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Hypoxia induces changes in expression of isoforms of the divalent metal transporter (DMT1) in rat pheochromocytoma (PC12) cells

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Abstract

Although hypoxia has been shown to increase the expression of a variety of proteins involved in iron homeostasis, including transferrin and its receptor, little is known about the effect of low oxygen on formation of isoforms of the major iron transport protein, divalent metal transporter 1, DMT1. Accordingly, we examined the effects of hypoxia on expression and subcellular distribution of the different isoforms of DMT1 in rat PC12 cells. Treatment with low oxygen modestly increased expression of protein and mRNA levels for both the +IRE and −IRE species of DMT1. In contrast, expression of the exon 1A containing species of DMT1 was greatly increased by hypoxia as indicated by Western blot and real-time RT-PCR analysis. Message levels for the 1A isoforms increased approximately 60-fold after exposure of PC12 cells to 1% oxygen for 5 h. The subcellular distribution of exon 1A isoforms of DMT1 remained consistently in the cytoplasmic milieu of the cell after hypoxic exposure, as also did the distribution of +IRE species of DMT1. The −IRE species of DMT1, however, responded to hypoxia by becoming increasingly associated with the regions adjoining the outer cellular membranes, while a portion partially colocalized with an early endosomal marker (EEA). Hypoxia also caused a significant increase in the uptake of manganese in PC12 cells. In summary, these results demonstrate that hypoxia selectively increases expression of exon 1A containing species of DMT1 with lesser increases in either the +IRE or −IRE isoforms the transporter.

Keywords: Divalent metal transporter 1; DMT1; PC12 cells; Hypoxia; HIF; Manganese; Iron response element

Transition metals such as manganese and iron are essential for normal homeostatic functioning of brain. Several abnormal neurological conditions, however, are associated with deposition of iron and/or manganese in the lesioned brain areas and these metals have been proposed to contribute to the observed neurological deficits [1,2]. It is not unlikely that the damaged regions of the brain may experience ischemic conditions [3] leading to compensatory changes in cellular processes controlling and maintaining normal balance of metals [4]. Since regulation of the physiological actions of metals within the CNS is dependent on their cellular transport across the blood–brain barrier and their subsequent uptake into neuronal cells, it is important to understand the influence that hypoxic conditions may have on transport of required transition metals within these lesioned areas. In this regard, prior studies have already demonstrated that hypoxic conditions are reported to increase cellular uptake of iron [5] and expression of transferrin (Tf) [6] and the transferrin receptor (TfR) [7,8].

The divalent metal transporter 1 (DMT1; NRAMP2, DCT1 or SLC11A2) along with both Tf and TfR plays a vital role in metal ion transport into cells. Several studies have reported that DMT1 functions as a transporter for a variety of metals including iron, manganese, cobalt, copper, cadmium and nickel [9–11]. DMT1 is highly hydrophobic, with twelve predicted transmembrane domains [12] and is ubiquitously expressed throughout the body [11]. There are two splice variants of DMT1 which differ in 3′-end of the message [9]. One form contains an iron responsive element (+IRE) in the 3′-untranslated region of the message capable of binding iron response proteins (IRP) resulting in the stabilization of the message.

Abbreviations: DMT1, divalent metal transporter 1; HIF, hypoxia inducing factor; HRE, hypoxic response elements; IRE, iron response element; IRP, iron response proteins; Tf, transferrin; TfR, transferrin receptor

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Accordingly, this form of DMT1 may be similar to the transferrin receptor (TfR) in that it potentially can be regulated by iron. The second mRNA form, lacking the IRE (−IRE), is presumably incapable of being regulated by iron, at least by an IRE/IRP interaction. The polypeptides produced by the +IRE mRNAs contain 18 amino acid residues at the carboxy-terminus distinct from that encoded by −IRE mRNAs; likewise the −IRE DMT1 has 25 unique amino acid residues at the carboxy-terminus. These two splice variants of DMT1 are differentially expressed in tissues and each form can be transcribed from two alternative promoters, presumably upstream of either exon 1A or exon 1B, resulting in four predicted DMT1 mRNA isoforms differing in both their 5′- and 3′-ends [13].

