Biochemical and Biophysical Research Communications 389 (2009) 177–180

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reduced formation of advanced glycation endproducts via interactions between glutathione peroxidase 3 and dihydroxyacetone kinase 1

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ARTICLE INFO

Article history:
Received 14 August 2009
Available online 26 August 2009

Keywords:
Advanced glycation endproduct
Dihydroxyacetone
Dihydroxyacetone kinase 1
Glutathione peroxidase 3
Oxidative stress

ABSTRACT

Dihydroxyacetone (DHA) induces the formation of advanced glycation endproducts (AGEs), which are involved in several diseases. Earlier, we identified dihydroxyacetone kinase 1 (Dak1) as a candidate glutathione peroxidase 3 (Gpx3)-interacting protein in *Saccharomyces cerevisiae*. This finding is noteworthy, as no clear evidence on the involvement of oxidative stress systems in DHA-induced AGE formation has been found to date. Here, we demonstrate that Gpx3 interacts with Dak1, alleviates DHA-mediated stress by upregulating Dak activity, and consequently suppresses AGE formation. Based on these results, we propose that defense systems against oxidative stress and DHA-induced AGE formation are related via interactions between Gpx3 and Dak1.

Introduction

Advanced glycation endproducts (AGEs), a heterogeneous group of non-enzymatic glycation products of proteins, play a critical role in protein deterioration during chronic diabetic complications, atherosclerosis, hypertension, Alzheimer's disease, and aging [1–4]. Methylglyoxal is a major intracellular reactive dicarbonyl intermediate derived from glycolysis. Spontaneous dephosphorylation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate at the triose phosphate isomerase step results in methylglyoxal formation. Earlier reports showed that increased methylglyoxal concentrations during diabetes promote AGE formation [5]. Moreover, dihydroxyacetone (DHA) stimulates the formation of AGEs [6,7] through conversion to methylglyoxal. The dihydroxyacetone kinase (Dak) enzyme catalyzes the conversion of DHA into the glycolytic intermediate, dihydroxyacetone phosphate. *Saccharomyces cerevisiae* contains two Dak enzymes that efficiently detoxify DHA. High concentrations of DHA are toxic and trigger AGE formation, both *in vivo* and *in vitro* [8].

Glutathione peroxidase 3 (Gpx3) functions as a major scavenger of peroxide in *S. cerevisiae* [9]. Several studies report that Gpx3 modulates the activities of a variety of redox-sensitive thiol proteins, particularly those related to signal transduction and protein translocation [9,10]. Our group has successfully identified several novel Gpx3-interacting proteins with the aid of two-dimensional electrophoresis/mass spectrometric analyses after immunoprecipitation [11–14], including Dak1 [12]. The observed interaction between Dak1 and Gpx3 is interesting, as there is no reported evidence of association between oxidative stress and DHA-induced AGE formation [6,7]. In the present study, we clearly demonstrate that Gpx3 physically interacts with Dak1 and enhances Dak activity in the presence of DHA, consequently reducing AGE formation.

Materials and methods

Strains and antibodies. The *S. cerevisiae* strain YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) and its isogenic derivatives were used in all experiments. Gene disruption of YIR037w (*Δgpx3*) was performed using PCR-mediated deletion, as described previously [11]. Anti-His from (IGtherapy, Chuncheon, Korea), anti-FLAG (Sigma), and anti-AGE (Fitzgerald Industries International, Inc., Concord, MA) antibodies were employed. Anti-Gpx3 antibody was produced and purified by Abfrontier, Inc. (Seoul, Korea) using mice.

Plasmid construction. DNA fragments encoding *gpx3* and *dak1* ORF were amplified from *S. cerevisiae* cDNA. The GST-Gpx3 construct (pGEX-6P-1-Gpx) was generated by inserting *gpx3* between the BamHI and SalI sites of pGEX-6P-1. The Dak1-His construct (pET-32a-Dak1) was produced by cloning *dak1* between the EcoRI and NotI sites of pET-32a(+). Additionally, pESC-LEU-Myc-Gpx3 and pYES2/NTC-His-Dak1 were generated by subcloning *gpx3*

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.116

between the BamHI and SalI sites of pESC-Leu and *dak1* between the EcoRI and NotI sites of pYES2–NTC, respectively.

