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Extracellular H_2O_2 and not superoxide determines the compartment-specific activation of transferrin receptor by iron regulatory protein 1

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Abstract

Iron regulatory protein 1 (IRP1) functions as translational regulator that plays a central role in coordinating the cellular iron metabolism by binding to the mRNA of target genes such as the transferrin receptor (TfR)—the major iron uptake protein. Reactive oxygen species such as H_2O_2 and $O_2^{\cdot-}$ that are both co-released by inflammatory cells modulate IRP1 in opposing directions. While H_2O_2—similar to iron depletion—strongly induces IRP1 via a signalling cascade, $O_2^{\cdot-}$ inactivates the mRNA binding activity by a direct chemical attack. These findings have raised the question of whether compartmentalization may be an important mechanism for isolating these biological reactants when released from inflammatory cells during the oxygen burst cascade. To address this question, we studied cytosolic IRP1 and its downstream target TfR in conjunction with a tightly controlled biochemical modulation of extracellular $O_2^{\cdot-}$ and H_2O_2 levels mimicking the oxygen burst cascade of inflammatory cells. We here demonstrate that IRP1 activity and expression of TfR are solely dependent on H_2O_2 when co-released with $O_2^{\cdot-}$ from xanthine oxidase. Our findings confirm that extracellular H_2O_2 determines the functionality of the IRP1 cluster and its downstream targets while the reactivity of $O_2^{\cdot-}$ is limited to its compartment of origin.

Keywords: *Reactive oxygen species, oxidative stress, iron metabolism, transferrin receptor, iron regulatory protein 1*

Introduction

Because of the flexible coordination chemistry of iron and its redox potential, cells and organisms utilize iron-containing proteins for vital metabolic functions, such as oxygen transport, electron transfer, and catalysis [1]. While these properties explain why iron is an essential constituent for a multitude of biochemical activities, they also render it potentially toxic for cells and tissues. In the presence of reactive oxygen species (ROS), iron catalyses the generation of hydroxyl radicals (Fenton/Haber–Weiss reactions) that damage membrane lipids, proteins, and nucleic acids [2]. Considering that ROS, including H_2O_2 and $O_2^{\cdot-}$, are inevitable byproducts of aerobiosis, cells have to tightly control intracellular iron levels to minimize iron toxicity and satisfy its metabolic needs.

One of the fascinating triumphs of evolution is that iron sulphur clusters not only function in electron transport during oxidative phosphorylation, but can also serve as iron and oxygen sensors [3]. For instance, iron regulatory protein-1 (IRP1) exerts its dual activity through the disassembly of its cubane iron–sulphur [4Fe–4S] cluster. While the iron cluster containing holoprotein is an cytosolic aconitase, the apoprotein functions as RNA-binding translational regulator [4–6] that plays a central role in coordinating the cellular iron metabolism in eukaryotes. The activity of IRP1 as a translational regulator is affected not only by iron levels, but also ROS such as H_2O_2.

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Both, iron and H_2O_2 activate IRP1 for binding to mRNA iron-responsive elements (IREs) located on the 5′ and 3′ untranslated regions of target genes [7]. Thus, binding of IRP1 to five IRE′s in the 3′ untranslated region of the transferrin receptor (TfR) mRNA prevents its degradation and, subsequently, induces expression of TfR which is a major iron uptake mechanism in iron depleted cells. In addition to H_2O_2, several other reactive oxygen and nitrogen species have been identified that modulate IRP1 activity at low levels suggesting a direct signalling function of ROS [8–14]. However, the IRP1 response towards these reactants is complex: While NO [13,14] and H_2O_2 [8,12,15] induce the activity of IRP1, $O_2^{\cdot-}$ [16–20] and HOCl [11] have been shown to block its mRNA binding activity.