The 1B promoter region of human DMT1 has been proposed to contain two motifs that are similar to a sequence recognized by HIF-1 (hypoxia-inducible factor) in human erythropoietin gene [14], suggesting that, like erythropoietin, DMT1 may also be regulated by hypoxia. HIF-1, along with HIF-2 and HIF-3, belongs to bHLH-PAS family of transcription factors [15] that are induced by hypoxia and by some hypoxia mimicking iron chelators [16]. Under hypoxic conditions, HIF-1 alpha dimerizes with constitutively expressed HIF-1 beta and the resulting complex binds to the core DNA recognition sequence within the hypoxic response elements (HRE) of targeted genes. HIF-1 regulates transcription of many genes, presumably, helping cells to adapt to reduced oxygen conditions [17] including several genes important in iron metabolism, e.g. TfR, erythropoietin, heme oxygenase-1 and cerruloplasmin [7,18,19].

Prior studies from our laboratory demonstrate that expression of both the ±IRE isoforms of DMT1 are upregulated in rat pheochromocytoma (PC12) cells by treatment with manganese under iron deficient conditions [20]. Since iron deficiency mimics hypoxia conditions in that it results in the increased expression of HIF and since human DMT1 promoter contains a putative HIF-1 recognition sequence, studies were performed to determine whether expression and distribution of DMT1 could be regulated by hypoxia. Results of these studies reveal that expression of the 1A containing species of DMT1 is selectively increased in hypoxic-treated PC12 cells with only a modest upward trend in both the +IRE and −IRE isoforms of the transporter.

1. Material and methods

1.1. Cell culture and hypoxia treatment

PC12 cells were cultured in DMEM (Gibco, Grand Is., NY, USA) media supplemented with 10% FBS (Hyclone, Logan, UT) and 5% HS (JRH Bioscience, Lenexa, KS, USA) in the presence of 100 U/ml of penicillin and 100 μg/ml streptomycin. Cells were maintained at 36.5 °C in a humidified atmosphere containing 5% O_2, 5% CO_2 and 90% N_2. Cells were allowed to attach for approximately 16 h (approximately 70% confluency) before exposure to hypoxic (1% O_2, 6% CO_2, 93% N_2) or normoxic conditions for 2 or 5 h prior to harvesting for analyses.

1.2. Antibodies

Rabbit affinity purified polyclonal antibodies were prepared by Affinity BioReagents, Golden, CO as described previously [21]. The following peptides corresponding to C-terminal sequence of each protein were used as immunogens: +IRE: SISKVLLSEDTSGGNTK, −IRE: TARPEIYLLNTVDAVSLVSR and 1A N-terminal sequence: NCELKSYSKSTDPQVST. The synthesized C-terminal peptide fragment for generating the rat +IRE antibody was similar to that used previously [22], whereas the peptide used to raise the −IRE antibody was two amino acids shorter. Rabbit polyclonal antibody against HIF alpha was purchased from Novus Biologicals (Littleton, CO, USA), rabbit antibody against EPAS1 (endothelial PAS domain protein 1; HIF 2 alpha) from Santa Cruz Biotech (Santa Cruz, CA, USA) and murine monoclonal antibody against EEA (early endosomal antigen; Transduction Labs, San Diego, CA, USA).

1.3. Western blot

Cells seeded in 100 mm cell culture dishes were harvested when they attained approximately 80% confluency. They were subsequently lysed in buffer containing 0.1% SDS, 0.5% sodium deoxycholate and 1% Nonidet P-40 plus protease inhibitor cocktail (Roche, Switzerland). Protein content was quantified for each sample using the BCA method (Pierce, Rockford, IL, USA). Equivalent amounts of protein (10–25 μg) were loaded in each well of a 7.5 or 12% SDS–PAGE gel and transferred onto nitrocellulose membranes after electrophoresis. Membranes were blocked with 5% nonfat milk in TTBS and probed overnight at 4 °C with the different primary antibodies (1:10000) described above. After multiple washes with TTBS, the membranes were exposed to secondary antibody which was coupled to horseradish peroxidase in 5% nonfat milk in TTBS for 1 h at room temperature. After extensive washing, the complexes were visualized using West Pico chemiluminescent kit (Pierce). Specificity of the antibodies was verified by blocking the detection of bands with the specific immunizing peptide used to raise the three antibodies.

1.4. Real-time RT-PCR

Different forms of DMT1 mRNA were quantified by real time RT-PCR using detection with SYBR Green dye. Briefly, total RNA from hypoxic or normoxic growing

cells was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Two micrograms of total RNA were used for each amplification and each experiment was repeated four times in triplicate. Reactions were carried out in volume of 50 μl using a QuantiTect SYBR Green RT-PCR kit from Qiagen (Valencia, CA, USA) according to the manufacturer protocol.

