulates proliferation and survival of multiple cell types, and its anti-apoptotic effects occur through regulation of the mitochondrial cytochrome-c/caspase pathway [14–16]. IGF-1 expression is controlled by certain miRNAs, a endogenous 22 nucleotide non-coding RNA that anneals to partial complementary sequences in the 3′UTRs of target mRNAs of protein-coding genes leading to specify translational repression and mRNA cleavage [17–19]. miRNAs are novel biomarkers, modulators and therapeutic targets for disease [20–22]. Among the known miRNAs, miR-1 is expressed in embryonic stem cells [23], and induces apoptosis in cancer cells [24]. We and others have identified that IGF-1 is the target of miR-1 in ventricular cells [25,26]. However, the role of miR-1 in IGF-1's action and H_2O_2-induced cell death has not been investigated in iPS cells.

This study examined the protective effects of IGF-1 on human iPS cells developed through reprogramming with nuclear factors and determined the roles of miR-1 in H_2O_2-induced mitochondrial dysfunction, cytochrome-c release and apoptosis in the iPS cells incubated with or without IGF-1.

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2. Materials and methods

2.1. Generation of iPS cells

Normal human foreskin fibroblast (SCRC-1041) was purchased from ATCC, and was maintained in fibroblast medium: DMEM (Sigma/Aldrich) supplemented with 15% FBS, NFAA, L-glutamine and penicillin/streptomycin. The fibroblast cells were infected by lentivirus (MOI = 10) containing six reprogramming factors (Lin28, c-Myc, Klf4, Nanog, Sox-2 and Oct4) (viPS™ Vector Kit, Thermo Fisher Scientific, Inc.) in gelatin coated culture plates. After 6 days, the cells were cultured on mitomycin-inactivated MEF feeder cells with media: DMEM/F12, 20% KSR, 4 ng/ml bFGF, nonessential amino acids, 0.1 mM β mercaptoethanol, L-glutamine and penicillin/streptomycin (Invitrogen). After 30 days, colonies were selected and expanded on mitomycin-inactivated MEF or matrigel-coated plate with mTesR1 OR2 medium (Stem Cell Technologies) for further experiments. The typical infection efficiency was 70–90%, determined by expression of a control GFP vector or expression of SSEA3 by flow cytometry.

2.2. Quantification of fragmented DNA in cell death by ELISA

DNA fragmentation in cell death was analyzed using a cell death detection ELISA kit (Roche) according to the manufacturer's instructions. To determine the effect of H_2O_2 on apoptosis, iPS cells were exposed to different concentrations of H_2O_2 (25 μM, 50 μM, 100 μM, and 200 μM) for different time periods (24, 48, and 72 h). After H_2O_2 exposure, cells were lysed in 100 μl of lysis buffer and centrifuged for 10 min at 1500 rpm. Triplicate 20 μl samples of supernatant were placed into the streptavidin-coated microtest plates for analysis. DNA fragmentation was quantified by measuring absorbance at 405 nm with a reference wavelength at 492 nm.

2.3. Determination of mitochondrial function

To determine the effects of H_2O_2 on mitochondrial function, iPS cells were exposed to H_2O_2 at the doses up to 200 μM for different time periods (8, 16, 24 and 32 h). After the experimental treatment, the cells were washed with PBS (pH 7.2, 1 mL) 3 times, then incubated for 30 min with 100 nM MitoTracker®Deep Red FM (Molecular Probes) in 1 mL of PBS. After 30 min the cells were washed 3 times with PBS, and 1 mL of PBS was then added to the plates. Cells were removed from the culture plates with a scraper and placed into cytometer tubes. The indicator fluorescence was measured (excitation/emission wavelengths 644/665 nm) by flow cytometer.

2.4. Cytochrome-c measurement

The cytosolic level of cytochrome-c was determined by using an ELISA (R&D Systems, Minneapolis, MN, USA). Briefly, after H_2O_2 exposure, iPS cell cultures were washed twice with PBS, and cell membrane permeabilized in PBS with 0.5% Triton X-100. The cell lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. Cytosolic proteins were determined using the Bradford assay, and the same amounts of proteins from each sample loaded into 96-well microplates with immobilized anti-cytochrome-c. After incubation for 2 h at room temperature, the plates were washed 3 times with washing buffer. A substrate solution containing tetramethylbenzidine was added to each well, and the plates were incubated for 30 min at room temperature. Then, a stop solution containing hydrochloric acid was added to terminate the reaction. Absorbance was determined by using a microplate reader at 450/575 nm dual wavelengths. The data were expressed as the mean optical density of the samples normalized to a percentage of the control value.