These findings have provided a strong link between iron metabolism and the immune response as ROS are important products of the oxygen burst cascade of inflammatory cells [21–23]. Moreover, other experimental findings strongly suggest a functional compartmentalization of ROS metabolism. In mammalian cells, only extracellular H_2O_2 induces a fast and potent activation of IRP1 [8,9,12,15]. Release of H_2O_2 from intracellular sources such as mitochondria or peroxisomes does not activate IRP1 [9]. The H_2O_2-mediated activation of IRP1 is associated with an increase in iron uptake via the TfR [10,24]. Compartment-specific reactivities have been also described for $O_2^{\cdot-}$ in other organisms. Studies in *Saccharomyces cerevisiae* have suggested an important role for cytosolic and mitochondrial superoxide dismutases (SODs) in iron metabolism [25,26]. In some models of oxidative stress, the mitochondrial superoxide dismutase *(SOD*2) has the ability to compensate for the lack of the cytoplasmic enzyme *(SOD*1) and vice versa [27,28]. Other aspects of the SOD1Δ phenotype, however, such as oxygen-mediated vacuolar fragmentation cannot be rescued by *SOD2* over-expression [29]. Moreover, only recombinant bacterial FeSOD that is targeted to yeast mitochondria can rescue Sod2Δ, but it cannot rescue Sod2Δ if the mitochondrial targeting sequence is omitted and FeSOD is expressed in cytosol [30,31]. In *Drosophil*a, IRP1 is strongly activated by silencing and genetic mutation of the cytosolic *Sod*1, but is unaffected by silencing of mitochondrial *Sod2* [32]. Conversely, mitochondrial aconitase activity is relatively insensitive to loss of *Sod1* function, but drops dramatically if *Sod2* activity is impaired.

Although these data demonstrate that IRP1 can be specifically inactivated by $O_2^{\cdot-}$ from cytosolic and to some extend from mitochondrial origin, the question of whether IRP1 reacts with extracellular $O_2^{\cdot-}$ that is derived from inflammatory cells has not yet been elucidated. In addition, it is not clear how an extracellular co-release of $O_2^{\cdot-}$ and H_2O_2 that is rapidly formed from $O_2^{\cdot-}$ via dismutation affects IRP1 activity and downstream targets such as TfR. To address this question, we studied IRP1 and TfR as compartment-specific markers of $O_2^{\cdot-}$-reactivity in conjunction with a tightly controlled biochemical modulation of $O_2^{\cdot-}$ and H_2O_2 levels in the extracellular compartment. Using cultured fibroblasts, we present evidence that the cellular membrane limits the reactivity of extracellular derived $O_2^{\cdot-}$ while its co-released dismutation product H_2O_2 determines the functionality of the IRP1 cluster and its downstream pathways.

Materials and methods

Materials

Luminol, hypoxanthine, cytochrome C, sodium hypochlorite, hydrogen peroxide, xanthine oxidase (XOX), glucose oxidase (GOX), superoxide dismutase (SOD) and tetrazolium salt (MTT) were from Sigma (Deisenhofen, Germany). Catalase was from Boehringer (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin were from Gibco (Karlsruhe, Germany), and foetal calf serum was from Greiner Labortechnik (Solingen, Germany).

Cell culture

Murine B6 fibroblasts were cultured in 10 cm culture dishes with Dulbecco's modified Eagle's medium containing 1000 mg/l glucose, 2 mM glutamine, 10% foetal calf serum, 100 units/ml penicillin and 0.1 ng/ml streptomycin. Cells were maintained in an incubator at 37°C with 5% CO_2.

