# A NOVEL NRF2-MEDIATED ANTIOXIDATIVE AND ANTI-INFLAMMATORY MECHANISM THROUGH FERROPORTIN 1 INDUCTION: A POTENTIAL CYTOPROTECTIVE MECHANISM AGAINST ISCHEMIA-REPERFUSION INJURIES

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**Abstract** The long-term function of a graft after transplantation is dependent upon ischemia-reperfusion (I/R) injury, which represents the most important alloantigen-independent damage processes during transplantation. Basic leucine zipper (b-Zip) transcription factor NF-E2-related factor 2 (Nrf2) is protective against I/R injury by promoting antioxidative and anti-inflammatory mechanisms via up-regulation of antioxidant-responsive element (ARE)-regulated genes such as heme oxygenase 1 and γ-glutamylcysteine ligase. We previously demonstrated that macrophage Nrf2 plays an important role in an elastase-induced acute lung injury model. To further clarify the mechanisms that act downstream of Nrf2, we performed microarray analysis in the RAW264.7 murine macrophage cell line using endogenous electrophilic prostaglandin 15-deoxy-$\Delta^{12,14}$-prostagrandin $J_2$ (15d-PGJ$_2$). As a result, we identified ferroportin 1 (Fpn1) as a novel Nrf2 target gene. Fpn1 is the sole iron exporter in mammals and regulates iron homeostasis. On the other hand, free intracellular iron is detrimental to cells by enhancing both inflammation and Fenton reaction-mediated oxidative stress. We demonstrated that Nrf2 activation by several electrophilic compounds commonly result in the up-regulation of *Fpn1* mRNA in bone marrow-derived and peritoneal macrophages obtained from wild-type, but not from *Nrf2* knockout mice. Furthermore, Nrf2 activation enhances iron release from the murine macrophage cell line J774.1. It is previously reported that LPS suppresses *Fpn1* mRNA expression, which leads to iron retention in monocytes and macrophages. We showed that while LPS suppress *Fpn1* mRNA expression in human macrophages, Nrf2 activation restores the expression of *Fpn1*. Thus, we propose that iron-metabolism regulation may be an important factor of the Nrf2-mediated cytoprotective mechanism.

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## 1. Iron homeostasis is important for normal cell function.

Iron is an essential micronutrient for cell survival and proliferation in mammal. Excess cellular free iron generates reactive oxygen species (ROS) through the Fenton reaction and damage lipid membranes, proteins, and nucleic acids, while iron deficiency affects cell growth and leads to cell death[1-3]. Therefore, the cellular iron level is strictly regulated at the levels of uptake, storage and export.

## 2. Ferroportin 1 is the sole iron exporter in mammalian cells.

Ferroportin 1 (Fpn1) is the sole non-heme iron exporter in mammals and is highly expressed in duodenal enterocytes and tissue macrophages[1]. Fpn1 is localized to the basolateral membranes of duodenal enterocytes and plays an important role in iron absorption from diet[4]. Since dietary iron

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intake is restricted, a large amount of iron for daily requirement is recycled from the senescent red blood cells by the reticuloendothelial macrophages[1, 2]. After the phagocytosis of senescent erythrocytes by macrophages, the heme-derived iron is subsequently exported to circulation by the cooperation of heme oxygenase 1 (HO-1) and Fpn1[4]. Recently, it is suggested that Fpn1 is also expressed in the brain and might be involve in iron export from neural cells[5, 6]. Fpn1 expression is controlled by both transcriptionally and post-transcriptionally. *Fpn1* mRNA expression levels are transcriptionally regulated by erythrophagocytosis or iron overload in macrophages[7-9], but the mechanisms are not completely understood. Post-transcriptionally, Fpn1 protein expression is regulated either through translational regulation by an iron-responsive element (IRE) within the mRNA[10] or through protein degradation by hepcidin, a peptide hormone for iron homeostasis mainly produced by hepatocytes in response to iron, inflammatory stimuli, and hypoxia[11-13].

