PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells

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Received 21 April 2003; accepted 23 April 2003

First published online 26 May 2003

Edited by Jesus Avila

Abstract **Oxidative stress and ferrous metabolism are important in the pathogenesis in Parkinson's disease. In dopaminergic neurons, several stress proteins are upregulated under oxidative stress. To clarify this mechanism, we investigated hemin-related signal transduction and the induction of oxidative stress-related proteins in SH-SY5Y cells. We identified phosphatidylinositol 3-kinase (PI3K) and Nrf2 as important molecules in the induction of heme oxygenase-1, thioredoxin, and peroxiredoxin-I. PI3K-related signal controlled Nrf2 activation, and consequently, PI3K inhibitors blocked the nuclear translocation of Nrf2 and induction of stress proteins. These observations suggest that PI3K and Nrf2 are key molecules in maintaining suitable conditions under oxidative stress and ferrous metabolism.**

Key words: Phosphatidyl inositol 3-kinase; NF-E2-related factor 2; Antioxidant responsive element; Heme oxygenase-1; Thioredoxin; Peroxiredoxin

1. Introduction

Oxidative stress is one of the most important factors in the pathogenesis of idiopathic Parkinson's disease (PD) [1,2]. Because the accumulation of iron is observed in neurons of the substantia nigra in the post-mortem brain from PD patients [3,4], understanding ferrous metabolism and oxidative stress may be significant in examining the pathogenesis of PD. Heme oxygenase-1 (HO-1), which is well known as a stress protein and antioxidative protein, has been observed in the nigral neurons of the PD brain [5]. This suggests that neurons may express HO-1 to alleviate unsuitable conditions. Therefore, understanding the mechanisms used to induce HO-1 under stressed conditions is important.

HO-1 [6,7], thioredoxin (Trx) [8], and peroxiredoxin-I (PrxI) are antioxidative proteins that are induced by oxidative stress. HO-1 is required to catalyze heme to bilirubin and carbon monoxide in cells under stressed conditions [6,7]. In addition, a deficiency in HO-1 results in a vulnerability of cells to various stresses and a disorder in ferrous metabolism [9,10]. In fact, bilirubin is well known as a cellular antioxidant, while carbon monoxide is considered to play a role as a second messenger [11,12]. These observations suggest that HO-1 may play important cellular roles under stressed conditions. Trx is a ubiquitous protein with a redox-active disulfide/dithiol in its conserved active site sequence, CGPC [13]. Trx has been shown to scavenge singlet oxygen and hydroxyl radicals [14] and hydrogen peroxide in conjunction with peroxiredoxin [15]. Trx protects cells against cytotoxicity by hydrogen peroxide [16] and 1-methyl-4-phenylpyridinium [17]. Trx is induced under stress conditions or [16,18] by specific drugs [19].

Recently, Nrf2 has been reported to act as a common transcription factor in the induction of antioxidative proteins under stress conditions [20,21]. The inducible expression of HO-1, Trx, and PrxI is partially controlled by Nrf2 activation [20–25], and the promoter or enhancer region of each gene involves antioxidant responsive element (ARE) as a target of Nrf2.

In this study, we examined the signal associated with the induction of HO-1, Trx, and PrxI by hemin in human dopaminergic neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Cell culture and application of reagents

The human dopaminergic neuroblastoma cell line, SH-SY5Y, was purchased from ATCC. SH-SY5Y cells were maintained at 37°C in 5% CO_2 in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Culture medium was exchanged twice a week during cell growth. For immunoblot analysis and immunohistochemistry, experiments were carried out using 1×10^5 cells in six-well culture plates and 200–500 cells in eight-well chamber slides, respectively.

Hemin dissolved in DMSO was added to the medium. Cells were incubated for the duration indicated in the figure legends. In order to determine which signal pathway is important for the activation of Nrf2, the phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin (1 μM) (Alomone labs) and LY294002 (10 μM) (Alomone labs), an Erk inhibitor, PD98059 (10 μM) (Alexis), p38 inhibitor, SB203580 (10 μM) (Alexis), and JNK inhibitor SP600125 (10 μM) (BIOMOL) were added to the medium at 1 h before hemin administration.