Determination of H_2O_2 and H_2O_2-related enzyme activities

H_2O_2 concentrations and enzyme activities of GOX, XOX and catalase were measured using a sensitive non-enzymatic chemiluminescence assay as described earlier with some modifications [33,34]. Briefly, luminescence measurements were performed using an AutoLumat LB 953 luminometer (Berthold, Wildbad, Germany). Luminol (final concentration of 10^{-5} M) was premixed with DMEM medium and GOX or XOX in polystyrene tubes. 5 mM glucose or 1 mM hypoxanthine were used to determine GOX and XOX activity. At the appropriate time, an adjusted concentration of NaOCl (final concentration between 10^{-6} and 10^{-5} M) was injected and the luminescence was measured immediately. 10 μM H_2O_2 was used for calibration. Catalase activity is given in s^{-1} as described earlier [35,36]. Activity of XOX and GOX is indicated in M H_2O_2 per second. In some experiments, the Xylenol method was used as an independent H_2O_2 assay to confirm equal H_2O_2

generation in both the XOX/CAT and the GOX/CAT system [37]. Briefly, 200 μl of sample or H_2O_2 standard were mixed with 200 μl 25 mM H_2SO_4. 400 μl of reaction mixture was then added containing 0.5 mM $(NH_4)Fe(SO_4)_2$, 200 μM xylenol orange and 200 mM sorbitol in 25 mM H_2SO_4. Absorbance was read at 550 nm using a Fluostar (BMG Labtechnologies, Offenburg, Germany).

Superoxide anion generation by XOX

Superoxide anion generation by XOX was determined using the ferricytochrome C reduction technique modified from McCord and Fridovich [38]. The reaction buffer contained 1 mM hypoxanthine, 100 μM cytochrome C and xanthine oxidase in PBS pH 7.4. The reduction of cytochrome was measured at 550 nm.

Cytotoxicity studies

Cell viability was determined using the MTT assay as described [11]. Briefly, cells were treated with 1 mM hypoxanthine/XOX or 5 mM glucose/GOX in DMEM medium for 24 h in 96-well plates at 37°C using varying enzyme activities. After a washing procedure with PBS (to remove all enzymes), MTT was added to each well (0.5 mg/ml) and cells were incubated for 4 h at 37°C. Finally, 10% SDS in 0.01 M HCl was added to complete cell lysis and absorbance was measured after overnight incubation at 570 nm.

H_2O_2 and $O_2^{\cdot-}$ treatment protocols

B6 cells were cultured in 10 cm culture dishes until a confluence of 50%. Cells were treated with different XOX and GOX activities. They then were harvested either after 60 min for IRP1 activity or after 24 hours for TfR expression. 100 μM of H_2O_2, desferal or hemin, respectively, were used as positive and negative controls. In some experiments, external purified SOD or catalase were added to cultured cells to modulate levels of ROS.

Western blotting

Cells were lysed in RIPA lysis buffer and lysates were immediately boiled for 10 min. Equal aliquots of 25 μg protein were resolved by SDS/PAGE on 8% gels and proteins were transferred on to nitrocellulose filters. The blots were saturated with 5% non-fat milk in PBS and probed with antibodies against TfR (Zymed, San Francisco, CA, USA) or β-actin (Sigma). Dilutions of primary antibodies were 1:4000 (TfR) and 1:200 (β-actin). After washing with TBS containing 0.05% (*v*/*v*) Tween 20, the blots with anti- TfR monoclonal antibodies were further incubated with rabbit anti-mouse IgG (1:6000 dilution). The blots with anti-β-actin antibodies were incubated with goat anti-rabbit IgG (1:10000 dilution). Detection of the peroxidase-coupled secondary antibodies was performed with the ECL® method (Amersham, Piscataway, NJ, USA). The blots were quantified by densitometric scanning using the TotalLab software version 1.11 (Nonlinear Dynamics Inc., Durham, NC, USA).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described recently using a radiolabeled human ferritin H-chain IRE probe [39]. Briefly, samples were centrifuged, supernatants were chilled on ice for up to 1 h, and 10 μl (2.5 μg/μl) were analysed by EMSA with 25,000 cpm of ^{32} P- labeled IRE probe in the absence or presence of 2% 2-mercaptoethanol (*2-ME*). RNA–protein complex formation was quantified by densitometric scanning of the depicted autoradiographs.

