Low-Dose Radiation Suppresses Pokemon Expression under Hypoxic Conditions

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Our previous data demonstrated that $CoCl_2$-induced hypoxia controls endoplasmic reticulum (ER) stress-associated and other intracellular factors. One of them, the transcription factor Pokemon, was differentially regulated by low-dose radiation (LDR). There are limited data regarding how this transcription factor is involved in expression of the unfolded protein response (UPR) under hypoxic conditions. The purpose of this study was to obtain clues on how Pokemon is involved in the UPR. Pokemon was selected as a differentially expressed gene under hypoxic conditions; however, its regulation was clearly repressed by LDR. It was also demonstrated that both expression of ER chaperones and ER stress sensors were affected by hypoxic conditions, and the same results were obtained when cells in which Pokemon was up- or down-regulated were used. The current state of UPR and LDR research associated with the Pokemon pathway offers an important opportunity to understand the oncogenesis, senescence, and differentiation of cells, as well as to facilitate introduction of new therapeutic radiopharmaceuticals.

Key words: Low-Dose Radiation (LDR), Hypoxia, Pokemon

Introduction

Oxygen is mandatory to sustain aerobic life. It is absolutely involved in oxidative phosphorylation and ATP generation in the mitochondrial respiratory chain, which are indispensable to maintain cell viability. Therefore, decreasing the normal oxygen supply (hypoxia) to a level insufficient to maintain cellular function is fatal to cells. Hypoxia-related diseases have recently become a problem. Although our understanding of hypoxia in physiology and pathophysiology has increased in recent years, the mechanisms that contribute to the positive and negative regulation of hypoxia are poorly understood (Sunderram and Androulakis, 2012).

The effects of low doses ($\leq$ 0.1 Gy) and low levels of radiation on living organisms including mice and humans are well studied. In particular, low-dose radiation (LDR) stimulates immunity to cancer and biological defences against DNA damage (Rosenberg and Knox, 2006). It has also been reported that LDR controls hypoxia-inducible factor (HIF) expression during hypoxia treatment, and thus cures/prevents hypoxia. We used the differential display-polymerase chain reaction (DD-PCR) method to explore the differentially expressed genes that are induced or repressed by LDR under hypoxia conditions (Ryu *et al.*, 2012).

The central functions of the endoplasmic reticulum (ER) – *e.g.*, lipid biosynthesis; ion homeostasis; and translocation, folding, processing, and traf-

ficking of secreted and membrane-bound proteins – are highly conserved and crucial to many metazoan processes, including development and function of the immune, neuronal, and endocrine systems. The unfolded protein response (UPR) in mammalian cells involves three distinct ER stress sensors – IRE1 (inositol-requiring enzyme 1), PERK [PKR (protein kinase regulated by RNA)-like ER-associated kinase], and ATF6 (activating transcription factor 6) – which are downstream components of ER chaperones, and transmit stress signals from the ER to the nucleus in response to perturbations in protein folding in the ER (Walter and Ron, 2011). Activation (autophosphorylation and dimerization) of IRE1 activates the endonuclease domains, which cleave X-box DNA-binding protein (XBP) mRNA, generating an activated form of XBP1, whereas activation of PERK results in phosphorylation of the α subunit of eukaryotic translation initiation factor 2 and inhibits translation initiation. ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, causing nuclear translocation of the N-terminal cytoplasmic domain, which contains the DNA-binding, dimerization, and transactivation domains, and subsequent binding to both ER stress response element (ERSE) and ATF6 sites to enhance the expression of ER chaperone genes (Lee *et al.*, 2011). In recent studies, ER stress has been revealed to mediate various neuronal cell death processes (Ogawa *et al.*, 2007). The expression of ER stress-related molecules may have an important role in transient ischemic injury. We demonstrated previously that hypoxia induced by $CoCl_2$ is strongly associated with apoptosis through the ER stress response (Kim *et al.*, 2010). We also studied the relevant genes associated with and without ER stress.

The results of DD-PCR experiments showed that a gene encoding *Pokemon* was differentially expressed – about twofold – by LDR under hypoxic conditions. Pokemon is a member of the POZ and Krüppel (POK) protein family of transcriptional repressors, which has an NH_2-terminal POZ/BTB domain and COOH-terminal Krüppel-type zinc fingers. Some characteristics of potential interacting POK family partners are expected to lead to a great diversity in their cellular functions beyond simple target gene repression. Pokemon mainly modulates important tumourigenesis cellular functions/pathways and acts as an ARF tumour suppressor (Maeda *et al.*, 2005). Pokemon also mediates several pathways in the expression of some chaperones which are related to ischemia/hypoxia, and thus protective effects against ischemia/hypoxia (Yuan *et al.*, 2012). Although we observed that Pokemon was aberrantly down-regulated in a cellular hypoxic model, little is known about the mechanism by which it becomes down-regulated under hypoxic conditions. Thus, we investigated the roles of Pokemon in ischemic injury. Because of the relationship between down-regulated Pokemon and LDR treatment, we investigated the Pokemon expression in cellular hypoxia by molecular biological techniques.