Primers designed for real time RT-PCR were as follow: For the −IRE form, the forward primer was AAGGCGAAGAAAGATCTGGAG; the reverse primer was CCACAGGCCGCTGTTTG; for the +IRE form, the forward primer was GCTGAGCGAAGATACCAGCG; the reverse primer was TGTGCAACGGCACATACTTG; for exon 1A, the forward primer was TCCGATGGGGAAGAAGCAG0CC; the reverse primer was GGATCTGTGCTCTTAGAATAGG; for rat β-actin which served as the internal control, the forward primer was CTCATTGCCGATAGTGATGAC; and the reverse primer was AAGAGAAGCTGTGCTCTATGTTGC. PCR products were electrophoresed in 2% Agarose-1000 (Invitrogen) to confirm that PCR yielded a single product of the expected size. The expected sizes were 110 bp (+IRE), 140 bp (−IRE) and 84 bp (1A).

The results were analyzed by the ΔCt method which reflects the difference in threshold for the target gene relative to that of β-actin in each sample. To ensure validity of our calculations, we confirmed that primers sets used in this study have the same efficiencies as ascertained by varying template concentrations. In each case, the log of the template concentration when plotted against ΔCt yielded values of less then 0.1 for the slope.

1.5. Immunofluorescent staining

Cells were initially seeded on coverslips treated with poly-L-lysine (50 mg/ml) for approximately 20 h at 4 °C. Cells were subsequently fixed with fresh 4% paraformaldehyde for 20 min, washed with PBS three times, permeabilized with Triton X-100 (0.1%) for 5 min, blocked with 5% BSA in PBS for 1 h at room temperature and incubated overnight at 4 °C with primary antibody diluted (1:2000) in PBS containing 5% BSA. Cells were washed and incubated for 1 h with secondary antibody (anti-rabbit conjugated to Alexa 568, Molecular Probes, Eugene, OR) in PBS containing 5% BSA at room temperature. After washing, the coverslips were mounted using Vectashield medium (Vector, Burlingame, CA, USA), sealed with nail polish and viewed under confocal microscope (60× objective). Double staining was performed using antibody to DMT1 along with primary monoclonal mouse antibody against EEA. The secondary anti-mouse antibody used was conjugated with Alexa 488 (Molecular Probes).

1.6. $^{54}Mn^{2+}$ uptake assay

PC12 cells were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. When the cultures reached 60–70% confluency, they were exposed to hypoxic condition or normal O_2 tension for an additional 5 h at which time they were assayed for $^{54}Mn^{2+}$ uptake. Cells were washed twice with prewarmed (37 °C) incubation buffer, pH 7.4, containing 10 mM Hepes, 150 mM NaCl, 1% glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were subsequently resuspended in a similar buffer, although at pH 6.0, containing 80 nM $^{54}Mn^{2+}Cl_2$. Cells were incubated at 37 °C in a CO_2 incubator for various times (0, 2.5, 5, 10, 12.5 and 15 min) after which ice-cold incubation buffer, pH 7.4, was added to stop the reaction. Cells were then washed with cold buffer two more times, then detached with a cell scraper and the radioactivity determined using an LKB γ-counter.

2. Results

2.1. Hypoxia effect on DMT1 expression

To confirm that our experimental conditions (1% oxygen) resulted in a hypoxic environment, Western blots were performed to ascertain whether the HIF alpha transcription factor was indeed induced. Results of these experiments, shown in Fig. 1, demonstrate that both EPAS1 and HIF-1 alpha were increased (stabilized) by the hypoxic conditions employed.

To determine whether hypoxic-induced changes occurred in DMT1 mRNA expression, real time RT-PCR were performed on RNA isolated from extracts of PC12 cells maintained under normoxic or hypoxic conditions. Results of these studies, shown in Fig. 2, demonstrate that hypoxia modestly increased message levels for either the −IRE and +IRE isoforms of DMT1 in PC12 cells whereas expression of the 1A containing species of the transporter was increased approximately 60-fold.

Studies were also conducted to determine the influence of hypoxia on expression of the +IRE, −IRE and 1A isoforms of DMT1 protein. As illustrated in Fig. 3, both the +IRE and −IRE antibodies yielded multiple bands similar to those reported in prior papers [21–26]. Exposure of the PC12 cells to hypoxia for 5 h resulted in the upregulation of the −IRE forms of DMT1 protein, whereas the +IRE species remained relatively unchanged. The 1A isoform of DMT1, detected as a single band at approxi-

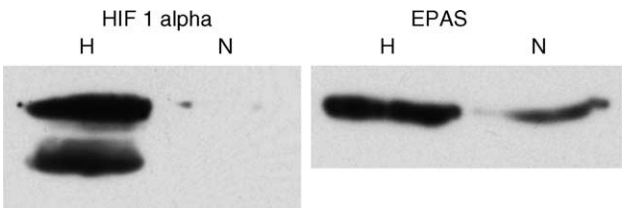

Fig. 1. Expression of HIF1α and HIF2α in PC12 cells exposed to normoxia or hypoxia. Western Blot analysis was used to determine protein levels of HIF1α and HIF2α in extracts from PC12 cells exposed for 5 h to normoxia (N) or hypoxia (1% oxygen; H).

