Regulation of xanthine oxidoreductase by intracellular iron

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Regulation of xanthine oxidoreductase by intracellular iron. Am J Physiol Cell Physiol 283: C1722–C1728, 2002. First published August 22, 2002; 10.1152/ajpcell.00280.2002.—Xanthine oxidoreductase (XOR) may produce reactive oxygen species and play a role in ischemia-reperfusion injury. Because tissue iron levels increase after ischemia, and because XOR contains functionally critical iron-sulfur clusters, we studied the effects of intracellular iron on XOR expression. Ferric ammonium citrate and FeSO4 elevated intracellular iron levels and increased XOR activity up to twofold in mouse fibroblast and human bronchial epithelial cells. Iron increased XOR protein and mRNA levels, whereas protein and RNA synthesis inhibitors abolished the induction of XOR activity. A human XOR promoter construct (nucleotides +42 to −1937) was not induced in vitro in human embryonic kidney cells. Hydroxyl radical scavengers did not block induction of XOR activity by iron. Iron chelation by deferoxamine (DFO) decreased XOR activity but did not lower endogenous XOR protein or mRNA levels. Furthermore, DFO reduced the activity of overexpressed human XOR but not the amount of immunoreactive protein. Our data show that XOR activity is transcriptionally induced by iron but post-translationally inactivated by iron chelation.

deferoxamine; gene regulation; iron-sulfur proteins; reactive oxygen species; ischemia-reperfusion injury

XANTHINE OXIDOREDUCTASE (XOR) catalyzes the last two reactions of purine catabolism by oxidizing hypoxanthine to xanthine and further to urate. The enzyme occurs in two forms; the dehydrogenase form (XDH, EC 1.1.1.204), using NAD+, and the oxidase form (XO, EC 1.1.3.22), using molecular oxygen as the electron acceptor. In the latter reaction, reactive oxygen species (ROS), i.e., superoxide anion (O₅⁻) and hydrogen peroxide (H₂O₂), are formed (20). The dehydrogenase form can be converted into the oxidase form either reversibly by sulfhydryl oxidation or irreversibly by proteolysis (29). During ischemia-reperfusion, the dehydrogenase form is converted into the ROS-producing oxidase form. On this basis, XOR has been suggested to contribute to the pathogenesis of ischemia-reperfusion injury (28).

Increased XOR activity after hypoxia is interesting in view of the putative role of XOR in postischemic injury (19, 32). Cytokines also induce XOR activity (14, 16, 31), suggesting that XOR may contribute to ROS production in inflammatory conditions. Deferoxamine (DFO), an iron chelator, has been shown to reduce XOR activity in bovine pulmonary artery endothelial cells, which was proposed to account for the protective role of DFO in lipopolysaccharide-induced cytotoxicity (34).

In considerations of the pathophysiological role of XOR in vivo, its tissue expression is crucial. In humans, only the liver, mammary gland, and intestine express high levels of XOR (26, 35), whereas in rodents, the enzyme is strongly expressed in several organs (24). In the mammary gland, XOR protein level clearly increases during lactation (26), and the enzyme is present in human milk (38), where it has been proposed to have a microbicidal function (41).

Even though iron is a prerequisite for normal cellular functions, an increase in catalytically active iron after tissue ischemia can exacerbate ischemia-reperfusion injury by favoring the formation of intracellular free radicals and lipid peroxidation (5, 10, 40). A marked deposition of iron can be detected in rat brain after focal ischemia (6), and another study, using perfused rabbit lung as a model, showed that after prolonged ischemia iron can be released from its intracellular stores into the vascular space (21).

XOR is a homodimer with two equivalent active sites. Electrons are transferred from purine substrate to molybdopterin cofactor and further through two nonidentical [2Fe-2S] centers of the ferredoxin type to FAD cofactor (30). Studies on the relationship between intracellular iron levels and XOR activity have not been reported. However, because XOR contains iron-sulfur clusters, and because it may play a pathophysiological role under conditions of varying tissue iron levels, the effect of iron on XOR regulation is of interest. In this study, we tested whether and by what mechanism intracellular iron levels influence XOR expression and activity.