Results

A titrated mixture of hypoxanthine/xanthine oxidase/catalase (XOX/CAT) allows the continuous co-release of superoxide and H_2O_2 at physiologically relevant concentrations

Based upon previously described models that mimic the continuous release of H_2O_2 [34] and HOCl [11] by inflammatory cells, we set up in establishing a well-defined superoxide releasing enzymatic model to study small differences in the regulation of IRP1 and TfR by ROS. XOX and GOX were eventually chosen as both enzymes show comparable kinetics of H_2O_2 production but clearly differ in their ability to generate superoxide (Figure 1(A) and (B)). Figure 1(A) shows XOX-mediated production of $O_2^{\cdot-}$ as measured by the reduction of cytochrome C [38]. H_2O_2 release was determined using a sensitive chemiluminescence assay [34]. XOX-generated $O_2^{\cdot-}$ is significantly degraded by additional SOD while catalase does not affect superoxide production at all. The latter indicates that H_2O_2—the dismutation product—does not affect the dismutation rate. Under these conditions, XOX generates H_2O_2 at a rate of 18 nM s^{-1} corresponding very well to quantities that are released by activated neutrophils [33].

Further studies established conditions that would allow a comparable release of H_2O_2 either by GOX or XOX (Figure 1B). The Xylenol assay was used as an alternative method to confirm these results. In this experiment, enzymes are diluted to adjust their H_2O_2 generation rates to 10 nM s^{-1}. Comparable to observations in suspensions of neutrophils [33], addition of SOD to the XOX system does not affect the generation of H_2O_2 which confirms the fast spontaneous dismutation of $O_2^{\cdot-}$ at pH 7.4. In the

presence of catalase ($k = 3.7\,s^{-1}$), H_2O_2 decreased to undetectable levels (bottom lane).

We next studied whether a XOX/CAT system would be able to form stable H_2O_2 steady-state concentrations. As H_2O_2 may accumulate in a pure XOX solution up to toxic concentrations, catalase has been previously introduced to maintain stable steady-state H_2O_2 concentrations over a long time [9,11,34,40]. Figure 2A shows the formation of H_2O_2 in both the conventional GOX/CAT and the XOX/CAT system. In the presence of 1 mM hypoxanthine, the steady state concentration of H_2O_2 could be maintained over 3–4 h at a generation rate of $k_{XOX} = 20$ nM/s. In contrast to GOX/CAT, a XOX/CAT system allows the formation of H_2O_2 at a defined rate and concentration but additionally releases superoxide anions (Figure 2B). Under special conditions (e.g. at very low oxidase activities) the H_2O_2 degradation by cellular catalase or glutathione peroxidase suffice to prevent accumulation of H_2O_2 and no external catalase is necessary (not shown).

H_2O_2 mediates the cytotoxicity of XOX in B6 fibroblasts

Using both the GOX and XOX systems, we next studied whether the additional release of superoxide would influence the cellular proliferation rate as

