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Identification of GCN2 as new redox regulator for oxidative stress prevention *in vivo*

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ABSTRACT

Constitution of oxidative defense systems and, correspondingly, oxidative stress prevention are highly dependent on amino acid supply. *In vitro*, experiments have demonstrated that amino acid availability participates to the homeostasis of reactive oxygen species. However the molecular mechanisms involved in the maintenance of redox homeostasis responsive to circulating amino acid levels remain unclear. As GCN2 is a protein kinase considered to be an important sensor for amino acids availability and a potential regulator of redox homeostasis, we hypothesized that this kinase can modulate redox homeostasis *in vivo*, in response to an amino acid-imbalanced diet.

We investigated the response of GCN2+/+ and GCN2−/− mice to a long-term (24 weeks) leucine-imbalanced diet (EDΔLeu). In order to evaluate the oxidation level in each group of mice, we determined the degree of protein oxidation in the liver. Interestingly, GCN2−/− mice exhibited an increase in protein carbonylation, a marker of oxidative stress, in response to the EDΔLeu diet. These data correlate with a decrease in hepatic GPX1 expression, a major antioxidant enzyme, and a decrease in total GPX activity in the liver. Our results suggest that GCN2 and its downstream signaling pathway have an important role in the protection against oxidative injuries induced by an amino acid-imbalanced diet, and that it can play a critical role in the prevention of oxidative damage.

1. Introduction

Reactive oxygen species (ROS) have been shown to be involved in a wide variety of cellular functions [1]. It is essential, but can also be highly toxic if there is a misregulation in the quantities produced [2,3]. The term "oxidative stress" refers to a cell's state as characterized by excessive production of ROS and/or reduction in the efficiency of antioxidant defenses responsible for their metabolism. Oxidative stress is associated with cytotoxic effects such as peroxidation of membrane phospholipids and protein oxidation and has been shown to be implicated in the etiology of various diseases [4–6].

There are both endogenous and exogenous sources of ROS, the majority originating as a bioproduct of oxidative phosphorylation. ROS can be effectively metabolized in order to counterbalance its potentially damaging effects by different mechanisms including several enzymes (SOD, GPX, catalase, etc.) and antioxidant molecule (glutathione). The balance between ROS production and antioxidant capacity is carefully controlled by several regulatory processes. Several articles demonstrate that phosphorylation of the translation initiation factor 2 (eIF2a) can modulate cellular resistance to oxidative stress. Indeed, it has been shown that antioxidant status correlates with the basal level of the phosphorylated form of eIF2a [7]. In addition, cellular expression of a mutant form of eIF2a (S51D), which acts as a constitutively phosphorylated form of the protein, was shown to bring about an increase in the resistance to oxidative stress [7]. Conversely, expression of a non-phosphorylated form of eIF2a (S51A) was shown to lead to oxidative damage [8,9]. These results were confirmed with another approach in a different study [10].

Various forms of cellular stresses induce phosphorylation of eIF2a through four known eIF2a kinases: protein Kinase R (PKR), Heme-regulated eIF2a Kinase (HRI), Protein Kinase R-like Kinase (PERK) and the General Control nonderepressive 2 kinase (GCN2). Phosphorylation of eIF2a partially inhibits general protein synthesis while promoting translation of specific mRNAs, including that the transcription factor ATF4. It has been proposed that ATF4 protects cells against oxidative damage by inducing expression of genes involved in amino acid biosynthesis and transport, antioxidative stress response, and protein folding and secretion [8,11]. One of the protein kinases that regulate the eIF2a/ATF4 signaling pathway, GCN2, is an important sensor of amino acid availability [12,13]. A recent publication by Arriazu et al. [14] suggests that

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GCN2 could play a role in the regulation of redox homeostasis. Indeed, they have shown a correlation between amino acid deprivation, GCN2 activity and the intracellular levels of ROS in isolated hepatic cells. However, at the level of the entire organism, the role of GCN2 with regard to redox homeostasis has never been established. We hypothesized that amino acid supply from food could regulate GCN2 activity which, in response, could control the balance between pro and antioxidant molecules. In the present work, we investigate the effect of a moderate dietary amino acid imbalance on protein carbonylation in the liver, a marker of oxidative stress, in wild type or GCN2−/− mice. Our data show that GCN2 ablation in combination with an amino acid imbalanced diet dramatically increases protein carbonylation in the liver. Theses results demonstrate that an amino acid-imbalanced diet affects the equilibrium between pro and antioxidant compounds, and that this equilibrium is reestablished upon GCN2 activation.