## 3. Transcription factor Nrf2 regulates cytoprotective genes.

NF-E2-related factor 2 (Nrf2) is a basic leucine zipper (b-Zip) transcription factor and plays an important role in the cytoprotection against oxidative stress through the induction of antioxidant responsive element (ARE) -regulated genes[14]. Under unstimulated conditions, Nrf2 is bound by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and subject to the proteasomal degradation. The cysteine residues in Keap1 are modified by oxidative stress or electrophiles and Nrf2 is liberated from Keap1-mediated degradation. Subsequently, Nrf2 translocates from the cytoplasm into the nucleus and binds to AREs in the regulatory regions of the genes that encode antioxidant enzymes that protect the cell from the oxidative

damage. Thus, Nrf2 protein level is regulated by Keap1-mediated degradation process, and the stabilization of Nrf2 is required for the cellular protective response to oxidative and electrophilic stresses[15, 16]. Many bioactive food components such as sulforaphane (SFN), an isothiocyanate from broccoli sprouts, resveratrol from grapes, curcumin from turmeric, and carnosic acid from rosemary also activate Nrf2[17-20]. Nrf2 is ubiquitously expressed in mammalian tissues and Nrf2/ARE system regulates a battery of cytoprotective genes such as drug-detoxifying enzyme (glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1)), antioxidant defense enzymes such as HO-1, enzymes used in glutathione synthesis (subunits of glutamate-cysteine ligase), enzymes for NADPH generation (malic enzyme, etc), and phase III drug transporters[21] (Figure 1). Ablation of Nrf2 increases the cellular sensitivity to oxidative stress and inflammation[22-24]. The activation of Nrf2 signaling pathway has a protective effect against oxidative stress including ischemia-reperfusion injury and inflammation in the brain[25, 26].

## 4. Activation of Nrf2 up-regulates *Fpn1* mRNA expression and enhances iron efflux through Fpn1 induction.

Recently, we and others independently reported that Nrf2 regulates *Fpn1* mRNA expression in macrophages[27, 28]. One report comes from the study regarding the heme-mediated regulation of iron homeostasis. It was previously reported that heme activates *Fpn1* transcription in an iron-independent manner during erythrophagocytosis[29]. Marro S. *et al.* found that heme-mediated *Fpn1* mRNA induction is achieved through Nrf2 and BTB and CNC Homology 1 (Bach1) [27]. Bach1 also belongs to the CNC transcription factor family. It bind to ARE-like sequences and acts as a
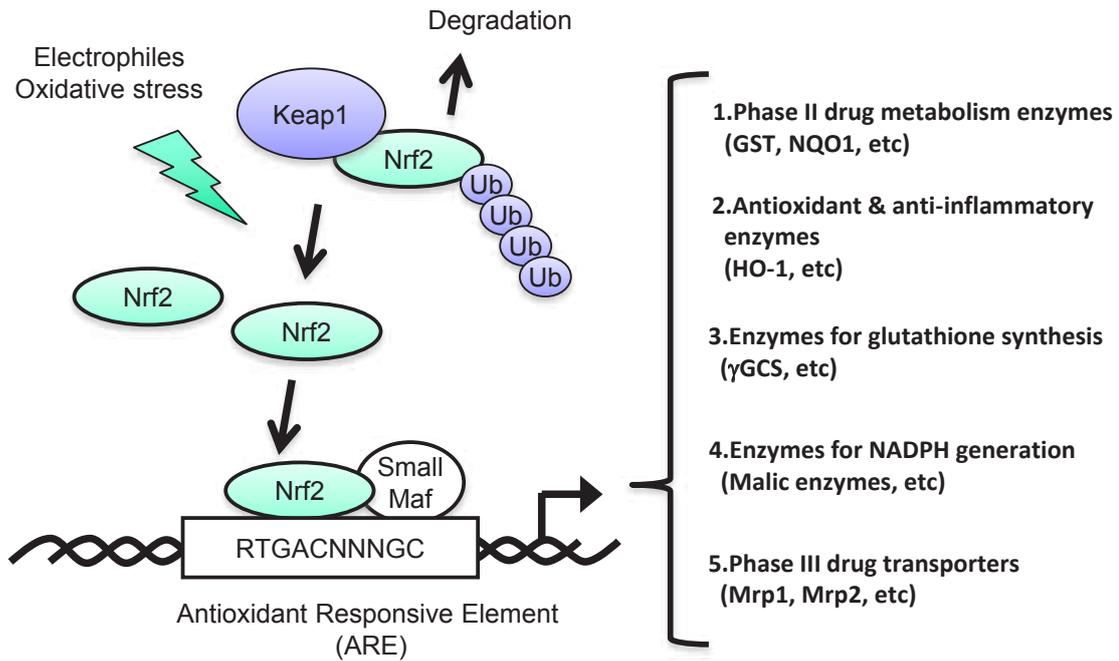
**Figure 1** Nrf2/ARE system regulates a battery of cytoprotective genes.