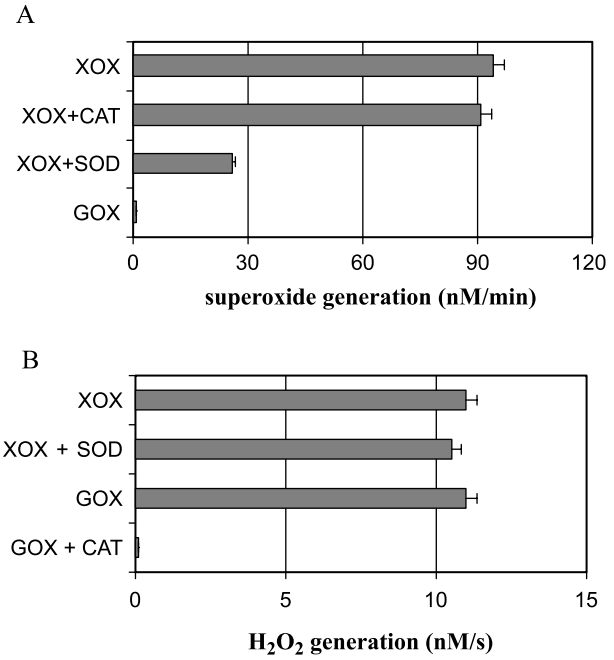

Figure 1. Generation of superoxide (A) and H_2O_2 (B) in a XOX- or GOX -system (A) Superoxide release by XOX was measured using the cytochrome C assay. XOX-generated $O_2^{\cdot-}$ is not affected by catalase but significantly reduced in the presence of SOD. No $O_2^{\cdot-}$ is produced by GOX. (B) XOX and GOX generate equal amounts of H_2O_2 that are not affected by SOD while catalase completely removes H_2O_2 from the system. Values represent mean of three determinations. Enzyme activities: $k_{XOX} = 11$ nM H_2O_2 s^{-1}, $k_{GOX} = 11$ nM H_2O_2 s^{-1}, $k_{CAT} = 3.7\,s^{-1}$, SOD with 100 U/ml.

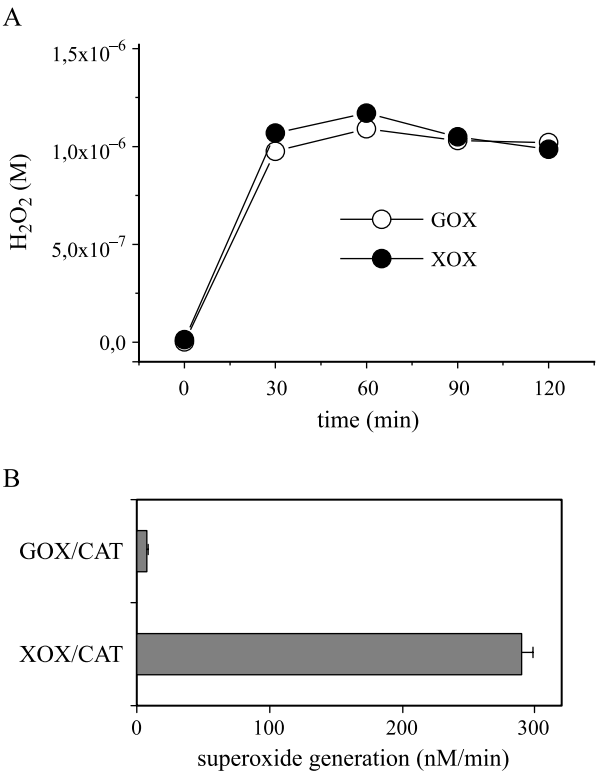

Figure 2. Generation of identical concentrations of H_2O_2 (A) but different amounts of $O_2^{\cdot-}$ (B) in a XOX/CAT or GOX/CAT system—Identical concentrations of H_2O_2 (1 μM) are formed in both systems allowing to study $O_2^{\cdot-}$-mediated functions independent of H_2O_2. $O_2^{\cdot-}$ was detected using cytochrome C reduction. Further conditions: $k_{XOX} = 18\,nM\,s^{-1}$, $k_{CAT} = 0.015\,s^{-1}$; GOX/CAT system: $k_{GOX} = 18\,nM\,s^{-1}$, $k_{CAT} = 0.015\,s^{-1}$

a general indicator of environmental stress. Interestingly, the proliferation rate did not depend on the enzymes used (Figure 3). Exposure to H_2O_2 over 24 h by both XOX and GOX did not affect cellular growth at rates lower than 10^{-8} M H_2O_2 s^{-1}. Production of H_2O_2 at rates higher than $10^{-8}\,M\,s^{-1}$ significantly reduced cell survival of B6 cells with about 70% surviving cells at a rate of $10^{-7}\,M\,s^{-1}$. No cells survived at a H_2O_2 rate more than $10^{-6}\,M\,s^{-1}$. Addition of external purified SOD with an activity that completely prevented cytochrome C reduction (100 U/ml) did not protect the cells. To prevent XOX-mediated hypoxanthine depletion, medium with enzymes and substrates were changed every 8 h. Further studies confirmed that neither the substrate (hypoxanthine) nor the product (urate) affect B6 cell proliferation at the concentrations used (not shown). These studies show that only H_2O_2 determines the cell survival rate in B6 cells when exposed to XOX.

