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Original Contribution

Glutathione peroxidase 8 is transcriptionally regulated by HIFα and modulates growth factor signaling in HeLa cells

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ABSTRACT

GPx8 is a mammalian Cys-glutathione peroxidase of the endoplasmic reticulum membrane, involved in protein folding. Its regulation is mostly unknown. We addressed both the functionality of two hypoxia-response elements (HREs) within the promoter, *GPx8* HRE1 and *GPx8* HRE2, and the GPx8 physiological role. In HeLa cells, treatment with HIFα stabilizers, such as diethyl succinate (DES) or 2-2′-bipyridyl (BP), induces GPx8 expression at both mRNA and protein level. Luciferase activity of pGL3^{GPx8wt}, containing a fragment of the *GPx8* promoter including the two HREs, is also induced by DES/BP or by overexpressing either individual HIFα subunit. Mutating *GPx8* HRE1 within pGL3^{GPx8wt} resulted in a significantly higher inhibition of luciferase activity than mutating *GPx8* HRE2. Electrophoretic mobility-shift assay showed that both HREs exhibit enhanced binding to a nuclear extract from DES/BP-treated cells, with stronger binding by *GPx8* HRE1. In DES-treated cells transfected with pGL3^{GPx8wt} or mutants thereof, silencing of HIF2α, but not HIF1α, abolishes luciferase activity. Thus *GPx8* is a novel HIF target preferentially responding to HIF2α binding at its two novel functional *GPx8* HREs, with *GPx8* HRE1 playing the major role. Fibroblast growth factor (FGF) treatment increases GPx8 mRNA expression, and reporter gene experiments indicate that induction occurs via HIF. Comparing the effects of depleting GPx8 on the downstream effectors of FGF or insulin signaling revealed that absence of GPx8 results in a 16- or 12-fold increase in phosphorylated ERK1/2 by FGF or insulin treatment, respectively. Furthermore, in GPx8-depleted cells, phosphorylation of AKT by insulin treatment increases 2.5-fold. We suggest that induction of GPx8 expression by HIF slows down proliferative signaling during hypoxia and/or growth stimulation through receptor tyrosine kinases.

In excess, hydroperoxides (ROOH)[1] have a proinflammatory role and are toxic to cells. However, when produced in limited amounts associated with physiological signaling, ROOH appear to modulate redox-sensitive processes, including growth, differentiation, and proliferation [1–4]. Emerging knowledge suggests that ROOH are required for receptor tyrosine kinase (RTK) signaling, with the intriguing function of amplifying the RTK signaling cascades [1,5].

The glutathione peroxidase (GPx) family of proteins encompasses distinct gene products that efficiently reduce ROOH into corresponding alcohols. Inverse genetic studies suggest that they are nonredundant enzymes, despite catalyzing a similar reaction and often exhibiting an overlapping cellular distribution [6]. In humans, the family of glutathione peroxidases includes members that may contain either the rare amino acid selenocysteine (Sec) or the more common cysteine (Cys) as the redox-active moiety. Apart from that, in both subfamilies the redox-active residue is included in a conserved catalytic tetrad (Fig. 1) [7].

Abbreviations: AKT, protein kinase B; BP, 2,2′-bipyridyl; CysGPx(s), glutathione peroxidases(s) containing cysteine as the redox-active moiety; DES, diethyl succinate; EPO, erythropoietin; ER, endoplasmic reticulum; ERK1/2, extracellular-regulated kinases 1 and 2; ERO-1, ER oxidoreductin 1; FGF, fibroblast growth factor; GPx(s), glutathione peroxidase(s); HIF, hypoxia-inducible factor; h*GPx8*, gene encoding human glutathione peroxidase 8; HRE, hypoxia-response element; JNK, Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase, NOX4, NADPH oxidase family member 4; PLOOH, phospholipid hydroperoxide; PTP, protein tyrosine phosphatase; ROOH, hydroperoxide; P-AKT, phosphorylated protein kinase B; PGK, phosphoglycerate kinase; PDI, protein disulfide isomerase; P-ERK1/2, phosphorylated extracellular regulated kinases 1 and 2; TK, thymidine kinase; SeGPx(s), glutathione peroxidase(s) containing selenocysteine as the redox-active moiety; RTK(s), receptor tyrosine kinase(s).