Pull-down assays. To confirm the interactions between Gpx3 and Dak1 *in vitro*, *Escherichia coli* cells were transformed with pET-32a derivatives coding for full-length, N-terminal and C-terminal domains of Dak1-His. After induction with IPTG, cell extracts were prepared by centrifugation at 13,000 rpm for 30 min following lysis. Soluble fractions were incubated with 100 μl of Ni–NTA agarose for 4 h at 4 °C with rotation. Beads were collected by centrifugation at 3000 rpm for 1 min and washed three times in lysis buffer. Next, cell lysates overexpressing the Gpx3 protein were mixed and incubated for 2 h at 4 °C with rotation, followed by three washes. Bound proteins were eluted using SDS–PAGE sample buffer and separated by SDS–PAGE, followed by immunoblotting with anti-His antibody. Protein bands were visualized with the ECL detection system (PIERCE and Millipore). The *in vivo* interaction test was performed using a His-pull-down experiment, as described previously [11,12].

Determining the effects of Gpx3 on cell growth after DHA treatment. Yeast cells were cultured to the mid–late-exponential phase in YNB medium (1% yeast extract, 2% peptone, and 2% galactose) supplemented with the appropriate amino acids or bases. For spotting experiments, the number of yeast cells was adjusted and spotted on YEPD agar supplemented with 0.2 M DHA. For growth curve titration analysis, yeast cells were grown in YNB, and cell numbers adjusted with fresh YNB (OD_{600} of 1.0). Next, cells were treated with 50 mM DHA and their growth monitored by measuring OD_{600} until 168 h after treatment. Data are representative of three separate experiments.

Dak activity assay. The Dak activity assay was performed as described earlier by the Blomberg group [15]. Extracts for assays were generated using cells harvested from 50 ml of culture grown to the mid-exponential phase (OD_{600} = 0.35). Cells were washed in ice-cold 20 mM MES buffer, pH 6.5, and disrupted by vortexing with acid-washed glass beads. DAK activity was recorded as the amount of NADH oxidized/unit of time in a coupled reaction with excess glycerol-3-phosphate dehydrogenase. During DAK activity measurements, 4 mM DHA was added to initiate the reaction. Protein concentrations of extracts were determined using the Bradford assay (Bio-Rad) with bovine serum albumin as the standard.

AGE formation assays. The AGE assay for total protein was adopted from a total protein carbonylation assay [16]. Proteins were extracted from 2 ml of culture. Glass beads (0.3 mm) and sample buffer were added and protein extraction performed as described in a previous report [17]. Dot blotting of 0.5 mg protein/slot onto membranes (Protran™, Whatman, UK) was performed using Bio-Dot SF (Bio-Rad). We employed a primary monoclonal mouse anti-AGE antibody (1:5000 dilution) (Fitzgerald Industries International, Inc.) [18] and a secondary anti-mouse IgG antibody conjugated to HRP. Antibody and chemiluminescence substrate-treated membranes were exposed in Hyperprocessor (Amersham Life Science). All samples to be compared were loaded onto the same filter.

Statistical analysis. Data are presented as means ± SE (standard error) of three independent experiments performed in triplicate. Data were analyzed for statistical significance using the Student's *t*-test. The minimum level of significance was set at $P < 0.05$.

Results

Gpx3 interacts with the N-terminal region of Dak1

Initially, we examined whether the Gpx3 protein in fact interacted with Dak1 using an *in vitro* pull-down assay. After pull-down using Ni–NTA agarose beads from lysates of *E. coli* overexpressing Dak-His, crude extracts from *E. coli* containing Gpx3 were added under normal conditions. Following incubation for 2 h, immunoblotting analysis using anti-Gpx3 was performed. Our results showed that Gpx3 interacts with Dak1 *in vitro* (Fig. 1A). To confirm whether the Gpx3 protein binds Dak1 *in vivo*, Dak1-His was expressed in the YPH499 wild-type, ΔGpx3, and ΔGpx3-overexpressing Gpx3 strains. Lysates were prepared and used in a pull-down assay with Ni–NTA agarose beads. As shown in Fig. 1B, Dak1 interacted with endogenous Gpx3 in the wild-type YPH499 strain and Gpx3 induced in ΔGpx3-overexpressing Gpx3 strain. To identify the specific region(s) involved in interactions with Gpx3, we constructed and expressed two Dak1 fragments. Proteomic analysis of fragmented Dak1 disclosed the presence of two domains [19]. Accordingly, the protein was cleaved into two fragments, specifically, an N-terminal fragment containing residues at positions 16–344 (Dak1-N) and a C-terminal fragment incorporating residues at positions 412–582 (Dak1-C). Interactions between Gpx3 and the two Dak1 fragments were examined. Our data clearly