2.5. Synthesis of miRNAs mimics and transfection of miRNAs

miR-1 mimics were synthesized based on 5′-UGGAAUGUAA-AGAAGUGUGUAU-3′ for mature human miR-1 (Amibon, Texas). miR-1 mimic negative control (Amibon, Texas) was used as additional control in some experiments. After 4 h starvation in serum-free medium, cells (1×10^5 per well) were transfected with miR-1 mimic or miR-1 mimic negative control, with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

2.6. Micro-RNA isolation and expression

The mirVana™ qRT-PCR miRNA Isolation and Detection Kit (Ambion, Inc.) were used. Reactions contain mirVana qRT-PCR primer sets specific for miR-1 or 5S rRNA. 5S rRNA was used as an internal control. qRT-PCR were performed for 40 cycles. To verify PCR product identity, PCR products were confirmed by agarose gel electrophoresis.

2.7. Statistics

Data were presented as mean ± SEM. Statistical analysis was performed by using ANOVA or Student's *t* test when appropriate. *P* value <0.05 was considered significant. All experiments were performed at least 3 times.

3. Results

3.1. H_2O_2 exposure induces apoptotic cell death in iPS cells

To determine whether H_2O_2 could induce apoptosis, the iPS cells were exposed to the specified concentration of H_2O_2 for 24, 48, and 72 h, and cells were assessed for apoptosis by analyzing histone-associated DNA fragments. Analysis of histone-associated DNA fragments showed that incubation of the iPS cells with H_2O_2 caused a concentration- and time-dependent DNA fragmentation and cell loss in the cultures over the 72 h interval (Fig. 1A). Significant cell death occurred in the cultures exposed to H_2O_2 at 200 μM for 72 h. The untreated iPS cells showed little changes in DNA fragmentation when cultured under the same conditions except for H_2O_2 treatment.

3.2. IGF-1 prevents H_2O_2-induced apoptosis

To determine whether IGF-1 exerts a protective effect on iPS cells against a oxidative stress agent, such as H_2O_2, IGF-1 (20 ng/ml) and H_2O_2 (200 μM) were added into the iPS cells. The death ELISA showed that iPS cells with IGF-1 had significantly lower levels of histone-associated DNA fragments than the control cells when exposed to 200 μM H_2O_2 for 72 h (Fig. 1B).

3.3. IGF-1 restores the mitochondrial function in iPS cells exposed to H_2O_2

Mitochondrial dysfunction characterizes apoptosis induced by cytotoxic substances. To measure mitochondrial function in iPS cells, iPS cells exposed to H_2O_2 showed a decrease in Deep Red fluorescence signal in a concentration- and time-dependent manner (Fig. 2C and G). At the cytotoxic dose (200 μM) H_2O_2 decreased mitochondrial function at 32 h, but not at 8, 16 and 24 h (Fig. 2G). IGF-1 protected iPS cells against H_2O_2-induced mitochondrial dysfunction (Fig. 2D).