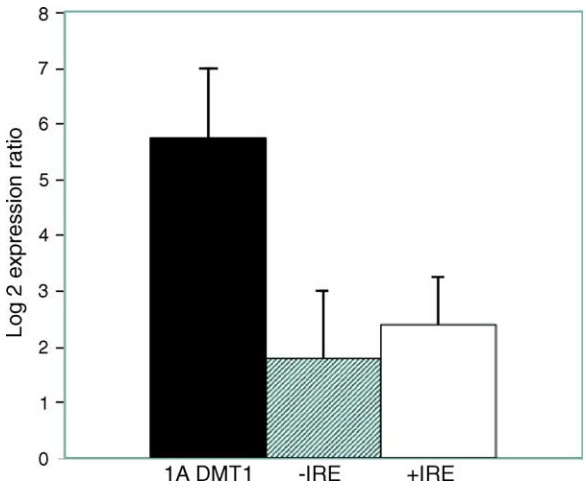

Fig. 2. Real-time RT-PCR analysis of mRNA levels for 1A, +IRE and −IRE containing isoforms of DMT1 in PC12 cells maintained under hypoxic and normoxic conditions. PC12 cells were incubated for 5 h under hypoxic conditions to determine changes in expression of different forms of DMT1. Expression of DMT1 was determined as described in Section 1 and normalized against β-actin mRNA for each sample. Results were analyzed by the ΔCt method which reflects the difference in threshold for the target gene relative to that of β-actin in each sample.

mately 50 kDa, was strongly upregulated by exposure to hypoxia when compared to control samples, where the 1A form was undetectable under the Western Blot conditions used. Since the −IRE and +IRE antibodies to DMT1 recognize only the C-terminal end of the protein, this 50 kDa band may represent either the +IRE or −IRE isoform of DMT1, a combination of both species or possibly even a truncated form missing the carboxy tail. Shorter exposure to hypoxia for only 2 h failed to alter the expression of any form of the DMT1 (data not shown).

2.2. Hypoxia effect on DMT1 distribution

Because prior studies [22] from our laboratory revealed that the −IRE form of DMT1 resides not only in the cytoplasm of PC12 cells but also in the nucleus, we investigated the effects of hypoxia on the subcellular distribution of this form of DMT1 as well as of other species of DMT1. The data reported in Fig. 4 reveals that the distribution of the −IRE isoforms of DMT1 in PC12 cells is not altered by hypoxia in that both cytoplasmic and nuclear staining is seen in both normoxic and hypoxic cultures. Hypoxia, however, resulted in the majority of the −IRE isoform clustering closer to the outer cellular membrane with little change in the fraction associated with the nucleus. The location of +IRE forms of DMT1 is consistently in the cytoplasm of the cells and remains essentially unchanged by hypoxia. Consistent with previous findings [27], the 1A isoform is restricted only to the cytoplasm and localization remains unchanged despite treatment with hypoxia. Although not readily discernable in this image, the 1A species of DMT1 is distributed in a punctuate pattern within the cytoplasm indicating that it may be expressed within the endosomal compartment. Lack of 1A staining in the nucleus may indicate that only forms of DMT1 that initiate in exon 2 are present in this cellular compartment. There was an increase in staining with this antibody after the cells were treated with hypoxia although the magnitude of this increase was not comparable to that expected based on Western blot analysis. This may be cause by the fact that immunofluorescent staining is not necessarily quantitative because of masking or differences in antibody saturation on epitopes of the protein in the intact cell.

In a previous publication from this laboratory [21], we confirmed the findings of Tabuchi et al. [28] that the −IRE isoforms of DMT1 colocalizes with early endosomal marker, EEA. Thus, we were interested to determine if these proteins would also colocalize in PC12 cells and whether hypoxia would alter this association. Results of these experiments, illustrated in Fig. 5, reveal that the extent of colocalization of the −IRE species of DMT1 with EEA was maintained even though there was an increase in the −IRE isoform which accumulated near the outer cellular membrane during hypoxia.