Material and Methods

PC12 cell culture

Rat pheochromocytoma PC12 cells were routinely cultured in Dulbecco's modified Eagle's media supplemented with 10 % horse serum and 5 % fetal calf serum on collagen-coated dishes in a humidified 5 % CO_2 atmosphere at 37 °C. Media were exchanged 2 – 3 times per week, and each passage took 7 – 10 d.

LDR and $CoCl_2$ treatment

The 80 % confluent PC12 cells were treated with 0.1 Gy LDR using a linear accelerator (Clinac 2100 C; Varian, Milpitas, CA, USA) and 6 MV radiography. Ischemic conditions were induced by 0.1 mM $CoCl_2$ treatment for 12 h.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using an SV Total RNA Isolation system (Promega, Madison, WI, USA), according to the company's instruction. RT-PCR was performed using the forward primer (F) (5′-ACCACCAGTCCATCGCCATT-3′) and reverse primer (R) (5′-CCACCCTGGACGGAAGTTTG-3′) for IRE1; F (5′-AGTGGTGGCCACTAATGGAG-3′) and R (5′-TCTTTTGTCAGGGGTCGTTC-3′) for Bip; F (5′-CTAGGCCTGGAGGCCAGGTT-3′) and R (5′-ACCCTGGAGTATGCGGGTTT-3′) for ATF6; F (5′-ATCGAGTTCACCGAGCAGAC-3′) and R (5′-TCACAGCTTTCTGGTCATCG-3′) for PDI; F (5′-GGTCTGGTTCCTTGGTTTCA-3′) and R (5′-TTCGCTGGCTGTGTAACTTG-3′) for PERK; F (5′-ACATCAAATGGGGTGATGCT-3′) and R (5′-AGGAGACAACCTGGTCCTCA-3′) for GAPDH; and F (5′-AAACAGAGTAGCAGCTCAGACTGC-3′) and R (5′-TCCTTCTGGGTAGACCTCTGGGAG-3′) for XBP1. The RT-PCR primers were supplied by

Bioneer Co. (Daejeon, Korea). RT-PCR conditions for 30 cycles were: 94 °C for 30 s; 58 °C for 30 s; and 72 °C for 1 min (10 min in the final cycle), using both primers mentioned above with T*aq* DNA polymerase. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Western blotting

PC12 cells were scraped, lysed by adding sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris/HCl, pH 6.8, 6% (w/v) SDS, 30% glycerol, 125 mM DTT, 0.03% (w/v) bromophenol blue], and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with primary antibodies overnight at 4 °C. The blots were developed using an enhanced chemiluminescence Western blotting detection system kit (Amersham, Uppsala, Sweden). Rabbit anti-eIF2 antibody, eIF2-P antibody, and goat anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

XBP1 splicing experiment

Total RNA was reverse-transcribed, and double-strand cDNA was synthesized by PCR using specific sense and anti-sense primers for the XBP1 gene (F, 5′-AAACAGAGTAGCAGCGCAGACTGC-3′; R, 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′). The amplified cDNA was treated with *Pst*I, and the resulting products were analysed by electrophoresis in a 2% agarose gel.

Results and Discussion

Hypoxic conditions occurred where there was an imbalance between oxygen supply and consumption by $CoCl_2$. It is well established that $CoCl_2$-induced hypoxia enhances HIF-1 protein expression in various cell lines (Kubis *et al.*, 2005). HIF-1 is the major transcription factor that responds to changes in available oxygen in the cellular environment and is specifically activated during hypoxia. The HIF-1 intracellular signal pathway is well established under hypoxic conditions (induced by $CoCl_2$ in this experiment) and involves induction of various genes to adapt to the newly hypoxic conditions (Wang *et al.*, 2012). Overcoming hypoxia by radiation may be a viable and important approach. Several data show that LDR is effective for cell survival under hypoxic conditions, which can be positive rather than negative for cell protection against hypoxia (Lerman *et al.*, 2010). DD-PCR was developed as a rapid, accurate, and sensitive method for the detection of the expression of temporally altered genes in any cell. We isolated *Pokemon* as a differentially expressed gene using the DD-PCR method, and found it to be repressed about two times by LDR under hypoxic conditions (data not shown).