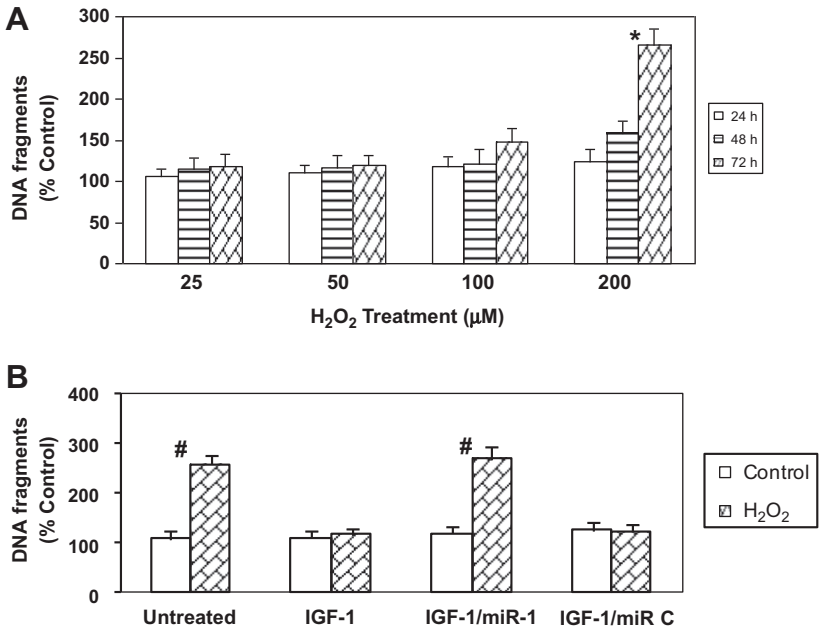

Fig. 1. (A) H_2O_2-induced iPS cell apoptosis. The iPS cells were exposed to the specified concentration of H_2O_2 for 24, 48, and 72 h, and cells were assessed for apoptosis by analyzing histone-associated DNA fragments. $^{*}P < 0.01$ compared to control. (B) iPS cells were exposed to 200 μM H_2O_2 with or without IGF-1, miR-1 mimic or miR-1 mimic negative control. After 72 h, apoptosis was analyzed for histone-associated DNA fragments. $^{\#}P < 0.01$ compared to control.

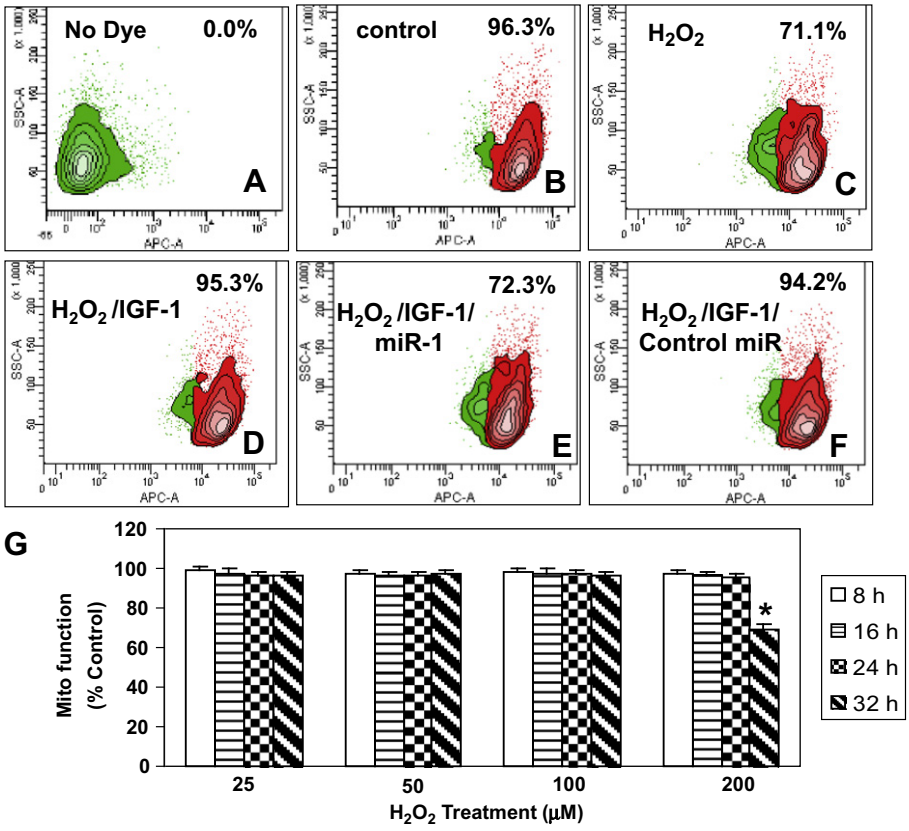

Fig. 2. (A–F) Effects of IGF-1 and miR-1 on H_2O_2-induced mitochondrial dysfunction. iPS cells were exposed to 200 μM H_2O_2 (C), and/or IGF-1(D), miR-1 mimic (E), or miR-1 mimic negative control (F) for 32 h, then loaded with 100 nM MitoTracker®Deep Red FM. After washing, the cells were analyzed for fluorescence by flow cytometry. (G) Effect of H_2O_2 on mitochondrial function in iPS cells. iPS cells were exposed to 25 μM, 50 μM, 100 μM and 200 μM H_2O_2 for 8, 16, 24, and 32 h, then loaded with 100 nM MitoTracker®Deep Red FM. After washing, the cells were analyzed for fluorescence at 644/665 nm (excitation/emission) by flow cytometry. $^{*}P < 0.05$ compared to control.