transcriptional repressor in a battery of ARE-regulated genes including *HO-1* [30]. Bach1 export from the nucleus and its protein degradation are enhanced by heme[31]. Thus, heme inhibits the repressor function of Bach1 and enhances *HO-1* gene expression. Furthermore, Nrf2 is also activated by heme and enhances *HO-1* gene expression. According to Marro *et al.*, heme also increases *Fpn1* mRNA in the RAW264.7 murine macrophage cell line and heme-mediated *Fpn1* up-regulation is controlled by an ARE element approximately 7kb upstream from the *Fpn1* transcription start site [28]. They also showed that Nrf2 positively regulates Fpn1 promoter activity while Bach1 negatively regulates its activity. Thus, they hypothesize that iron recycling in macrophages are transcriptionally co-regulated by heme through Bach1 and Nrf2. We have been interested in Nrf2-mediated anti-inflammatory mechanisms. We previously demonstrated that macrophage Nrf2 plays a critical role in an elastase-induced acute lung injury model [32]. To further elucidate the

mechanisms acting downstream of Nrf2, we performed microarray analysis in RAW264.7 macrophages using endogenous electrophilic prostaglandin 15d-PGJ$_2$. As a result, we identified *Fpn1* as a novel Nrf2 target gene[27]. Nrf2 activation by several electrophilic compounds commonly resulted in the up-regulation of *Fpn1* mRNA in macrophages derived from wild-type mice but not from *Nrf2* knockout mice. Further, Nrf2 activation enhanced iron release from the murine macrophage cell line J774.1. Previous investigations showed that inflammatory stimuli, such as LPS, down-regulates macrophage *Fpn1* expression by transcriptional and hepcidin-mediated post-translational mechanisms leading to iron sequestration by macrophages[33-36]. We showed that two Nrf2 activators, diethyl maleate and SFN, restored the LPS-induced suppression of *Fpn1* mRNA in macrophages[27]. These results suggest that Nrf2 regulates iron efflux from cells through *Fpn1* gene transcription and Nrf2 may control iron metabolism during inflammation. Recent studies revealed that the

decrease of intramacrophage iron levels due to Fpn1 up-regulation attenuates TLR4-mediated inflammation by inhibiting expression of pro-inflammatory cytokines, such as IL-6, TNFα, and INF-β[37, 38].

## 5. An emerging role of Fpn1 as an anti-inflammatory factor

In addition to the above-mentioned anti-inflammatory role of Fpn1 through iron metabolism, it is recently reported that Fpn1 activates intracellur signaling for inflammatory gene expression upon hepcidin binding[39]. Hepcidin induces Fpn1 degradation and internalization *via* activation of Jak2 and the subsequent phosphorylation of Fpn1. De Domenico *et al.* demonstrated that Jak2 activated upon hepcidin binding also phosphorylates Stat 3 that results in the upregulation of genes such as *Interleukin (IL) 7, IL17 receptor* and *SOCS*. Hepcidin pretreatment inhibits LPS-induced TNFα and IL-6 gene expression via SOCS induction. *In vivo*, hepcidin pretreatment rescues LPS-induced lethality.