Pokemon is an essential factor for oncogenesis, in which its main role is that of a specific repressor of the ARF/p53 pathway (Taneja *et al.*, 2010). However, the role of Pokemon in hypoxia is unknown; thus we tested the Pokemon expression under $CoCl_2$-induced hypoxia conditions. As shown in Fig. 1a, although Pokemon showed higher expression (about two times) during hypoxia, expression of both ARF and p53 was down-regulated by about half. Interestingly, LDR after induction of hypoxia produced results opposite to those shown in Fig. 1b. That is, LDR under hypoxic conditions down-regulated Pokemon, but up-regulated ARF and p53, respectively. The results in Fig. 1 show that LDR appears to regulate the hypoxia signal pathway in an opposite manner to Pokemon, and may provide some clues for clinical treatment of hypoxia using LDR.

A number of abnormal pathophysiological factors (hypoxia, nutrient deprivation, redox or calcium unbalance) lead to the accumulation of unfolded/misfolded proteins in the ER and cause ER stress. Cells adapt against ER stress via the UPR mechanism. It has been demonstrated that hypoxia perturbs the redox status of the ER lumen and leads to activation of the UPR and up-regulation of ER chaperones, and furthermore, that ER stress up-regulates HIF-1 expression in ischemia (Fels and Koumenis, 2006). We were interested in whether LDR under hypoxic conditions changes UPR-associated factors, such as ER chaperones (Bip and calnexin) and ER stress sensors (ATF6, IRE1, and PERK) (Walter and Ron, 2011). Bip, also known as GRP78 or HSPA5, is a heat-shock protein 70 molecular chaperone located in the ER lumen that binds newly synthesized proteins for folding and assembly. It is an essential component of the ER stress signaling pathway. Calnexin is a 67-kDa integral protein of the ER membrane. Its main function is to retain unfolded or unassembled N-linked glycoproteins for ER quality control in the ER lumen. The UPR is mediated by three ER transmembrane proteins (ER stress sensors) that sense ER stress and signal down-stream pathways. Several previous reports have suggested a link

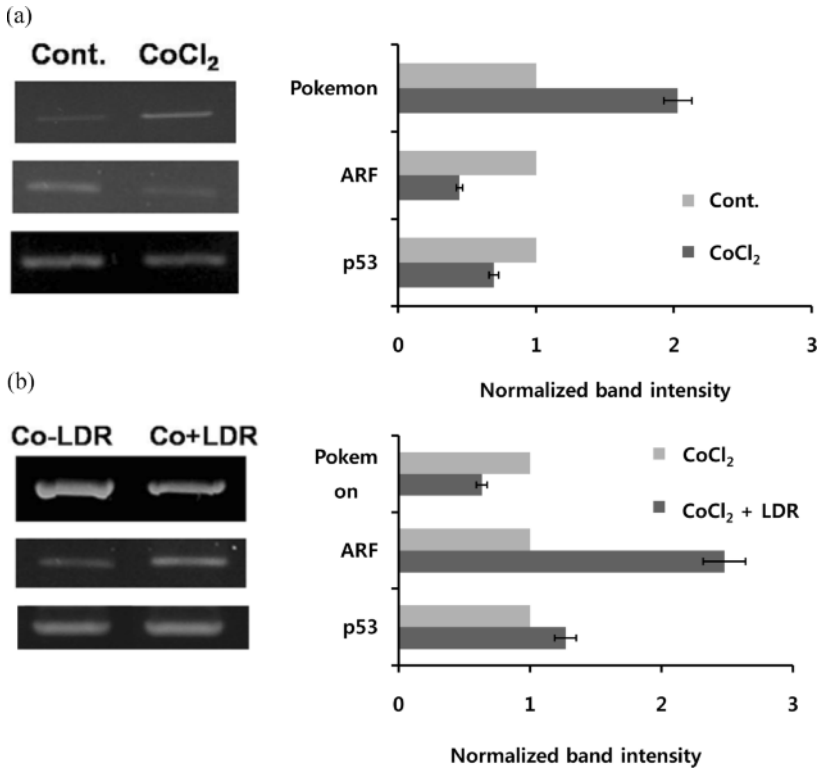

Fig. 1. Expression of Pokemon and factors (ARF/p53) induced by $CoCl_2$ and low-dose radiation (LDR). (a) PC12 cells were treated with 0.1 mM $CoCl_2$ for 12 h. (b) $CoCl_2$-treated cells were additionally treated with 0.1 Gy of LDR. Bar graphs represent the average of three RT-PCR values.