3.4. IGF-1 reduces the release of cytochrome-c from mitochondria into the cytoplasma

IGF-1 reduces the release of cytochrome-c from mitochondria into the cytoplasma. Concurrently with the mitochondrial dysfunction, mitochondrial cytochrome-c may be released into the cytoplasma due to a partial leakage of mitochondrial membrane triggered by cytotoxic agents. iPS cells exposed to 200 μM H_2O_2 showed increased cytosolic levels of cytochrome-c after 48 h of exposure but not after 16, 24 and 32 h (Fig. 3A). IGF-1 treatment attenuated this H_2O_2-induced increase in the cytosolic levels of cytochrome-c as shown in H_2O_2-treated iPS cells as compared with no-IGF-1 treated cells (Fig. 3B).

3.5. H_2O_2 exposure increases expression of miR-1 and miR-1 mimic blocks the anti-apoptotic effects of IGF-1

We found that H_2O_2 increased miR-1 determined by the mirVana qRT-PCR miRNA detection assay after 16 h exposure to 200 μM H_2O_2 (Fig. 4A). Prediction algorithms showed that 3′-untranslated regions of IGF-1 gene as a target of miR-1 (Fig. 4B). Moreover, over expression of specific miR-1 mimics, but not miR-1 mimic negative control, blocked the capacity of IGF-1 to prevent H_2O_2-induced apoptosis (Fig. 1B), mitochondrial dysfunction (Fig. 2E and F) and cytochrome-c release (Fig. 3B).

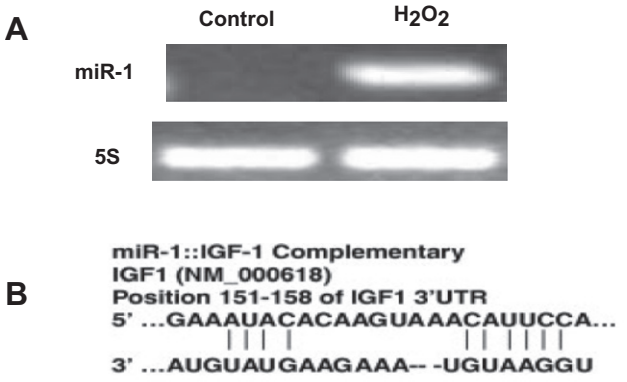

Fig. 4. (A) H_2O_2 increased expression of miR-1 in iPS cells. The micorRNA was isolated with mirVana miRNA Isolation Kit. RT-PCR was performed using the mirVana™ qRT-PCR miRNA Detection Kit containing mirVana qRT-PCR Primer sets specific for miR-1 or 5S rRNA. 5S rRNA was used as an internal control. The PCR products were confirmed by agarose gel electrophoresis. (B) The position of predicted miR-1 target sites along 3′UTR of IGF-1 is predicted by Targetscan.

4. Discussion

In the present study, we demonstrated that the effects of IGF-1 on preventing H_2O_2-induced mitochondrial dysfunction, cytochrome-c release and apoptosis are mediated by miR-1. To our knowledge, these novel findings in iPS cells have not been reported before.

Our findings that H_2O_2 increased miR-1 expression in iPS are consistent with previous reports that miR-1 is expressed in embryonic stem cells [23], and induces apoptosis in cancer cells [24]. Our studies that miR-1 mimics rather than miR-1 mimic negative control blocked the capacity of IGF-1 to prevent H_2O_2-induced mitochondrial dysfunction, cytochrome-c release and apoptosis in iPS cells provide substantial evidence that IGF-1 is the target of miR-1 in iPS cells in addition to the prediction algorithms analysis. Our novel findings in iPS cells are consistent with the results that miR-1 regulates apoptosis by targeting anti-apoptosis genes in somatic cells [27].