2. Materials and methods

2.1. Animals and diets

Seven-months-old mice were individually housed in wire-bottom cages in a temperature-controlled room (22 ± 0.8 °C) with a 12 h light−dark cycle and 55 ± 10% relative humidity. They were all fed a standard breeding diet A03 (Safe) for 2 weeks before the beginning of the experiment. After this 2 week adaptation period, the mice were randomly divided into three groups ($n = 7$ per group) and fed for 24 weeks with a 12 h fed-not fed cycle. The diets used to study the response were semiliquid and manufactured in INRA using purified ingredients (Louis François) and free L-amino acids (Jerafrance). All diets contained free L-amino acids (calculated on the base of the amino acid composition of a 20% lactoserum diet as the sole protein source). The EDΔLeu diet was adjusted with alanine. Moreover, in this diet, isoleucine and valine levels were also reduced in order to keep the blood concentration of these amino acids constant after eating [21]. At the end of the experimental phase, after an overnight fasting period, mice were weighed and sacrificed. Blood was collected and the liver and adipose tissue were frozen and stored at −80 °C. All procedures were carried out according to the European Community for the use of experimental animals (L358-86/609/EEc) and were approved by the Institut National de la Recherche Agronomique (INRA-France). INRA animal facilities were approved by the French veterinary department (C634514).

2.2. Plasma biochemical compounds measurement

Amino acids: Blood samples were withdrawn from the aorta. Plasma samples were treated with sulfosalicylic acid and thiodiglycol. Free amino acid proportions were determined using ion-exchange liquid chromatography followed by postcolumn detection with ninhydrine (Bio-Tek system). The internal standard, norleucine, allowed for the evaluation of sample treatment efficiency in order to normalize the raw values [24].

Glucose, triglycerides, total-, LDL-, HDL-cholesterol, urea, lactate blood measurements were realized on Modular analyzer (Roche Diagnostics) using the manufacturer's recommendations. Insulinemia was measured on Immulite analyzer (Siemens Health Diagnostics) after verification of immune-reactivity and -compatibility.

2.3. Western-blot analysis of protein carbonyls and ATF4 expression

Mice livers were lysed in a buffer containing 200 mM Tris pH 7,5, 2 mM EDTA, 150 mM NaCl, 10 μg/ml protease inhibitor cocktail (Sigma–Aldrich). Liver proteins (15 μg) were denatured with 12% SDS and incubated 15 min with 2,4-dinitrophenylhydrazine (DNP) at room temperature. DNP-derived proteins were loaded on a 4–12% SDS–PAGE and detected by Western-blotting using an anti-DNP antibody according to the manufacturer's protocol (Oxyblot protein oxidation detection kit, Millipore-Chemicon).

ATF4 expression was measured by western blot as previously described [19].

2.4. Analysis of GPX1 expression using real-time RT-PCR

Total RNA was prepared using an RNeasy mini kit (Qiagen) and treated with DNase I, Amp Grade (InVitrogen) prior to cDNA synthesis. RNA integrity was electrophoretically verified by ethidium bromide staining. RNA (0.5 mg) was reverse transcribed with 100 U of Superscript II plus RNase H-Reverse Transcriptase (Invitrogen) using 100 μM random hexamer primers (Amersham Biosciences), according to the manufacturer's instructions. We used the following primers: mouse GPX1 primer: forward primer, 5′-GTCCACCGTGTATGCCTTCT-3′; reverse primer, 5′-CTCCTGGTGTCCGAACTGAT-3′; mouse β-actin, forward primer, 5′-TACAGCTTCACCACCACAGC-3′; reverse primer 5′-AAGGAAGGCTGGAAAAGAGC-3′. Real-time quantitative PCR was carried out using a LightCycler TM System (Roche Applied Science) as described previously [25,26]. Results were displayed as relative levels of GPX1 per β-actin. Each experiment was repeated at least four times.

