Nitrated Proteome in Human Embryonic Stem Cells

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Abstract: Post-translational modifications (PTMs) of proteins regulate self-renewal and differentiation in embryonic stem cells (ESCs). Nitration of tyrosine residues of proteins in ESCs modulates their downstream pathways, which can affect self-renewal and differentiation. However, protein tyrosine nitration (PTN) in ESCs has been rarely studied. We reviewed 23 nitrated sites in stem cell proteins. Functional enrichment analysis showed that these nitrated proteins are involved in signal transduction, cell adhesion and migration, and cell proliferation in ESCs. Comparison between the nitrated and known phosphorylated sites revealed that 7 nitrated sites had overlapping phosphorylated sites, indicating functional links of PTNs to their associated signaling pathways in ESCs. Therefore, nitrated proteome provides a basis for understanding potential roles of PTN in self-renewal and differentiation of ESCs.

Keywords: Tyrosine nitration, Nitrotyrosine enrichment, LC-MS/MS, Proteomics, Embryonic stem cells

Introduction

Nitration of tyrosine residues modulates the activity of proteins, thereby affecting their downstream processes.¹–³ Protein tyrosine nitration (PTN) plays key roles in regulating signaling and metabolic pathways under diseased conditions.²–³ This PTN occurs through the action of reactive nitrogen species (RNS) under conditions of oxidative stress.⁴ A growing body of evidence suggests that PTN can play roles in self-renewal and differentiation of embryonic stem cells (ESCs)⁵,⁶ during early embryogenesis, nitric oxide (NO) synthases are expressed in the growing embryo, implying the presence of RNS gradients in the developing organs.⁷–⁹ The exposure of ESCs to a high concentration of NO promotes differentiation of ESCs though repression of NANOG.¹⁰–¹² Nitration of pyruvate kinase isoform M2 mediated by RNS impairs proliferation of neural progenitor cells. These data collectively indicate that PTN can affect cellular processes related to self-renewal and differentiation of stem cells.¹⁵–¹⁶ However, PTN in ESCs and its roles in self-renewal and differentiation of ESCs have been rarely studied.

Recently, mass spectrometry (MS)-based profiling methods have been employed to identify post-translationally modified proteins and understand their roles in stem cells.¹⁷ For example, Brill et al. profiled phosphorylated proteomes of pluripotent cells and their differentiated cells and found that the phosphorylation of receptor tyrosine kinases plays significant roles in maintaining self-renewal of ESCs.¹⁸ Therefore, MS-based profiling of nitrated proteomes in ESCs and their differentiated cells is critical to identify nitrated proteins and to understand their potential roles in self-renewal and differentiation of ESCs. However, liquid chromatography (LC)-MS/MS analysis of nitrated proteomes has been hampered due to a limited number of proteins undergoing PTN and low abundance of nitrated proteins.¹⁰ To resolve this problem, we previously developed a fluorine-fluorine interaction-based affinity purification method to selectively isolate nitrated peptides.¹⁹

Here, we briefly reviewed a total of 23 nitrated sites from nitrated proteomes of stem cells and their differentiated cells. PTN exerts its functions by modulating phosphorylation of the nitrated tyrosine or neighboring serine/threonine residues.²⁰ Thus, the comparison between the detected nitrated and known phosphorylation sites can reveal functional implication of the nitrated sites.
Furthermore, PTN modulates the activity of the proteins, thereby affecting activities of their interacting proteins in the downstream pathways. In this review, we reviewed these aspects to understand potential roles of the nitrated proteome in self-renewal and differentiation of stem cells. Therefore, our nitrated proteome provides a basis for understanding potential roles of PTN in self-renewal and differentiation of stem cells.