Superoxide does not prevent H_2O_2 -mediated IRP1 activation and subsequent TfR expression

Cultured B6 fibroblasts were exposed to identical and continuous H_2O_2 levels using both XOX and GOX as

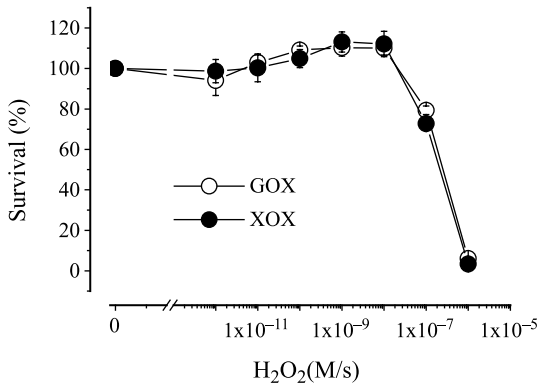

Figure 3. H_2O_2 determines the XOX- or GOX-mediated cytotoxicity in cultured B6 fibroblasts; B6 fibroblasts were exposed to H_2O_2 and $O_2^{\cdot-}$ that were continuously released by the XOX/CAT or GOX/CAT system over 24 h. Cells were treated with XOX and GOX using the appropriate dilutions to generate H_2O_2 from 10^{-6} to 10^{-12} M s^{-1}. Cell survival was determined using the MTT assay. Medium including enzymes and 5 mM of enzyme substrates (hypoxanthine and glucose) was changed every 8 h to prevent substrate depletion. Experiments were carried out in triplicates.

established above (Figures 1–3). IRP1 activity was measured by EMSA after 60 min [9] and TfR expression was determined by Western blotting after 24 h [10]. No significant difference could be found between both enzymatic models (Figure 4). Treatment of cells either with GOX or with XOX induces almost identical levels of IRP1 activity (up to 3.5 times) and TfR expression (up to 1.7 times) in a dose-dependent manner.

Addition of external purified catalase but not SOD affects XOX-induced TfR expression

We next studied whether the presence of external SOD or catalase would modulate XOX- or GOX-mediated TfR expression as important downstream target of IRP1 (Figure 5). Positive and negative controls include bolus treatment with H_2O_2 (lane 2), desferal (lane 3) and hemin (lane 4). Treatment with a continuous H_2O_2 flux by GOX or XOX at enzymatic activities of 10 nM s^{-1} results in a strong increase of TfR expression (up to 3.1 times, lane 5 and 7) that is completely prevented in the presence of catalase (lane 6 and 9). In contrast, addition of SOD (100 U/ml) did not affect H_2O_2 -mediated induction of TfR expression at an activity that completely inhibited cytochrome C reduction. These findings further confirm that release of extracellular $O_2^{\cdot-}$ does not block IRP1 binding activity.