MATERIALS AND METHODS

Cell culture. NIH/3T3 mouse fibroblasts from American Type Culture Collection (ATCC; Manassas, VA) and human
embryonic kidney 293T cells (from Prof. K. Saksela, University of Tampere, Finland) were cultured as described previously (27). Transformed human bronchial epithelial BEAS2B cells (ATCC) were cultured in bronchial epithelial cell growth medium (Cytotect, Helleback, Denmark) supplied with 100 U/ml penicillin and 100 μg/ml streptomycin. For treatments with different compounds, the cells were grown to subconfluence. Ferric ammonium citrate (FAC) was from Riedel-de Haen (Seelze, Germany); FeSO₄, CuSO₄, and dimethyl sulfoxide (DMSO) were from Merck (Darmstadt, Germany); and actinomycin D, cycloheximide, DFO, 1,3-dimethyl-2-thiourea (DMTU), N-acetylcysteine (NAC), and 1,10-phenanthroline were from Sigma (St. Louis, MO).

**Intracellular iron measurement.** NIH/3T3 cells were treated with iron or iron chelators, washed three times with PBS, harvested in PBS, frozen and thawed twice, and centrifuged at 4°C at 15,800 × g for 8 min in an Eppendorf centrifuge. The iron content of the supernatant was measured by using Raauta kit 141010 (Reaega, Kuopio, Finland), in which iron is first released in denaturing conditions (pH 4.8) and then reduced by ascorbic acid to ferrous iron, which forms a complex with ferene. This complex is then measured spectrophotometrically.

**XOR activity measurement.** Total XOR and XO activities were determined by using [14C]xanthine (NEN Life Science Products, Boston, MA) as substrate in the presence or absence of NAD⁺, respectively, and separating the product (uric acid) by HPLC as described previously (36). For the activity measurements, cells were washed twice with PBS and harvested in 50 mM potassium phosphate buffer, pH 7.8, containing 0.5 mM dithiothreitol, 1 mM EDTA, 0.5 μM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride or 50 μl/male protease inhibitor cocktail (Sigma). Subsequently, the cell suspension was lysed as described above. The protein concentration of the supernatant was measured with a Bio-Rad DC protein assay (Bio-Rad, Hercules, CA), and the supernatant was stored at −70°C.

**Expression of XOR protein.** 293T cells were transiently transfected with pcDNA3-expression vector (Invitrogen, Croningen, The Netherlands) carrying the complete coding sequence of human XOR (36), named pcDNA3-XOR. When the cells in 10-cm-diameter dishes reached ~50% confluence, they were transfected with 2 μg of pcDNA3-XOR by using FuGene6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer, and after 17 h, fresh medium with FAC or DFO was added. After 24-h incubation, the cells were harvested as described above for the activity measurements.

**Western blot analysis.** Proteins from NIH/3T3 (10 μg) or 293T cells overexpressing XOR (2.5 μg), harvested as described above, were separated by 7.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The blotted membrane was blocked with 5% (wt/vol) skim milk in 0.1 M Tris, 1 M NaCl, and 0.1% (vol/vol) Tween 20 for 1 h. For detection of XOR protein, polyclonal human anti-XOR antibodies (38) at a dilution of 1:1,000 (for NIH/3T3 proteins) or 1:2,000 (for cells overexpressing XOR) were used, followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:5,000. To control for the protein loading, we applied monoclonal anti-β-tubulin antibody (Sigma) at a dilution of 1:1,000 on the same Western blot. The protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Amersham, UK).

**Northern blot analysis.** Total RNA was extracted by using the RNeasy Mini kit (Qiagen, Hilden, Germany) and separated on a 1% agarose-formaldehyde gel (10 μg/lane) for Northern blot analysis. RNAs were transferred to Bionyde A nylon filters (PALL Gelman Sciences, Portrait, UK) and fixed by UV cross-linking. Membranes were hybridized by using standard procedures (37) with 32P-labeled complementary RNA probe corresponding to nucleotides 321 to 3021 of the rat XOR cDNA (kindly provided by Prof. T. Nishino, Nippon Medical School, Tokyo, Japan) (3). The membranes were exposed to autoradiography films (Curex Ortho HT-L PLUS; Agfa, Mortsel, Belgium). The same membranes were reprobed with random primed (Prime-a-Gene labeling system; Promega, Madison, WI) mouse 18S ribosomal gene DNA probe (Ambion, Austin, TX) to control for RNA loading. The X-ray films were scanned (Hewlett Packard ScanJet 6300C) and analyzed with the Scion Image beta 4.0.2 analysis software (Scion, Frederick, MD).