2.3. Hypoxia effect on transport of manganese

An important aspect of these studies was to establish whether the elevated levels of the 1A species of DMT1

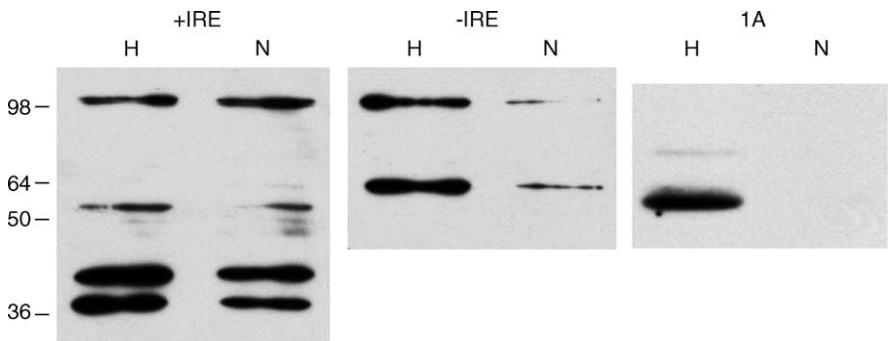

Fig. 3. DMT1 protein expression in PC12 cells under normoxic (N) and hypoxic (H) conditions. Western blot analysis of different species of DMT1 was performed on protein extracts from PC12 cells grown for 5 h under normoxic or hypoxic (1% of oxygen) conditions. Proteins were detected using affinity purified antibodies against 1A, −IRE or +IRE epitopes of DMT1.