between ER stress and various diseases (diabetes mellitus, cancer, atherosclerosis, inflammation, viral infection, bipolar disorder, and glomerular disease), in which expression of ER chaperones is disturbed (Wang and Kaufman, 2012). Previous reports have shown that ER stress is caused by hypoxia and activates ER stress sensors. As shown in Fig. 2a, $CoCl_2$-induced hypoxia induced both expression of ER chaperones and ER stress sensors. Among them, Bip and IRE1 expression were up-regulated approximately twofold. When cultured cells were LDR-irradiated after hypoxia, their expression pattern was opposite to the results presented in Fig. 2a; ER chaperones were up-regulated with ER stress sensors, except PERK (Fig. 2b). The result of Fig. 2 coincides with that of Fig. 1, such that the opposite expression pattern is induced by LDR under hypoxic conditions, as shown in Fig. 2. LDR is thus a regulator for UPR-associated factors, and a potential tool for ischemia treatment.

To assess whether regulation of ER stress involves Pokemon expression, both the expression of ER chaperones and ER stress sensors were tested under conditions of up- and down-regulated Pokemon gene expression, respectively. As shown in Fig. 1a, Pokemon expression was up-regulated under hypoxia, in which expression of both ER chaperones and ER stress sensors were up-regulated (Fig. 2a). Direct suppression of Pokemon by siRNA inhibited the expression of both ER chaperones and ER stress sensors (Fig. 3). These results demonstrate that regulation of the Pokemon expression directly controls the UPR, including up-regulation of both ER chaperones and ER stress sensors.

To understand whether Pokemon expression controls the down-regulation of the ER stress sensors eIF2 alpha and XBP1, cells were constructed with either up- or down-regulated expression of Pokemon. ER stress induces phosphorylation of eIF2 alpha, which prevents total protein synthesis of the cell for survival (Kimball and Jefferson, 2012). Figure 4a shows the results of Western blotting for eIF2 alpha and its phosphorylation by up- or down-regulation of the Poke-

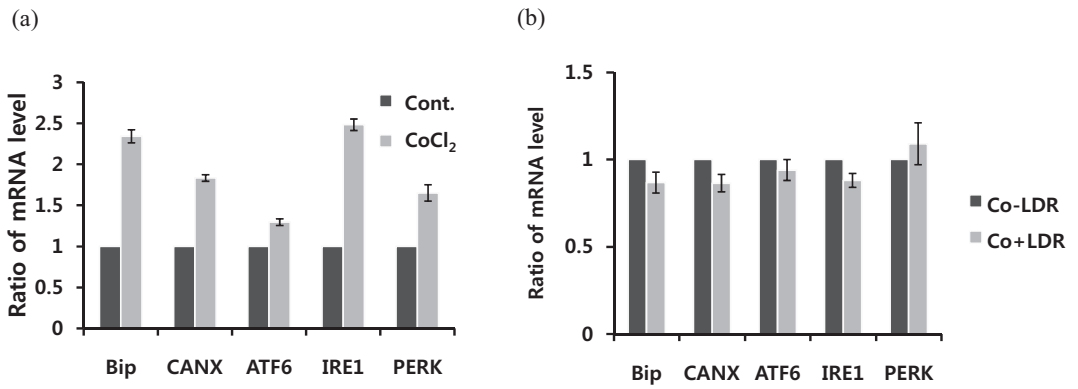

Fig. 2. Expression of (a) endoplasmic reticulum (ER) chaperones and (b) ER stress sensors. All experimental conditions were the same as those in Fig. 1. Bip, immunoglobulin heavy-chain binding protein; CANX, calnexin; ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, PKR (protein kinase regulated by RNA)-like ER-associated kinase.