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In humans, the Sec subfamily (SecGPx) comprises four tetrameric peroxidases (GPx1, GPx2, GPx3, and GPx6) that use glutathione (GSH) to reduce H_2O_2 and other small ROOH, including free fatty acid hydroperoxides, and one monomeric peroxidase, GPx4, which exhibits unusual preferences toward both the oxidizing and the reducing substrates. Indeed, GPx4 efficiently reduces, in addition to small ROOH that are substrates for the tetrameric SecGPxs, phospholipid and cholesterol or cholesterol ester hydroperoxides incorporated in membranes or lipoproteins [8]. Consequently, unique among the SecGPxs, GPx4 is a vital enzyme preventing ferroptotic cell death in vivo, in which lipid peroxidation is involved [9,10]. Nevertheless, GPx4 also accepts protein thiols as electron donors when GSH is limiting, a property related to its role in male fertility [11,12]. However, recent research suggests that all the SecGPxs are relevant players in inflammation, cancer, proliferation, and signaling, as reviewed in [13]. As a whole, the importance of the SecGPx homologs has contributed to the perception of a multifaceted role for ROOH in cells.

The Cys homologs (CysGPx) comprise, in humans, three members that have been much less studied than the SecGPxs. Structurally, two are monomeric proteins, namely GPx7 and GPx8 [14,15], and one, GPx5, is a tetrameric enzyme specifically secreted from the epididymis [16] (Fig. 1). GPx7 and GPx8 are the last discovered members and are thus among the most mysterious in terms of function. Evolutionary studies suggested that they derived from SecGPx4, which actually had a Cys-containing ancestor. Thus, human GPx7 and GPx8 represent a recent return to Cys usage in glutathione peroxidases, which is not easily rationalized [17].

Overall, steady-state kinetic studies of various Cys variants of glutathione peroxidases indicate that the step reducing ROOH is not dramatically affected by replacing Sec with Cys. This contrasts with the reducing step by glutathione regenerating the ground-state enzyme, which is much slower and severely affects the enzyme turnover [7,18]. Yet, an artificially constructed CysGPx4 can rescue the death phenotype of SecGPx4-lacking cells [19], demonstrating that a Cys-to-Sec replacement does not prevent the vital functions of the enzyme, at least in cells.

GPx7 and GPx8 are widely distributed in mammals. Unique among the glutathione peroxidases family members, they both contain an endoplasmic reticulum (ER) retention signal at the protein C-terminal end (Fig. 1). GPx7 is free in the lumen, whereas GPx8 is an intrinsic membrane peroxidase with its active site facing the lumen [15,20]. Peculiarly, they may accept protein disulfide isomerase (PDI) as a reductant more efficiently than GSH [15,18,21].

The ER location and reactivity with PDI prompted the proposal that GPx7 and GPx8 are involved in oxidative protein folding. This apparently occurs by reoxidizing PDI in the peroxidatic reaction, in which H_2O_2, produced by ER oxidoreductin (ERO-1), is the oxidant [15,21]. Peroxiredoxin 4, a peroxidase present in the ER in most tissues as a secretory protein [22], is apparently much less efficient in removing ER H_2O_2 compared with GPx7 and GPx8 [15,21,23]. Furthermore, kinetic analysis shows that recombinant GPx7 may reduce both H_2O_2 and phospholipid hydroperoxide (PLOOH) by both GSH and PDI, where kinetic constant measurements and concentration of the two reductants within the ER comply with the proposal that the actual GSH concentration within the ER might modulate PDI oxidation [18]. On the other hand, whether reduction of PLOOH is a functional role in GPx7 physiology remains to be addressed.