## 6. Nrf2 may have a beneficial effect for ischemia-reperfusion injury through Fpn1 induction.

Ischemia-reperfusion injury occurs when blood flow to a tissue is disturbed and subsequently recanalized. It is related to a various disease states including myocardial infarction, stroke, and post-transplantation injury [40-42]. Hypoxia during ischemia and subsequent reoxygenation by reperfusion are thought to be the major factors contributing to ROS production and the subsequent cellular damage[43]. Iron is believed to play a critical role in neuronal injuries caused by oxidative stress in cerebral ischemia[44]. In the brain, it is well known that free iron significantly increases during ischemia and is responsible for oxidative damage. Increased amount of free iron and ferritin have been observed in ischemic brain[45, 46], while the iron chelator desferrioxamine (DFO) diminish the damaging role of free iron in the ischemic brain[47, 48]. Recently, Ding *et al.* have shown that ischemia increases intracellular iron contents by reducing Fpn1 expression in the brain[49]. Furthermore, Leonard *et al.* demonstrated that reoxygenation-specific activation of the Nrf2 mediates cytoprotective gene expression in ischemia-reperfusion injury[50]. Thus, we propose that the regulation of iron metabolism may be an important aspect of the Nrf2-mediated cytoprotective mechanism and it may have the protective role against ischemia-reperfusion injury (Figure 2).

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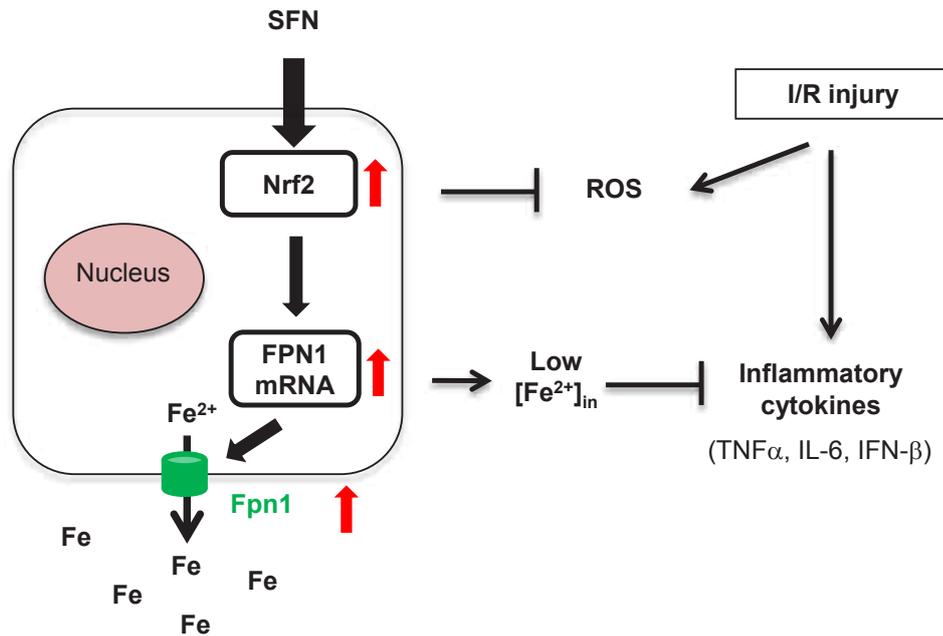
**Figure 2** Nrf2 might be capable of improving iron homeostasis during I/R-induced inflammation by increasing *Fpn1* transcription along with the genes that have anti-inflammatory functions.

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