**Transfection and reporter gene analysis.** Human XOR promoter luciferase reporter gene constructs (XOR1, from +42 relative to the translational start site to −1937, and XOR5, from +42 to −142) were generated, and 293T cells were transiently transfected with XOR promoter constructs and β-galactosidase expression vector as described previously (27). Medium containing FAC (1 or 2 mM) was changed 16 h after transfection, and the cells were further incubated for 24 h. Luciferase activity was determined as described previously (27).

**Statistical analysis.** Data are expressed as means ± SD, and means were compared by using a two-tailed t-test with unequal variations. P values <0.05 were considered significant.

**RESULTS**

**Intracellular iron.** The culture medium contained 2.3 μM iron, and the basal iron concentration of NIH/3T3 cells was 0.7 nmol/mg protein. Compared with cells grown in normal medium, the intracellular iron content in NIH/3T3 cells increased progressively up to 12-fold when FAC was present at concentrations between 180 μM and 1.8 mM in the medium and up to 19-fold when 1 mM FeSO₄ was added to the cell culture (Table 1). The effect of iron chelators on intracellular iron content could not be reliably determined because the concentration of iron in the control cells was near the lower limit of the assay.

**Intracellular XOR activity.** The basal XOR activity in NIH/3T3 cells was 849 ± 288 nmol·min⁻¹·mg protein⁻¹. Total XOR activity in NIH/3T3 cells incubated with FAC at concentrations between 180 μM and 1.8 mM increased progressively up to twofold and some-

<table>
<thead>
<tr>
<th>Table 1. Intracellular iron content of NIH/3T3 cells</th>
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<tr>
<td>Fe, nmol/mg protein</td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>FAC (180 μM)</td>
</tr>
<tr>
<td>FAC (1 mM)</td>
</tr>
<tr>
<td>FAC (1.8 mM)</td>
</tr>
<tr>
<td>FeSO₄ (1 mM)</td>
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<tr>
<td>DFO (100 μM)</td>
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<tr>
<td>Phen (5 μM)</td>
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Cells were treated with or without ferric ammonium citrate (FAC), FeSO₄, deferoxamine (DFO), or phenanthroline (Phen) for 24 h, and intracellular iron was determined. Data are means ± SD of triplicate samples, each measured twice.
what less upon incubation with FeSO$_4$ (1 mM) (Fig. 1A). The increase in enzyme activity was not detectable after 12 h of incubation with iron but was clearly seen after 24 h (Fig. 1B). Neither iron compound altered the ratio of XDH to XO.

To explore the mechanism of induction of XOR activity, we incubated cells with actinomycin D and cycloheximide to inhibit RNA and protein synthesis, respectively, for 6, 12, and 24 h. Both compounds inhibited the iron-induced increase in enzyme activity, suggesting that XOR transcription and translation are needed for the elevation in XOR activity (Fig. 1C).

To study the effect of iron depletion on XOR activity, we treated NIH/3T3 cells with iron chelators. The Fe(III) chelator DFO (100 μM) had already decreased enzyme activity after 6 h, and the decrease progressed until 24 h (Fig. 1B). The Fe(II) chelator 1,10-phenanthroline (5 μM) reduced XOR activity to 60% of the basal activity in 24 h.

To explore whether the effect of iron on XOR activity is demonstrable in a human cell line with endogenous XOR activity, we incubated BEAS2B cells with 180 μM and 2 mM FAC for 24 h. XOR enzyme activity increased ~40% with 2 mM FAC, whereas iron chelation by DFO decreased the activity (Fig. 2). The basal XOR activity in BEAS2B cells (174 ± 24 nmol·min$^{-1}$·mg protein$^{-1}$) was about one-fifth that in NIH/3T3 cells.

Neither FAC nor DFO had an effect on the activity of purified bovine XOR (data not shown), and they did not consistently influence the total protein of the cells after 24 h of exposure, indicating that the viability of the cells was not compromised by the treatments.

Intracellular XOR protein. The amount of XOR protein in NIH/3T3 cells was assessed by Western blot analysis. The ~150-kDa band corresponding to XOR protein intensified after 24 h of incubation with FAC, whereas DFO did not change the level of immunoreactive XOR protein (Fig. 3A). The increase in XOR protein was inhibited by cycloheximide and actinomycin D. This finding is in accordance with the effect of cycloheximide on XOR activity, indicating that new protein synthesis is needed for the iron-induced increase in XOR activity.