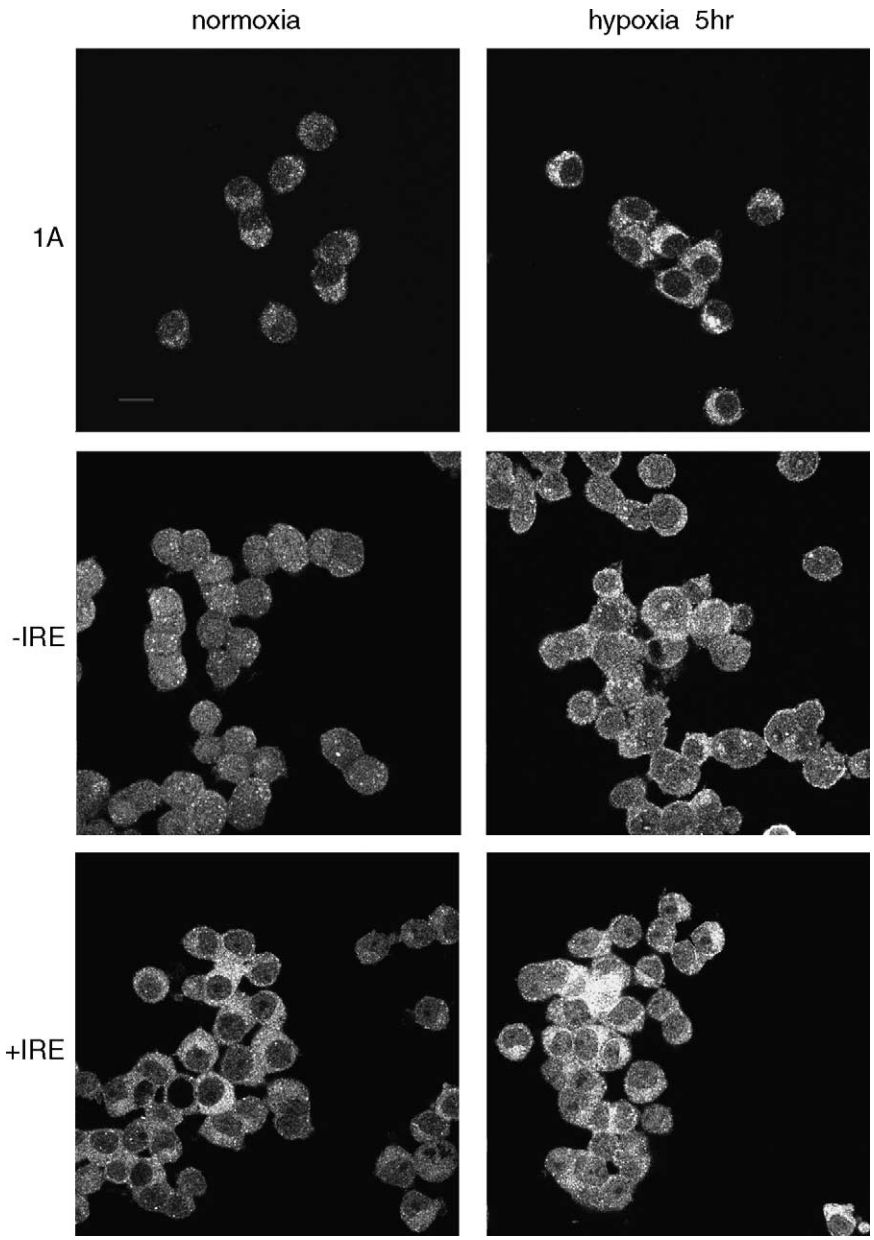

Fig. 4. Subcellular distribution of DMT1 in normoxic or hypoxic treated PC12 cells. PC12 cells were treated for 5 h under normoxic or hypoxic conditions. Immunofluorescent staining was accomplished using affinity purified antibodies recognizing either the −IRE and +IRE epitopes on the carboxy terminal end of the protein and exon 1A epitope on the N-terminal portion of DMT1. Confocal images were obtained using a Bio-Rad confocal laser microscope at 60× magnification. Scale bar = 10 μm.

seen after hypoxia resulted in increased transport of divalent metal ions. Accordingly, studies were performed to determine the effect of hypoxia on transport of manganese. Results of a typical experiment illustrated in Fig. 6 demonstrate that exposure of PC12 cells for five h to hypoxia significantly increased uptake of manganese by approximately 40%. In this experiment the rate for incorporation of Mn was 1.4-fold that for controls, with the difference highly significant by multiple regression analysis. Because the difference was modest, we repeated this experiment nine times and the results confirmed that a modest difference existed. In none of experiments, however, did the increase in transport correspond to the magnitude of change as that seen for mRNA of the 1A isoform of DMT1.

3. Discussion

The expression of a variety of proteins involved in iron homeostasis, such as erythropoietin, ferritin, cerruloplasmin, transferrin and TfR, has been reported to be induced during hypoxia [16–18,29]. These changes are compensatory to the low oxygen environment and presumably restore metabolism towards normal or functionally accep-

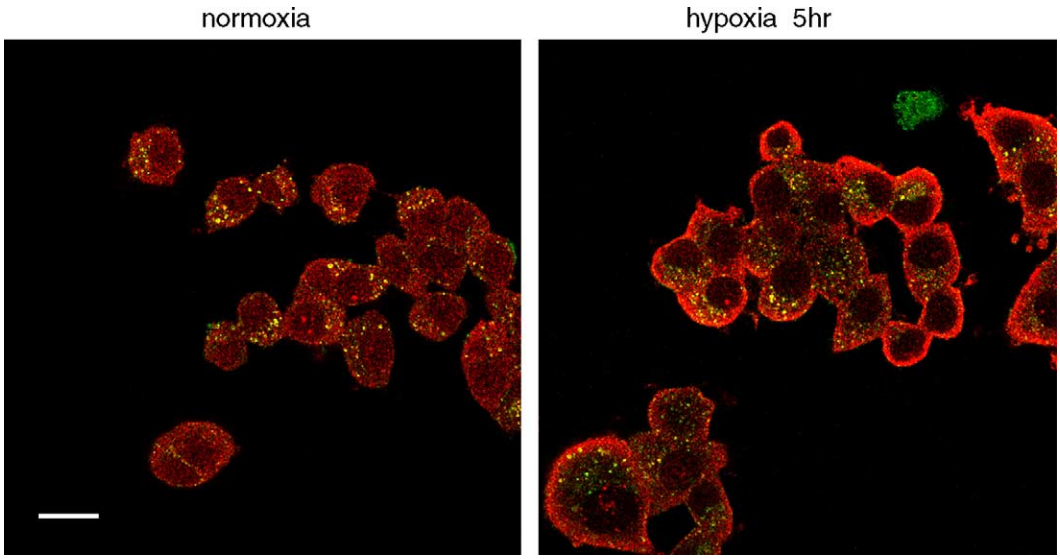

Fig. 5. Subcellular distribution of EEA and the −IRE form of DMT1 and in PC12 exposed to hypoxic or normoxic conditions. Localization of the −IRE form of DMT1 (red: Alexa 568) and EEA (green: Alexa 488) was determined in PC12 treated for 5 h under hypoxic or normoxic conditions. Confocal images were obtained using a Bio-Rad confocal laser microscope at 60× magnification. Orange or yellow color indicates colocalization. Scale bar = 10 μm.

table homeostatic conditions required for cell survival. As reported in this paper, DMT1, the principal transport protein for iron and other transition metals, behaves in an analogous fashion to these other essential components involved in iron homeostasis in that its expression is modified by hypoxia in a compensatory manner presumably to help preserve normal iron balance in vivo.