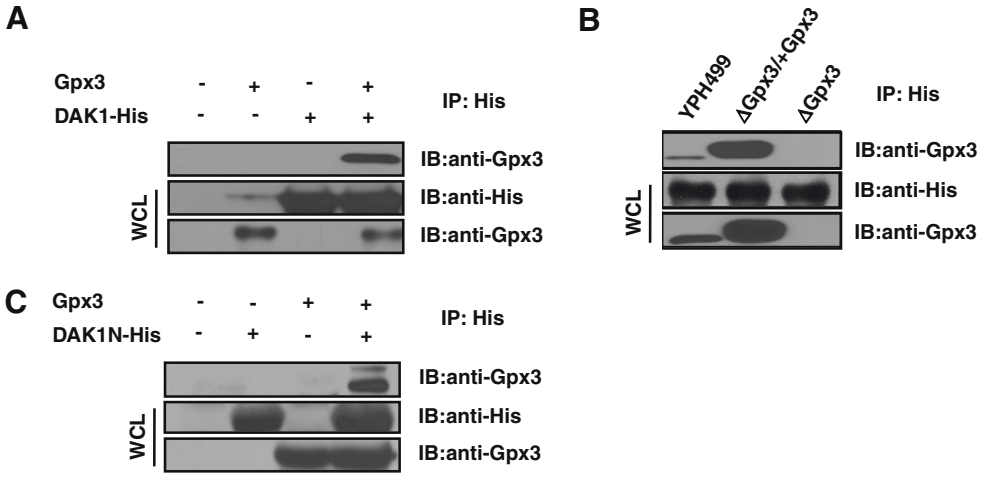

Fig. 1. Gpx3 interacts with Dak1 *in vitro* and *in vivo*. (A) Gpx3 associates with Dak1 *in vitro*. Protein lysates from *E. coli* overexpressing His-tagged Dak1 or Gpx3 used for pull-down assays with Ni–NTA agarose beads. Protein interactions were detected by immunoblotting with an anti-Gpx3 antibody. (B) Gpx3 interacts with Dak1 *in vivo*. Dak1-His was induced in YPH499, ΔGpx3, and ΔGpx3-overexpressing Gpx3 strains. Lysates were used for pull-down assays with Ni–NTA agarose beads. (C) The N-terminal fragment of Dak1 was involved in interactions with Gpx3. An N-terminal fragment containing residues at positions 16–344 (Dak1-N) was expressed in YPH499. Lysates were used for pull-down assays with Ni–NTA agarose beads.

showed that the N-terminal fragment of Dak1 was involved in interactions with Gpx3 (Fig. 1C).

Deletion of Gpx3 leads to retarded growth in the presence of DHA

We further examined whether interactions between Gpx3 and Dak1 influenced the growth of *S. cerevisiae* in the presence of DHA. DHA is toxic to yeast cells, and detoxification is dependent on functional Dak [8,19]. No significant changes in growth were evident between wild-type and ΔGpx3 strains under normal growth conditions (Fig. 2A). On the other hand, growth was retarded in the ΔGpx3 strain in the presence of DHA, compared to wild-type (Fig. 2B). In particular, deletion of Gpx3 induced an elongated lag phase in DHA-treated cells. However, growth recovered to level similar to that of the wild-type strain (approximately 144 h after culture commencement). The spot assay additionally disclosed involvement of Gpx3 in the detoxification of DHA, although this effect was not significant (Fig. 2C).

Gpx3 promotes increased Dak activity and consequently suppresses AGE formation

To establish the involvement of Gpx3 in DHA detoxification of Dak1, we examined Dak activity in wild-type, ΔGpx3, and Gpx3-overexpressing ΔGpx3 strains treated with DHA or left untreated.

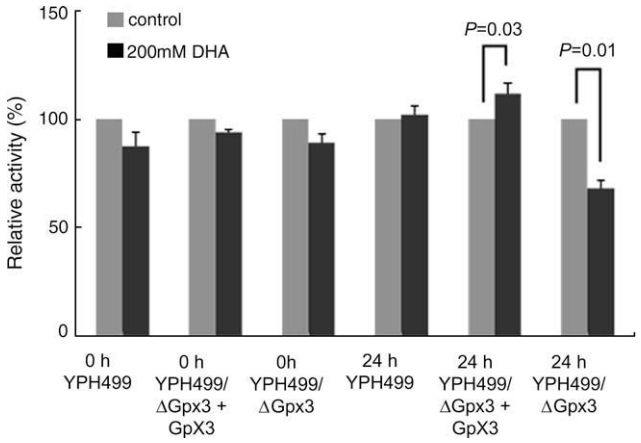

Fig. 3. Gpx3 induces an increase in intracellular Dak activity in the presence of DHA. Dak activity was measured after treatment with DHA for 24 h in YPH499, ΔGpx3, and ΔGpx3-overexpressing Gpx3 strains.