2.2. Immunoblot analyses

For immunoblot analysis, cells were lysed in sodium dodecyl sulfate (SDS)-sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 mM EDTA), and denatured by boiling at 100°C for 5 min. The nuclear fraction for Nrf2 analysis was prepared by centrifugation (15 000 × *g*, 10 min) after lysis in NP40 buffer (0.5% NP40, 1 mM Tris–HCl, pH 7.9, 500 μM dithiothreitol, 100 μM EDTA), followed by lysis in SDS-sample buffer. The samples were stored at −20°C until use. Dye and 2-mercaptoethanol were added to the specimens, and the mixture was boiled just before

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Abbreviations: PI3K, phosphatidyl inositol 3-kinase; Nrf2, NF-E2-related factor 2; ARE, antioxidant responsive element; HO-1, heme oxygenase-1

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00517-9

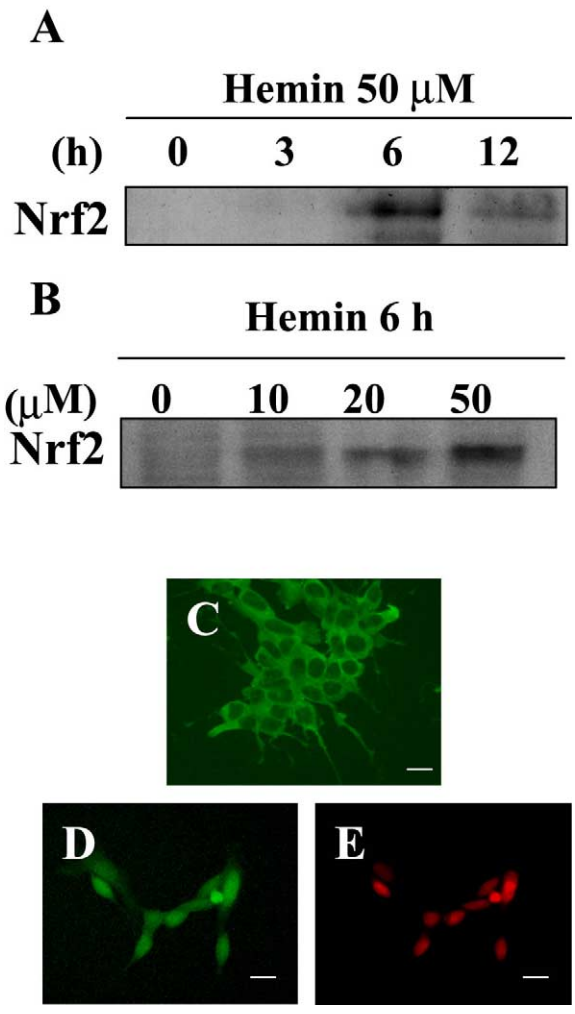

Fig. 1. Translocation of Nrf2 is induced by hemin administration in SH-SY5Y cells. A: Immunoblot analyses of Nrf2 in the nuclear fraction of SH-SY5Y cells. A time course experiment demonstrating the translocation of Nrf2 to the nucleus is shown. B: Dose–response of the translocation of Nrf2 by hemin. C: Immunohistochemistry of Nrf2 in SH-SY5Y cells. Nrf2 is shown labeled with FITC, and is localized in the extranuclear portion under normal conditions. D: Translocation of Nrf2 to the nucleus by hemin. Immunohistochemical signal is observed principally in the nucleus at 6 h after hemin administration. E: PI staining to localize nuclei. PI signal location coincides with that of Nrf2 staining in D. Scale bar = 10 μm.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Aliquots (20 μg) were used for SDS–PAGE. Each protein was separated by size on 8–13% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham), and the blots were incubated in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and either anti-human HO-1 antibody (Santa Cruz), human Trx antibody(Cosmo), human PrxI antibody (Santa Cruz), human Nrf2 antibody (Santa Cruz), Erk or phospho-Erk antibodies (Santa Cruz), p38 or phospho-p38 antibodies (Santa Cruz), JNK or phospho-JNK antibodies (Santa Cruz), and PI3K or phospho-PI3K antibodies (Santa Cruz). The bound primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Amersham) and ECL detection reagents (Amersham). Total protein content was determined using bicinchoninic acid protein assay reagent (Pierce).

2.3. Immunohistochemistry

For immunohistochemical analyses, cells in eight-well chamber slides were washed twice with PBS, and fixed in 4% paraformaldehyde. After incubating in ice-cold 95% ethyl alcohol/1% acetate and washing with PBS, the cells were incubated with each antibody overnight at 4°C, followed with an incubation with FITC-conjugated secondary antibody (Santa Cruz). For nuclear localization, counterstaining was performed using propione iodide (PI; ICN Biomedical Inc.).

3. Results and discussion

Nuclear translocation is considered to be an important mechanism for the activation of the transcription factor Nrf2. Therefore, we examined the nuclear expression of Nrf2 in the presence of hemin. Western blot analysis of the nuclear fraction demonstrated the translocation of Nrf2 by hemin (Fig. 1A,B). The nuclear translocation of Nrf2 mediated by hemin was dose-dependent (Fig. 1B), and a maximal level was observed at 6 h after hemin administration (Fig. 1A). At 12 h after hemin administration, nuclear Nrf2 level was lower than that of 6 h suggesting that 12 h is in off-peak of nuclear translocation. These translocations were detected as 100 kDa actin-binding form described by Kang [26]. Immunostaining also showed the translocation of Nrf2 by hemin in SH-SY5Y cells (Fig. 1C–E). Although Nrf2 was localized outside the nuclei under normal conditions (Fig. 1C), Nrf2 was translocated into PI-labeled nuclei (Fig. 1D,E) at 6 h after hemin administration.