DMT1 exists in at least four different isoforms all of which appear to be capable of transporting divalent metals

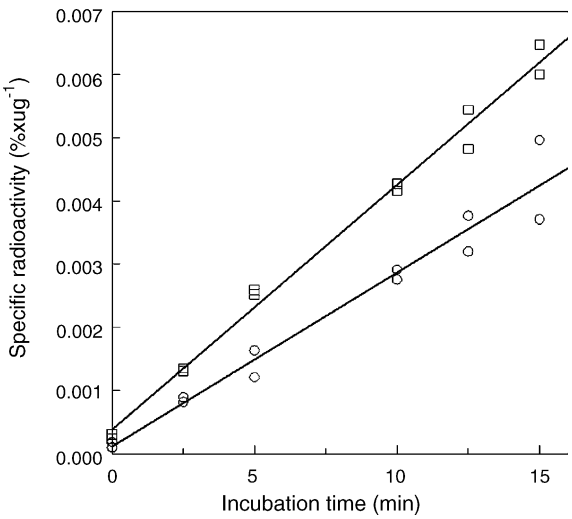

Fig. 6. Mn transport into PC12 cells exposed to hypoxic or normoxic conditions. PC12 cells cultured for 5 h under normoxic (○) or hypoxic conditions (□), then $^{54}Mn^{2+}$ was incubated with the cells at 37 °C in a CO_2 incubator for the times indicated, after which ice-cold incubation buffer, pH 7.4, was added to stop the reaction. Radioactivity was converted to percent uptake/μg protein. The rate for hypoxic incorporation was 1.4-fold that for control, with $P < 0.0005$ by multiple regression analysis in the data shown. This experiment is representative of nine independent replicates. Hypoxic incorporation of Mn exceeded that seen under normoxic conditions in all nine experiments (range 1.2–3.1), reaching significance in seven of them.

[13]. There is increasing evidence that the four species reside in distinct subcellular compartments but the function of each and what controls their expression in any given cell is not known [21,22,27]. Here we show that the +IRE isoforms of DMT1 exhibit little, if any, response to hypoxia arguing against the reported outcome as being a consequence of post-translational regulation via the iron response proteins 1 or 2. Thus, the regulatory mechanisms controlling DMT1 expression clearly go beyond the functional utility of the IRE in the message. For other examples, contrary to the expected change if only the IRE was regulating expression, Wang et al. [30] and Scheiber-Mojdehkar et al. [31] have demonstrated the selective increase of the −IRE form of DMT1 in rat pulmonary airway and HepG2 cells, respectively, upon exposure to iron. Studies in our laboratory [20] have similarly demonstrated increases in protein levels of both ±IRE forms of DMT1 in PC12 cells exposed simultaneously to both manganese and the iron chelating agent, desferrioxamine (DFO). Similarly, other studies reported the upregulation of DMT1 under treatment with DFO alone in other cell lines [32,33]. These results clearly imply that regulation of DMT1 is much more complex than previously thought, especially since there are two discrete promoter regions controlling DMT1 transcription, one for exon 1A and the other for exon 1B.

Our findings that 1A isoforms of DMT1 are selectively increased to a much greater extent than either of the two carboxy terminal species are difficult to rationalize based on current knowledge of the structures of DMT1 isoforms. It is interesting to note that a recent paper by Bedrine-Ferran et al. [34] reported a similar change in DMT1 expression in human Caco-2 cells undergoing differentiation. These investigators observed approximately a 40-fold upregulation of DMT1 mRNA(s) containing exon 1A in

differentiated Caco-2 cells compared to only a 7-fold change in the +IRE and 5-fold change in the −IRE isoforms of the transporter as analyzed by QRT-PCR. Interestingly, they also observed a lack of change in the 1B exon mRNA. Considering the fact that Caco-2 cells undergoing differentiation are normally tightly packed into discrete bundles, it is possible that the cells within the inner layers may experience mild hypoxic conditions. Whether hypoxia accounts for the selective change in the 1A containing species of DMT1 in these cells or whether the actual process of differentiation is responsible for this change is not known. Regardless of the mechanism, these data suggest that factors regulating the 1A forms of the transporter are distinct from those controlling the 1B isoforms and suggest the 1A forms may have a unique function required to maintain normal homeostatic control during hypoxic conditions.

Although the human 1B promoter contains a putative HRE [14], the relevant region in rats (based on our data) is that of the 1A promoter. Based on the relevant part of the rat genome project (accession number NW_047784), we were able to ascertain the putative promoter region from the rat genome and demonstrate that it contains a putative HRE as predicted by the MatInspector [35] program (Genomatix). Thus, the results reported in this manuscript suggest this putative HRE may be responsible for upregulating expression of this transporter although we can not rule out the possibility that HIF interactions with other nuclear transcription factors may also be involved [17,29].