GPx7 has been described as a tumor suppressor. Together with human *GPx3*, the human *GPx7* promoter was found to be hypermethylated and thus downregulated in some premalignant lesions of the esophagus [24]. Furthermore, in the epithelial cells of the esophagus, GPx7 could protect against oxidative damage of DNA and regulate oxidative signals that depend on the mitogen-activated protein kinases (MAPKs) p38MAPK and c-Jun NH_2-terminal kinase (JNK) upon exposure to pH 4 and bile acids [25]. More recently, it was documented that loss of GPx7 resulted in systemic oxidative damage, shortened life span, and increased carcinogenesis in mice [26]. Furthermore, GPx7 deficiency has been linked to obesity and preadipocyte differentiation [27] by controlling the dimerization of protein kinase A and activating the CCAAT/enhancer binding protein β.

Compared to GPx7, knowledge about GPx8 is much more limited. The enzyme has not been fully characterized kinetically, although it seems less efficiently reduced by PDI than GPx7 [15]. Apparently, however, GPx8 efficiently prevents the spillover of H_2O_2 generated from the ER by ERO-1 [23]. GPx8 was described as one of the cellular substrates of the hepatitis C virus NS3–4 A protease [20] and, according to a transcriptomic and proteomic profiling of KEAP1, it is downregulated in breast epithelial sulforaphane-treated cells, which suggests that nuclear factor (erythroid-derived 2)-like 2 indirectly dampens GPx8 expression [28].

The studies presented here were designed to address the physiological function of GPx8 by examining the functionality of two hypoxia-inducible factor (HIF) binding sites in the promoter. Indeed little is known about the link between HIF and glutathione peroxidases. Only the human plasma GPx3 was described to contain a binding site for HIF-1 and it is indeed induced by hypoxia [29]. Similarly the expression of the human GPx1 was found to be linked to oxygen sensing, but through the action of two oxygen-responsive promoter elements (OREs) [30]. The ORE is distinct from the hypoxia-response element (HRE) and apparently responds to a milder hypoxia [31]. Notably, however, the HIF subunits are stabilized not only by hypoxia, but by signaling as well, in which most likely the common triggers are reactive nitrogen and oxygen species [32]. We discovered that *GPx8* is indeed a HIF target and, as such, upregulated by chemical hypoxia and fibroblast growth factor (FGF) treatment. Furthermore, GPx8 depletion in cells affected FGF and insulin signaling. All together these findings link the expression of GPx8 to HIF stabilization and expand its function to the control of RTK signaling cascades.

1. Material and methods

1.1. Cell culture and treatments

HeLa cells (ATCC CCL-2™) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (Life Technologies). Treatments were performed on 70% confluent cells after overnight starvation in the absence of FBS. To stabilize HIFα subunits, 20 mM diethyl succinate (DES) or 0.1 mM 2,2′-bipyridyl (BP) (Sigma) was added and the cells were further incubated in serum-free medium for approximately 16 h. In some experiments, 100 ng/ml recombinant human FGF acidic (Life Technologies) was used in serum-free medium for 30 min and cells were collected after 24 h in complete medium.

GPx8-silenced HeLa cells contained a vector for stable expression of a small interfering RNA (siRNA) addressed to *GPx8* (SilenciX technology) and were purchased from Tebu-bio, which also provided HeLa cells transfected with a control short hairpin RNA. The percentage of silencing was 97%, as quantified by the manufacturer. GPx8-silenced cells and controls thereof were grown as above except that the medium contained 4 mM L-glutamine, 110 mg/L sodium pyruvate, and 125 μg/ml hygromycin B (Life Technologies). These were treated with 100 ng/ml FGF acidic or 20 μg/ml insulin for 10 min in serum-free medium and lysed as described below. Proteins were quantified by the Bradford reagent (Sigma).