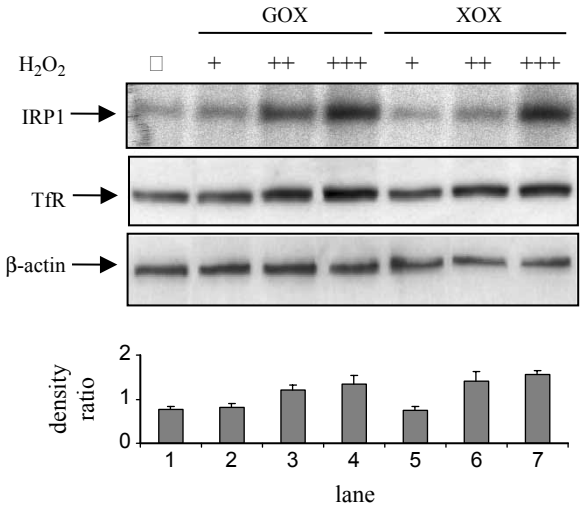

Figure 4. H_2O_2-mediated IRP1 activation and expression of the downstream target TfR is independent of co-released $O_2^{\cdot-}$ —B6 cells were treated with different amounts of H_2O_2 either released by XOX or GOX. IRP1 activity was determined after 1 h by EMSA (top panel). TfR expression (middle panel) and β-actin expression (bottom panel) were analyzed by western blotting after 24 h. *Lanes:* lane 1—control, lanes 2, 3 and 4—GOX with a H_2O_2 generation rate of 5 nM s^{-1}, 10 nM s^{-1} and 20 nM s^{-1}. lanes 5, 6 and 7—XOX with a H_2O_2 generation rate of 5 nM s^{-1}, 10 nM s^{-1} and 20 nM s^{-1}. GOX and XOX were used at such small amounts that intracellular catalase prevented H_2O_2 accumulation. Hypoxanthine and glucose were used at concentrations of 1 and 5 mM, respectively. Medium (including enzymes and enzyme substrates) was changed every 8 h. The depicted experiment is a representative of three independent measurements. TfR/β-actin densitometric ratios are indicated below.

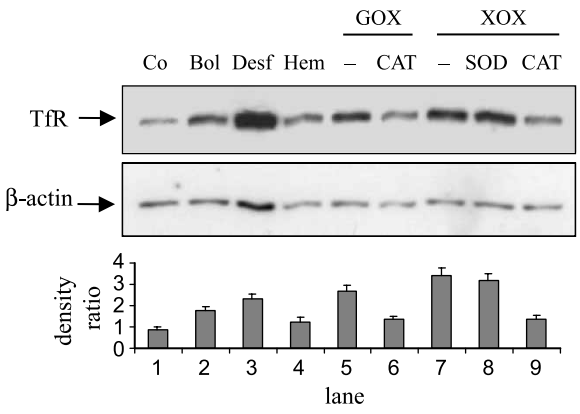

Figure 5. External catalase but not SOD block XOX- or GOX-mediated TfR induction—Cultured B6 cells were continuously exposed to H_2O_2 either released by XOX or GOX for 24 h in the presence of additional catalase or SOD. TfR expression (upper panel) and β-actin expression (bottom panel) were analyzed by western blotting after 24 h. *Lanes:* lane 1—control, lane 2—bolus of H_2O_2 100 μM, lane 3—100 μM desferal, lane 4—100 μM hemin, lane 5 and 6—GOX with a H_2O_2 generation rate of 10 nM s^{-1} without and with additional catalase (CAT, $k_{CAT} = 1\ s^{-1}$); lanes 7,8 and 9—XOX with a H_2O_2 generation rate of 10 nM H_2O_2 s^{-1} w/o additional SOD (100 U/ml) and catalase (CAT, $k_{CAT} = 1\ s^{-1}$). GOX and XOX were used at such small amounts that intracellular catalase prevented H_2O_2 accumulation. Hypoxanthine and glucose were used at concentrations of 1 and 5 mM, respectively. Medium (including enzymes and enzyme substrates) was changed every 8 h. The depicted experiment is a representative of three independent measurements. TfR/β-actin densitometrical ratios are indicated below.