In order to confirm whether antioxidative proteins, which contain ARE in their promoter or enhancer region, are co-induced, immunoblot analysis against antioxidative proteins was performed in cells at a later phase than that of Nrf2 activation. Immunoblotting clearly demonstrated the induction of HO-1, Trx, and PrxI in SH-SY5Y cells by hemin administration (Fig. 2). The expression level of HO-1 reached maximum levels at 12 h after the hemin administration. Although the expression of Trx and PrxI was observed under normal conditions to some degree, both proteins reached the maximum level at 12 h after hemin administration, as did HO-1 (Fig. 2). The increases of Trx and PrxI were slight compared with the increase in HO-1 expression.

In order to identify which signal cascade controlled Nrf2 activation, mitogen-activated protein kinases (MAPKs) and PI3K pathways were investigated. PI3K was phosphorylated 60–120 min after hemin administration (Fig. 3A), whereas MAPKs were not phosphorylated (Fig. 3B). In addition, the effects of MAP kinase inhibitors and PI3K inhibitors were examined (Fig. 4). Neither the nuclear translocation of Nrf2 (Fig. 4A) nor the induction of oxidative stress-related proteins (data not shown) by hemin was inhibited by the MAPK in-

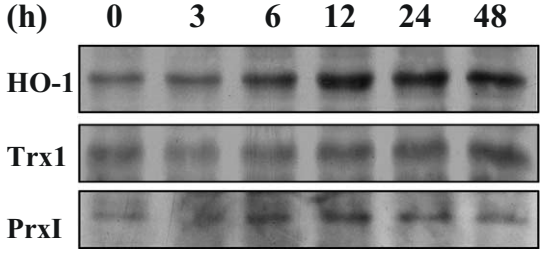

Fig. 2. Induction of HO-1, Trx, and PrxI in SH-SY5Y cells by hemin. A dramatic increase of HO-1 is observed in SY-SY5Y cells. Immunoblot analysis using HO-1 antibody demonstrates the induction of HO-1. Maximal induction is observed at 12 h after hemin administration. Increases in the expression of Trx and PrxI by hemin are also shown. Time course immunoblot experiment shows an upregulation of Trx and PrxI with minor additional induction on the basal expression level.

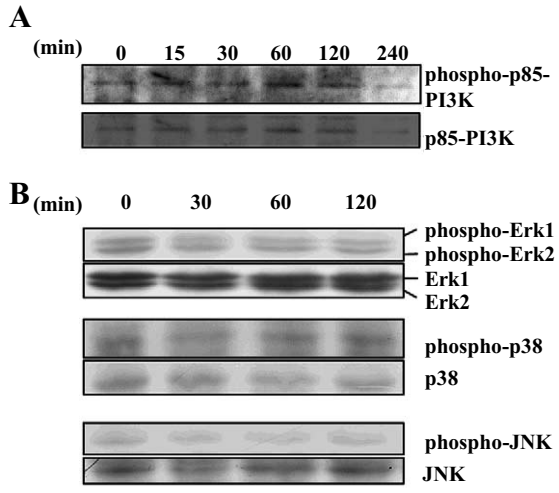

Fig. 3. Signal transduction from hemin to Nrf2 is regulated by PI3K. A: PI3K is phosphorylated by a hemin-derived signal. Immunoreactivity is detected using PI3K antibody and phospho-PI3K antibody. Phosphorylation of PI3K is shown at 60–120 min after hemin administration. B: MAPKs, Erk1/2, p38, and JNK are not significantly phosphorylated by hemin administration.

hibitors, PD98059, SB203580, and SP600125. In contrast, the PI3K inhibitors, wortmannin and LY294002, dramatically inhibited both the nuclear translocation of Nrf2 (Fig. 4A) and induction of oxidative stress proteins (Fig. 4B).

The regulation of antioxidative proteins is required for the protection of cells under stress conditions. We have shown that HO-1, Trx, and PrxI are induced in SH-SY5Y cells by hemin administration. Our present data indicate that the inductions of these antioxidative proteins are controlled by Nrf2 activation, and that the PI3K pathway may play an important role in the activation of Nrf2. On the other hand, the MAPK inhibitors, PD98059, SB203580, and SP600125, did not inhibit the Nrf2 activation by hemin.