Our data showed exposure to H_2O_2 for 32 h decreased mitochondrial function without affecting cytochrome-c release (measured 48 h after exposure to H_2O_2) or toxicity (measured 72 h after exposure to H_2O_2). These data suggest that the H_2O_2-induced decrease in mitochondrial function by itself is not sufficient to induce iPS cells toxicity. However, the finding that H_2O_2 affects mitochondrial function only at higher concentrations (200 μM) and not at lower concentrations (25 μM and 50 μM) indicates that H_2O_2-induced mitochondrial dysfunction in iPS cells is dose-dependent.

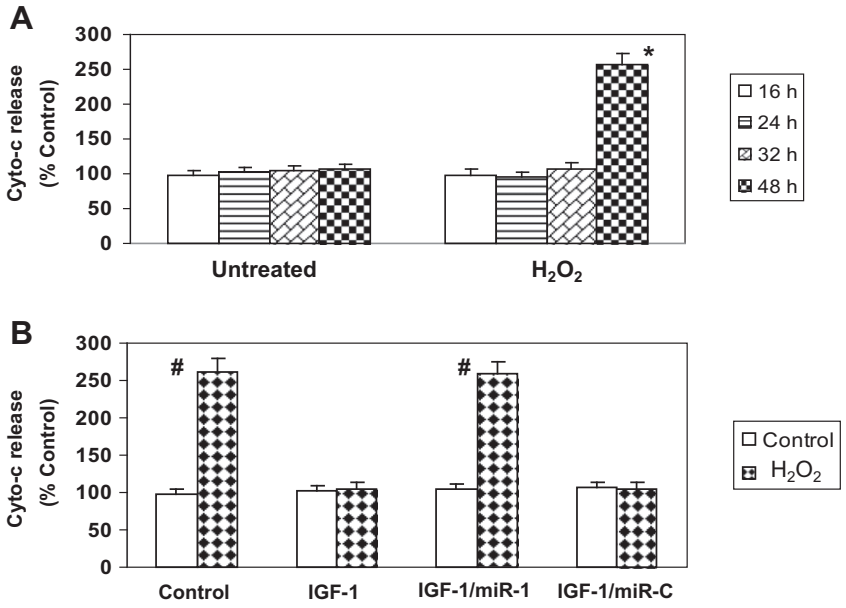

Fig. 3. (A) Effects of H_2O_2 on cytochrome-c release in iPS cells. iPS cells were exposed to 200 μM H_2O_2 for 16, 24, 32 and 48 h, and the cytosolic fractions were obtained by centrifugation. Cytochrome-c levels were assayed by ELISA, with absorbance determined using a microplate reader at 450/575 nm dual wavelengths. $^{*}P < 0.01$ compared to control. (B) Effects of IGF-1 and miR-1 on H_2O_2-induced cytochrome-c release in iPS cells. iPS cells were exposed to 200 μM H_2O_2 with or without IGF-1, miR-1 mimic or miR-1 mimic negative control. After 48 h, cytochrome-c levels were assayed by ELISA, with absorbance determined using a microplate reader at 450/575 nm dual wavelengths. $^{\#}P < 0.01$ compared to control.

Our observation shows that the H_2O_2-induced decrease in mitochondrial function (32 h) occurred before the release of cytochrome-c (48 h), suggesting that H_2O_2 might have attacked the mitochondria and increased the permeability of the mitochondrial membrane in iPS cells. This H_2O_2-induced cytochrome-c release may be a consequence of the opening of the mitochondrial permeability transition (MPT) channel. The finding in iPS cells is consistent with other reports that impaired mitochondria can release cytochrome-c into the cytoplasm, where it can bind to Apaf-1 and activate the caspase networks that induce somatic cell apoptosis [28–30]. Furthermore, the finding that only higher concentrations of H_2O_2 could induce cytochrome-c release and the fact that cytochrome-c release (48 h) occurred before cytotoxicity was induced (72 h) indicate that H_2O_2-induced iPS cells cytotoxicity requires the release of cytochrome-c from mitochondria, which is consistent with previous findings that cytochrome-c release is necessary for apoptosis in somatic cells [10,31,32]. However, the experiments that directly examine the requirement for cytochrome-c in apoptosis must await the generation of conditional null mutants of cytochrome-c that eliminate the death-promoting activity but maintain the essential function of cytochrome-c in oxidative phosphorylation [12].

In summary, these findings provide a new paradigm for biological effects of IGF-1 on iPS cells apoptosis which are mediated by miR-1, and have implications for the development of novel therapeutic strategies to prevent H_2O_2-induced iPS cell apoptosis.

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