As shown in Fig. 3, Gpx3 induced a slight increase in Dak activity in the wild-type strain following treatment with DHA. The ΔGpx3 strain displayed a dramatic decrease in Dak activity upon DHA treatment, whereas activity of the Gpx3-overexpressing ΔGpx3

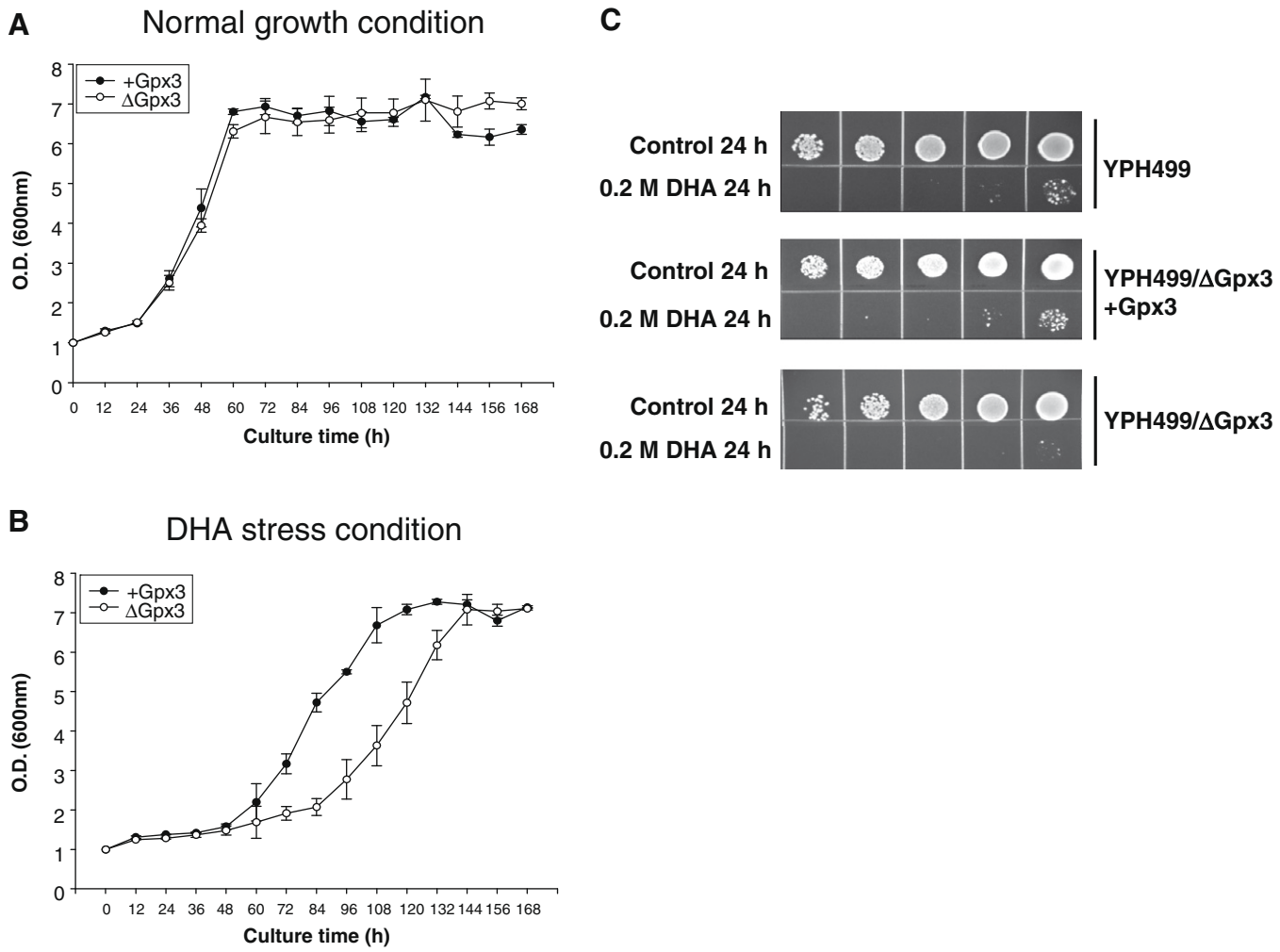

Fig. 2. Effects of Gpx3 on cell growth in the DHA-mediated stress. Growth curves of yeast strains under normal growth conditions (A) and in the presence of 50 mM DHA (B). Growth was monitored until 168 h after DHA treatment (see Materials and methods for details). Data are representative of three separate experiments. (C) Spot assays for monitoring the effects of Gpx3 on cell viability in DHA-mediated stress. The number of yeast cells was adjusted and spotted on YEPD agar supplemented with 0.2 M DHA. Cells were grown at 30 °C for 2 days.