Discussion

Earlier findings that ROS such as H_2O_2 and $O_2^{\cdot-}$ modulate the activity of IRP1 in tissue culture cells have raised intriguing mechanistic and physiological questions. They have linked iron homeostasis directly to inflammation as inflammatory cells are a major source of extracellular ROS. Similar to the cytokine-network during immunological responses, opposing agonists are targeting IRP1. Whereas extracellular H_2O_2 rapidly activates IRP1 *via* a signalling cascade [9,41], $O_2^{\cdot-}$ has been described in several reports to block its activity by a direct chemical attack [16–20]. This has drawn attention to the concerted action of ROS *in vivo* where H_2O_2 and $O_2^{\cdot-}$ usually coexist. In fact, the NADPH oxidase of leucocytes first produces $O_2^{\cdot-}$ that subsequently dismutates either spontaneously or enzymatically to H_2O_2 [42].

Using XOX and GOX in conjunction with catalase we have been able to study $O_2^{\cdot-}$ -mediated functions independent of H_2O_2. Our experimental approach is characterized by the following: First, introduction of additional catalase to XOX prevents an accumulation of H_2O_2 to toxic levels keeping H_2O_2 at constant concentrations for a long time. Second, the XOX/CAT system truly mimics the oxygen burst of inflammatory cells with respect to H_2O_2 and $O_2^{\cdot-}$ release in quantitative terms. In healthy individuals, leukocytes are able to generate H_2O_2 at a maximum rate of $0.2\,\mu M\,s^{-1}$ [33] and isolated suspensions of neutrophils can yield micromolar concentrations of H_2O_2 [15,34]. As demonstrated in Figures 1 and 2, these rates and concentrations can be faithfully obtained with the XOX or GOX/CAT system. Third, GOX/CAT alone serves as an appropriate $O_2^{\cdot-}$ -negative control [11,34].

On the basis of this methodological approach, we here present evidence that exposure of B6 cells to extracellular $O_2^{\cdot-}$ does not affect the activation of IRP1 and its downstream target TfR. Moreover, $O_2^{\cdot-}$ does not enhance the cell toxicity of the XOX/CAT system. The result directly applies to conditions *in vivo* as $O_2^{\cdot-}$ was released at a rate comparable to the generation in activated leukocytes. These findings are somewhat unexpected as $O_2^{\cdot-}$ has been shown to permeate cellular membranes [43] and to inhibit aconitase [44–47] as well as IRP1 activity [16–20]. On the other side, studies in other species such as *Saccharomyces cerevisiae* [29–31] or *Drosophila* [32] have clearly demonstrated a compartment-specific reactivity of intracellularly derived $O_2^{\cdot-}$ whether it is released in the cytosol or the mitochondria.

Two reasons might explain why extracellular $O_2^{\cdot-}$ does not modulate IRP1 activity when released from the extracellular compartment: First, fast dismutation prevents any possible competing side reaction, e.g. with cellular compounds. This is supported by the finding that neither in the cell nor in the cell-free XOX/CAT system SOD significantly changed H_2O_2 generation rate although clearly inhibiting cytochrome C reduction. It is in agreement with earlier observations on activated neutrophils [33] where addition of SOD did not alter the H_2O_2 concentration. Second, a direct effect on IRP1 is efficiently prevented by cellular SOD that enhances the spontaneous dismutation ($k = 5 \times 10^5\,M^{-1}\,s^{-1}$ at pH 7.4) by four orders of magnitude [48].

Taken together, we show that H_2O_2 clearly predominates IRP1 signalling, when $O_2^{\cdot-}$ and H_2O_2 are both released outside the cell. Comparable to HOCl [11], $O_2^{\cdot-}$ might only inhibit IRP1 when either produced outside the cell at unphysiological high concentrations or when produced directly inside the cell by a redox-cycling quinone such as menadione [49]. In confirmation, studies on SOD deficient yeast mutants support the idea that $O_2^{\cdot-}$ -mediated inactivation of cytosolic iron–sulphur proteins is confined to the intracellular generation of $O_2^{\cdot-}$ [32,50,51]. Our data set the stage for regarding H_2O_2 as the major positive regulator of IRP1 among the ROS.

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