**Backgrounds**

Post-translational modifications (PTMs) modulate the activity of most of proteins and a variety of biological processes. Specifically protein tyrosine nitration (PTN) has been proposed as possible modification which regulates enzymatic activity of the modified proteins, not just as a marker for oxidative stress.\(^1\) During PTN, nitrogen dioxide radical (NO\(_2^+\)) is attached at the ortho position of hydroxyl group on tyrosine residues. Peroxynitrite (ONOO\(^-\)), which is produced by the combination of nitric oxide (NO\(^+\)) and superoxide anion (O\(_2^-\)) is necessarily needed to initiate this modification.\(^2\)\(^-\)\(^3\) The reactive oxygen species (ROS) and the reactive nitrogen species (RNS) are key players to make proteins nitrated. The physicochemical properties of nitrated proteins have been reported to be affected as a consequence.\(^4\) PTN had been considered as an irreversible process, but many evidences that nitrative process is reversible have been suggested recently.\(^5\)\(^-\)\(^6\) Understanding that nitration is reversible process controlled by certain conditions or factors, PTN may play as a regulator of several signal transductions or protein-protein interactions.

Recently, Knyushko *et al.* reported that protein tyrosine nitration (PTN) of Ca-ATPase resulted in loss of Ca-ATPase activity and reduced transport function.\(^7\) It was suggested that nitration provides a mechanism for down-regulation of ATP utilization by the Ca-ATPase.\(^8\) It was reported that PTN was also associated with various pathological events, such as cardiovascular diseases and hypertension condition in kidney.\(^9\) Under hypertensive condition, induced nitric oxide signaling may increase nitrosative and oxidative stress, which could generate high concentration of proteins containing 3-nitrotyrosine. Tyther *et al.* identified numerous nitrated proteins in the medulla of hypertensive rats.\(^10\)

**Method developments for analysis of nitrated peptides**

Although the importance of PTN has been realized, analysis of PTN is difficult due to its low abundance and rapid turnover.\(^11\) Hence effective enrichment method has been requested to analyze nitro-proteome. Some research groups have reported to identify nitrated proteins by the combined use of antibody against nitrotyrosine, 2D-PAGE and mass spectrometry.\(^12\)\(^-\)\(^14\) Several publications on affinity enrichment methods for the low abundant nitrated peptides have been reported lately.\(^15\)\(^-\)\(^17\) Most of these methods are based on immunoprecipitation and chemical conversions followed by affinity chromatography. We previously introduced an enrichment method of nitrated peptides based on metal chelating of bis-pyridines introduced to nitro-tyrosine residues. The resulting nitrated peptides with bis-pyridines formed a complex with Ni\(^2+\) - NTA magnetic beads, so that the nitrated peptides or proteins could be isolated from the solution.\(^18\) Recently, we developed another enrichment method for nitrated proteins by using modified fluorous solid phase extraction (FSPE).\(^19\) FSPE utilized distinctive property of fluorine to isolate effectively fluorous-labeled peptides from non-labeled peptides mixture.\(^20\)\(^-\)\(^21\)

**Identification of nitrated proteomes from ESCs and their differentiated cells**

Reactive nitrogen species (RNS) plays roles in escaping pluripotent cells from the self-renewing state and then initiating the differentiation. Furthermore, protein tyrosine nitration (PTN) generated from the RNS appears to promote the differentiation of pluripotent cells.\(^22\) To investigate potential roles of PTN in ESCs, the nitrated proteome profiling is critical to identify nitrated proteins in ESCs and their differentiated cells, providing the difference in nitrated proteomes between undifferentiated ESCs and differentiated cells. Recent developments in high-throughput measurement technologies and equipment have offered new opportunities for research of human embryonic stem (ES) cells. The unique properties of stem cell were studied through a large amount of information from reported ES data. Many research groups focused on post-translational modifications (PTM) of proteins in ES cells. Especially, Jeroen *et al.* reported that effect of phosphorylation on differentiation of hES cells.\(^23\)

PTN has been reported to promote differentiation of pluripotent cells.\(^24\) To investigate this characteristic of PTN at the proteome level, we first selected a total of 23 nitrated peptides in ES cells and their differentiated cells (Table 1) from previous results and our own results.

**Cellular processes associated with nitrated proteins**

PTN often occurs in signaling molecules, thus modulating activities of their associated signaling pathways.\(^25\) To examine what cellular processes are associated with PTN in ESCs and their differentiated cells, we performed function enrichment analysis to identify GO biological processes (BPs), molecular functions (MFs), and cellular components (CCs) enriched by the 23 nitrated proteins (NPs) using DAVID software.\(^26\) The GOBP most significantly represented by the 23 NPs were ‘cell