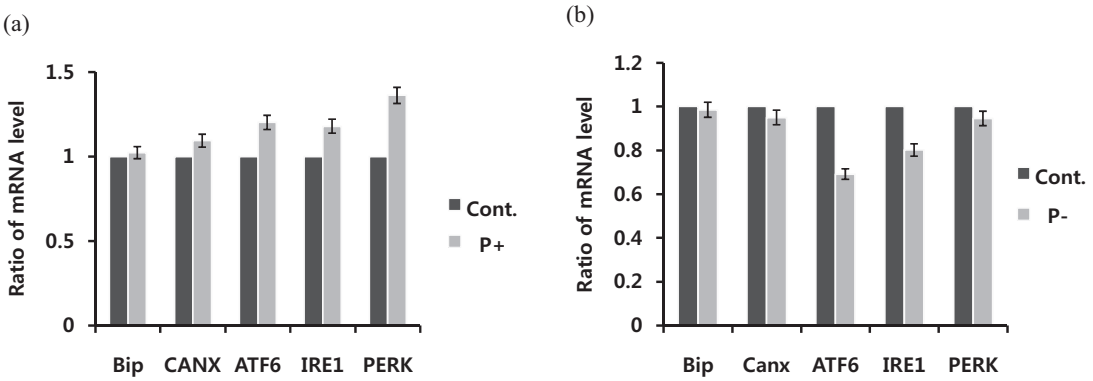

Fig. 3. Pokemon controls (a) endoplasmic reticulum (ER) chaperones and (b) ER stress sensors. Two kinds of cells were constructed; (a) over-expressing Pokemon (P+), (b) under-expressing Pokemon (P−) in the PC12 cells. mRNA preparations were as in Fig. 1.

mon expression. In Pokemon-down-regulated cells, eIF2 alpha phosphorylation was down-regulated, and the opposite was true for Pokemon-up-regulated cells. IRE1 is highly conserved in higher eukaryotes, and XBP1 is the mRNA substrate for IRE1 RNAse activity (van Schadewijk *et al.*, 2012). IRE1 removes a 26-nucleotide intron from unspliced XBP1 mRNA under UPR conditions, which leads to a translational frame shift. The IRE1 activity-dependent XBP1 mRNA splicing assay is one of the best methods to estimate ER stress. Figure 4b shows the results of the XBP1 RT-PCR splicing assay after ischemia induction or ischemia-LDR treatment. RNA isolated from triplicate wells of $CoCl_2$ or $CoCl_2$-LDR cells was amplified by RT-PCR, the resulting products were digested by *Pst*I, and the products of the digestion were analysed by agarose gel electrophoresis. *Pst*I was used to distinguish the unspliced from the spliced band, as it cuts only in the unspliced cDNA. In Fig. 4b, the arrow at the top indicates the hybrid (hXBP1) form of XBP1 mRNA, the bold arrow indicates the unspliced (uXBP1) form, and the lower two arrows at 290 bp and 183 bp indicate spliced (sXBP1) forms. These results demonstrate that Pokemon directly controls the ER stress sensors, eIF2 alpha and XBP1, through ER stress.

It is well known that ER stress mediates various processes in neuronal cell death. The regulation of ER stress-related molecules plays an important role to prevent ischemic injury through the UPR. Genes with a potential for ischemic recovery are characterized by various intracellular signaling pathways and may be in-

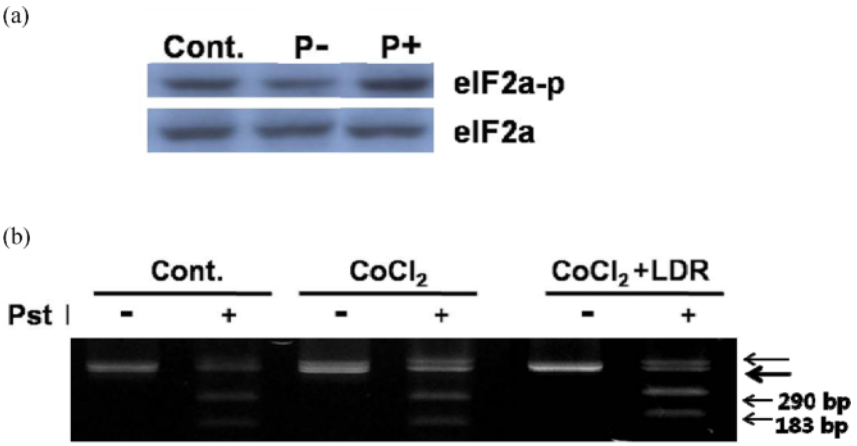

Fig. 4. Pokemon controls both eIF2 alpha phosphorylation and XBP1 mRNA splicing. (a) Western blotting was performed against the lysates of Fig. 3 using anti-eIF2 antibody and eIF2-P antibody. (b) RT-PCR was performed with the mRNAs of Fig. 1. The resulting PCR products were further digested by *Pst*I to reveal the restriction site that was lost upon splicing of XBP1 during ER stress. The resulting XBP1 cDNA products were revealed on a 2% agarose gel. Unspliced XBP1 mRNA produced the two lower bands indicated by arrows (upper, 290 bp; lower, 183 bp), whereas the spliced XBP1 mRNA is indicated by a bold arrow. XBP1, X-box DNA-binding protein 1.

teresting therapeutic targets for drug development. Additionally our results suggest that both ER stress and LDR are directly associated with the Pokemon pathway. These findings may lead to new clues for the treatment of ischemic injury, but further investigations are required to understand the underlying mechanism.

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