**Effect of intracellular iron on XOR transcription.** The effects of FAC and DFO on XOR mRNA levels in
NIH/3T3 cells were determined by Northern blot analysis. FAC (180 μM) raised the level of XOR mRNA 1.5-fold (Fig. 3, B and C). Surprisingly, DFO (100 μM) did not decrease but consistently somewhat increased the amount of XOR message (Fig. 3, B and C), suggesting that the suppression of XOR enzyme activity by DFO does not take place at the transcriptional level. Induction of XOR mRNA by iron occurred in the presence of cycloheximide (Fig. 3B), indicating that the transcriptional induction of XOR by iron is independent of de novo protein synthesis. Actinomycin D decreased the amount of XOR mRNA after 24 h of incubation and inhibited the rise in XOR mRNA in iron-treated cells (Fig. 3B). FeSO4 also increased the level of XOR mRNA (Fig. 3C). On the basis of these findings, we conclude that iron-induced increase in XOR activity is associated with transcriptional activation of the XOR gene.

To explore whether iron has an influence on the activity of the human XOR promoter, we applied FAC onto 293T cells transiently transfected with XOR promoter constructs. Iron did not, however, increase the activity of either XOR1, corresponding to ~2 kb of the XOR 5’-flanking region, or XOR5, representing the proximal promoter and being the most active of our XOR promoter constructs, in 293T cells (27).

**Effect of DFO on XOR expressed in 293T cells.** To study the possible direct effect of DFO on the formation of immunoreactive and active XOR protein, we expressed human XOR in 293T cells that have no measurable endogenous XOR activity. The plasmid pcDNA3-XOR produced enzymatically active XOR protein that could be detected as one band with human XOR antibody in Western blot analysis (Fig. 4), whereas nontransfected 293T cells exhibited neither detectable amounts of XOR protein nor activity (data not shown). During 24 h of incubation, DFO did not decrease the amount of immunoreactive XOR protein produced by pcDNA3-XOR as determined by Western blot analysis (Fig. 4). However, DFO reduced the enzyme activity of the expressed XOR protein to <20% of the activity found in cells grown in standard culture medium (Fig. 4), indicating posttranslational inhibition of XOR activity.

![Figure 2: XOR enzyme activity in BEAS2B cells. BEAS2B cells were incubated with 180 μM and 2 mM FAC or with 100 μM DFO for 24 h. XOR (filled bars) and XO (open bars) activities were determined as described in MATERIALS AND METHODS. The level of XOR activity in control cells was set as 100. Data are means ± SD of at least 3 independent experiments, each performed at least in duplicate. **P < 0.01; ***P < 0.001 compared with control cells.](http://ajpcell.physiology.org/)
inhibitors, we have shown that increased intracellular iron induces XOR in mouse fibroblast NIH/3T3 cells at the transcriptional level. These cells were used because they have relatively high endogenous XOR activity. In human bronchial epithelial BEAS2B cells, both the basal activity and the iron induction were smaller than in NIH/3T3 cells, which may reflect species- or tissue-specific differences in regulation of XOR expression or in iron metabolism.

Several proteins involved in iron metabolism are regulated at the posttranscriptional level by well-defined mechanisms involving sequence-specific mRNA-binding iron regulatory proteins (IRPs). In iron-depleted cells, IRPs bind to iron-responsive elements (IREs) and either promote mRNA stability (transferrin receptor) or inhibit protein translation (ferritin, 5-aminolevulinate synthase) depending on the location on the mRNA (15). The expression of proteins carrying iron-sulfur clusters may also be regulated by IRPs binding to IREs (17, 22). Furthermore, IRP1 itself is believed to be converted into non-RNA-binding cytosolic aconitase by assembly of a [4Fe-4S] cluster in iron-replete cells (23). Even though the rat XOR mRNA has been suggested to carry a 5'-IRE consensus sequence element (CAGUGA) (7), which is conserved in the human promoter, our data strongly support iron effects at the transcriptional rather than the mRNA level. Furthermore, actinomycin D prevented the iron-induced increase of both enzyme activity and mRNA, indicating that de novo RNA synthesis is required.