Nrf2 and Keap1 have been reported to be the primary sensors in the cellular response to oxidative stress [27,28]. The cytoplasmic protein, Keap1, interacts with Nrf2 and inhibits its nuclear translocation [27,28]. In addition, the disulfide bond in Keap1 is thought to be a sensor for oxidative conditions [28]. Signals associated with oxidative stress disrupt the binding between Nrf2 and Keap1, resulting in the translocation of Nrf2 into the nucleus [27,28]. In the nucleus, Nrf2 forms a heterodimer with small-Maf protein and activates the transcription of oxidative stress-related proteins via an ARE [20,27]. In Nrf2-deficient macrophages, the inducible expression of antioxidative proteins is not observed, suggesting that Nrf2 may play a significant role in inducing the transcription of genes encoding oxidative stress-related proteins [20,27].

In this report, we demonstrated that the PI3K pathway regulates the Nrf2-dependent inducible expression of HO-1, Trx, and PrxI. PI3K phosphorylates phosphatidylinositol at the D-3 position of the inositol ring and has been shown to form a heterodimer consisting of an 85 kDa and 110 kDa subunit [29]. The role of PI3K in intracellular signaling has been underscored by its implication in a plethora of biological responses such as cell growth, differentiation apoptosis, calcium signaling and insulin signaling [30]. Among the downstream targets of PI3K are phospholipase C and serine/threonine kinase Akt. Akt/PKB, one of the most well studied downstream targets of PI3Ks, protects cells from apoptosis through the phosphorylation and inhibition of the apoptotic signals [31]. Based on these diverse effects of PI3K, and because the induction of oxidative stress-inducible proteins is thought to be a protective response in cells, we were interested in determining whether PI3K is involved in ARE regulation [32]. The present experiments clearly demonstrated not only PI3K phosphorylation, but also the inhibition of Nrf2 activation by PI3K inhibitors.

Recently, some reports have referred to the association of Nrf2 nuclear translocation with the induction of antioxidative proteins [20,27]. These studies indicate that Nrf2 usually binds to Keap1 in the extranuclear space, and after suitable stimulation, Nrf2 translocates into the nucleus and acts as a transcription factor. Nrf2 forms a heterodimer with small-Maf and activates ARE. Since ARE is widely conserved in the enhancer or promoter regions of antioxidative protein genes, the activation of Nrf2 may induce the expression of ARE-driven genes [21,33]. Antioxidative proteins, such as the Trx super families, require mutual oxidation–redox systems between each other. Therefore, the co-induction of these proteins may be a reasonable mechanism to contribute to the tolerance of cells to oxidative stress. Ishii, using Nrf2 null mice, reported that Nrf2 activation associates with an inducible expression, and not a constitutive expression of oxidative stress-related proteins [20].

In this report, the co-induction of these proteins was shown, suggesting a reasonable mechanism of oxidative stress protection exists in SH-SY5Y cells. Clarifying the antioxidative mechanism associated with PI3K and Nrf2 is important to understand the resistance of cells to oxidative stress and oxidative stress-involved diseases such as PD.

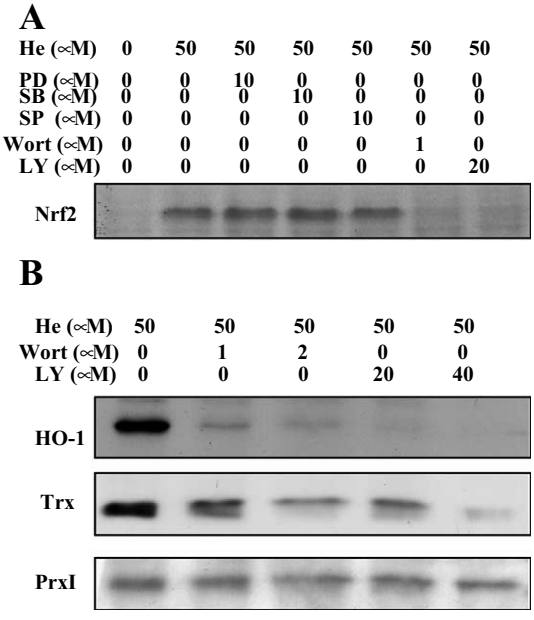

Fig. 4. The PI3K inhibitors, wortmannin and LY29004, block the Nrf2-related uptake of HO-1, Trx, and PrxI under hemin-treated conditions. A: Immunoblot by Nrf2 antibody of the nuclear fraction. Wortmannin and LY29004 inhibit the hemin-mediated nuclear translocation of Nrf2. B: Wortmannin and LY29004 inhibit the hemin-mediated induction of HO-1, Trx and PrxI. PD: PD98059, SB: SB203580, SP: SP600125, Wort: wortmannin, LY: LY294002.

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