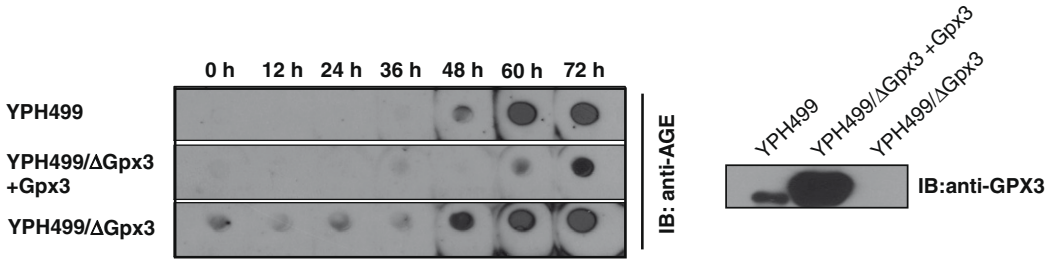

Fig. 4. Gpx3 influences AGE formation upon DHA treatment. Time-course analyses of AGE formation in YPH499, ΔGpx3, and ΔGpx3-overexpressing Gpx3 were performed after treatment with DHA. At the indicated times, lysates were prepared and immunoblotted using an anti-AGE antibody.

strain was significantly elevated (Fig. 3), implying that interactions between Gpx3 and Dak1 are involved in upregulation of Dak activity in the presence of DHA. In view of the finding that DHA induces AGEs, we examined whether the AGE level was affected by interactions between Gpx3 and Dak1. As expected, AGE formation was significantly increased in the ΔGpx3 strain, compared to wild-type (Fig. 4). Furthermore, a more significant reduction in AGE formation was observed in the ΔGpx3 strain overexpressing Gpx3, in comparison to wild-type. This may be attributed to the high Gpx3 protein level, compared to endogenous Dak1 of the wild-type strain (Fig. 4). Our results indicate that interactions between Gpx3 and Dak1 stimulate Dak activity, thereby reducing the generation of AGE that is toxic to yeast cells and concomitantly imparting greater resistance against DHA.

Discussion

Despite the critical involvement of AGEs in human diseases, limited AGE-related disease model systems are available at present. High levels of DHA induce AGE formation on proteins in *S. cerevisiae* [6,7]. Thus, *S. cerevisiae* treated with DHA represents a good model system for examining the mechanism of DHA-induced AGE formation. Dak catalyzes the conversion of the intracellular metabolite, DHA, into the glycolytic intermediate, DHAP, leading to alleviating of DHA-induced toxicity. Daks are present in various organisms, ranging from bacteria to humans. Two Dak enzymes have been identified in *S. cerevisiae*, specifically, a general stress-related Dak1 and glucose-repressed Dak2. Previously, we identified several novel candidate Gpx3-interacting proteins, one of which was Dak1, using proteomic tools [14]. This was an unexpected findings, as there is no evidence to support an association between oxidative stress and DHA-induced AGE formation [1,6,7]. In particular, Blomberg group reported that deletion of *gpx3* in a BY4742 strain background led to no significant changes in DHA tolerance [6]. However, our pull-down experiments clearly show that Gpx3 in fact interacts with the N-terminal region of Dak1, both *in vitro* and *in vivo*. Additionally, Gpx3 induces an increase in intracellular Dak activity following DHA treatment, although it is currently unclear whether Gpx3 also interacts with and stimulates Dak2. Increased Dak activity clearly influences the suppression of AGE formation in DHA-treated yeast cells. Based on these results, we propose that defense systems against oxidative stress and DHA-induced AGE formation are closely related via interactions between Gpx3 and Dak1. Our data provide a valuable step toward further clarifying the mechanism of AGE formation, and should thus aid in the development of drugs to treat AGE-related conditions, including diabetes and Alzheimer's disease.

Acknowledgments

We thank Drs. Do Hee Lee, Sang Chul Lee, Seung Jun Kim, and Sang J. Chung for careful reading of the manuscript and helpful advice. This work was supported by grants from KRIBB Initiative Program and the Basic Research Program (No. 2008-01050) of the Korea Science & Engineering Foundation, Republic of Korea.

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