The question as to why the 1A forms of DMT1 are upregulated without a proportional change in the ±IRE forms is a challenge to be explained, but a similar lack of proportionality for these forms of DMT1 was also observed previously [34]. It is possible that the 1A protein species are in relatively low abundance compared to the forms of DMT1 protein starting at exon 2 even after hypoxia treatment. If this were the case, then the relatively large increase in expression of the 1A species would not necessarily result in a proportional change in either the +IRE or −IRE isoforms as measured by either immunoblot or real-time RT-PCR. Comparisons of the different forms of DMT1 present in control cells from our real-time RT-PCR experiments, however, do not support this argument but actually demonstrate that message levels for the 1A and +IRE isoforms are similar under normoxia conditions while that for −IRE is only slightly lower (shows up ∼2 cycles later). It is also possible that while DMT1 encompassing the entire message from 1A to either the −IRE or +IRE carboxy terminus is upregulated, the corresponding 1B isoforms are proportionally down regulated such that total +IRE or −IRE remain relatively constant. It is equally possible that the 1A isoform of DMT1 expressed after treatment with hypoxia represents a new unidentified spliced variants of the message in which the +IRE or −IRE carboxy tail is removed.

The existence of unidentified spliced variants of DMT1 is also consistent with the multiple bands observed in the Western blot data presented in Fig. 1. Similar findings with other antibody preparations have previously been reported by others [21,23–25]. The observed high molecular weight species on our Western blot (∼100 kDa) with either the +IRE and −IRE antibody preparations may be indicative of a glycosylated form of the protein as described by Gruenheid et al. [10] and Tabuchi et al. [25]. This upper band was absent when 1A antibody was used suggesting that only isoforms of DMT1 that start in exon 2 may be glycosylated, at least under the conditions employed. It is not obvious, however, why different N-termini would affect glycosylation in a distal region of the peptide that is external to membranes while the N-termini themselves are internal in the predicted structure. We also considered the possibility that the higher molecular weight bands observed on the gels may correspond to aggregates of the transporter that form during preparation of samples for electrophoresis. Attempts, however, to eliminate the upper band by varying temperature, pH and reducing agents during preparations of our samples were ineffective. It is highly unlikely that these bands are artifacts since pretreatment of antibodies with immunization peptide totally abolished these signals. In addition, the strongest band for the +IRE form of DMT1, at about 40 kDa, is similar in size to the +IRE species of DMT1 (∼43 kDa) reported in rat duodenum by Yeh et al. [26]. Whether this protein possibly represents a degradation product of DMT1 is not known. As is becoming evident, the actual number of isoforms for DMT1 may actually exceed the four noted above especially in light of the findings of Lee et al. [12] identifying spliced variants missing either exon 10 or 12.

One of the major questions we wanted to address in this paper related to whether the observed increase in expression of the 1A isoform of DMT1 induced by hypoxia would translate to elevated cellular uptake of metals. Our results confirm that under hypoxic conditions manganese uptake was increased in PC12 cells when compared to cells maintained at normoxia. The apparent disparity, however, between the magnitude of the increase of manganese uptake (∼40%) and the increase expression of the 1A species of DMT1 (∼60-fold) produced by hypoxia may reflect the relative contribution that this form of the transporter supplies to the total uptake of manganese in these cells. As noted above, it is also possible that the small increase in manganese transport may depend on a compensatory decrease in the isoforms of DMT1 that start with exon 2 with the total production of DMT1 in the hypoxic treated cells not being very different than in control cultures. In addition, one also has to consider the possibility that other processes may also contribute to manganese uptake in PC12 cells and thus, changes in transport observed may not simply parallel differences in DMT1 expression [28].

In conclusion, our studies confirm that exposure of PC12 to short term hypoxic conditions strongly upregulates expression of the form of DMT1 possessing exon 1A peptide and base sequence. There was also some increase in expression of the −IRE species of DMT1 and a smaller one for the +IRE isoform. The reason for this selective increase in the 1A isoforms of DMT1 is not known, but studies are currently underway to analyze the structures of the different forms of DMT1 in control and hypoxic-treated PC12 cells and factors controlling HIF signaling of DMT1 in these cells. The increase in expression of the 1A isoforms of DMT1 seen upon hypoxia may also be responsible for elevated transport of iron and other essential divalent metals in order to maintain normal homeostatic conditions during ischemia.

Acknowledgments

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