Iron has been shown to directly modify the transcription of several genes, e.g., protein kinase C, cyclin-dependent kinase inhibitor p21, retinoblastoma susceptibility protein pRb, several stress proteins, and inducible nitric oxide synthase (iNOS), but little is known of the underlying mechanisms (1, 2, 12, 43). The transcription factor NF-κB has been implicated (25), and the human XOR promoter has been suggested to carry a consensus NF-κB binding site (42). Because our XOR promoter constructs failed to be activated by iron, this potential mechanism could not be studied further. NF-κB is also a major transcription factor mediating the effects of reactive oxygen metabolites on gene regulation (39). Increased intracellular iron may catalyze the formation of hydroxyl radicals (4, 18), which could account for increased transcription of the XOR gene. However, hydroxyl radical scavengers failed to prevent the induction of XOR by iron, thus lending no support for the role of oxidants and, indirectly, of NF-κB.

Iron chelation by the intracellular Fe(III) chelator DFO (33) substantially decreased XOR activity in both NIH/3T3 and BEAS2B cells. The rate of decrease in XOR activity caused by DFO was more rapid than the

Effects of hydroxyl radical scavengers and copper on XOR activity. To evaluate the possible role of ROS, generated in iron-catalyzed reactions, in the signaling pathway leading to increased XOR activity, we treated NIH/3T3 cells with the hydroxyl radical scavengers NAC (1 mM), DMTU (10 mM), or DMSO (30 mM) with or without iron. None of the hydroxyl radical scavengers inhibited the increase in XOR activity caused by iron (Table 2). To assess whether another transition metal, copper, could increase XOR activity, we treated NIH/3T3 cells with CuSO4. Instead of increasing XOR activity, CuSO4 decreased XOR activity (Fig. 1A).

DISCUSSION

Using XOR activity measurements, Western and Northern blotting, and protein and RNA synthesis

Table 2. Effect of hydroxyl radical scavengers and iron on xanthine oxidoreductase activity in NIH/3T3 cells

<table>
<thead>
<tr>
<th>FAC</th>
<th>OH scavenger</th>
<th>DMTU</th>
<th>DMTU</th>
<th>DMSO</th>
<th>NAC</th>
<th>NAC</th>
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</thead>
<tbody>
<tr>
<td>17 h:</td>
<td>XOR activity, nmol·min⁻¹·mg protein⁻¹</td>
<td>938</td>
<td>1,440</td>
<td>972</td>
<td>1,580</td>
<td>996</td>
</tr>
<tr>
<td>17 + 24 h:</td>
<td></td>
<td>1,640</td>
<td>914</td>
<td>1,310</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were incubated with FAC (180 μM) and dimethyl thiourea (DMTU; 10 mM), dimethyl sulfoxide (DMSO; 30 mM), or N-acetylcysteine (NAC; 1 mM) for 24 h, and xanthine oxidoreductase (XOR) activity was measured as described in MATERIALS AND METHODS.
induction of XOR enzyme activity by iron, suggesting that the underlying mechanisms may be different. Paradoxically, even though DFO decreased enzyme activity, XOR mRNA levels were increased in NIH/3T3 cells, whereas XOR protein remained unchanged. These data suggest that DFO influences XOR activity at the posttranslational level. This conclusion is supported by our findings in cells overexpressing XOR, in which DFO strongly suppressed XOR activity without causing a decrease in XOR protein levels. The phenomenon may be analogous to the inactivation of ribonucleotide reductase by iron chelation, which is associated with an increase in the proportion of the apoprotein lacking iron (9).

The mechanism of the increase in XOR mRNA caused by DFO was not studied further and remains unclear. In murine macrophages and NIH/3T3 cells, iron chelation upregulates iNOS expression by a mechanism involving the transcription factor NF-IL6 (C/EBP-β) (12). This factor has been shown to have a role in the transcriptional regulation of the rat XOR promoter (8), but it fails to bind onto human XOR promoter constructs (27).

We have explored the regulation of the XOR gene to better understand its potential pathophysiological role. In ischemic tissue, hypoxanthine, a substrate for XOR, accumulates, and during reperfusion XOR is converted into the ROS-producing oxidase form. Under the same conditions, tissue iron content increases. Our data, showing upregulation of XOR activity by iron, suggest another mechanism for exacerbation of tissue damage in ischemia-reperfusion injury and iron-overload states. Furthermore, because DFO rapidly and effectively suppresses XOR activity, the therapeutic potential of iron chelation in these clinical situations should be explored. Increased body iron stores are associated with an increased risk for cardiovascular events (11), and DFO has been shown to improve endothelial function in patients with coronary artery disease (13).

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