2.5. Measurement of total GPx activity in liver

Mice livers were lysed in a buffer containing 200 mM Tris pH 7,5, 2 mM EDTA, 150 mM NaCl, 10 μg/ml protease inhibitor cocktail (Sigma–Aldrich). Cellular debris were removed by centrifugation at 10,000*g*, 15 min, 4 °C. GPx activity was measured using the glutathione reductase-coupled test optimized for mouse tissue

Table 1
Body weight and blood biochemical parameters of mice at the end of the experiment. At the end of the experiment, mice were weighed and sacrificed. Sera were collected for glucose, triglycerides, cholesterol, urea, insulin and lactate analysis. Statistical analysis does not show significant differences between genotype and/or diet for any the biochemical compounds measured excepted for glucose. For glucose assays, values with no letter in common differ significantly ($P < 0.05$).

	Experimental diet (ED)	Experimental diet Δ Leu (EDΔLeu)
	GCN2+/+ GCN2−/−	GCN2+/+ GCN2−/−
Body weight (g)	29.9 ± 1.2 28.5 ± 1.6	30.1 ± 0.9 28.7 ± 1.2
Glucose (mmol/L)	7.2 ± 0.5[b] 8.6 ± 0.3[a]	7.9 ± 0.4[ab] 8.5 ± 0.6
Triglycerides (g/L)	0.92 ± 0.07 0.94 ± 0.07	0.98 ± 0.07 0.91 ± 0.08
Total cholesterol (mmol/L)	4.1 ± 0.3 3.7 ± 0.3	4.0 ± 0.4 3.7 ± 0.2
HDL cholesterol (mmol/L)	3.6 ± 0.3 3.2 ± 0.3	3.6 ± 0.1 3.2 ± 0.2
LDL cholesterol (mmol/L)	0.38 ± 0.03 0.35 ± 0.04	0.36 ± 0.03 0.34 ± 0.02
Urea (mmol/L)	8.7 ± 0.5 9.5 ± 0.3	9.8 ± 0.4 9.9 ± 0.4
Lactate (mmol/L)	2.7 ± 0.1 3.1 ± 0.3	3.5 ± 0.5 3.2 ± 0.4
Insuline (mUI/L)	70 ± 11 90 ± 16	85 ± 6 94 ± 8

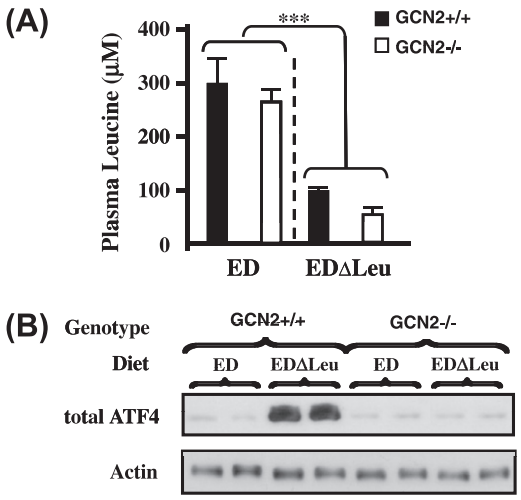

Fig. 1. Post-prandial injestion of a leucine imbalanced diet decreases blood leucine concentrations and activates the GCN2/ATF4 pathway. (A) Plasma leucine concentration of mice fed for four hours on ED or EDΔLeu diets. Values shown represent the means based on groups of seven animals. Values are expressed in µmol/L. (B) Western blot analysis of ATF4 protein expression in livers of GCN2+/+ and GCN2−/− mice fed ED or EDΔLeu diets for four hours. Actin serves as a loading control.

according to the manufacturer's protocol (Glutathione Peroxidase Assay Kit, Cayman).

3. Results and discussion

3.1. Experimental paradigm

The set of experiments presented in this article has been designed to explore the potential role of GCN2 in the control of redox homeostasis in the context of amino acid starvation and the subsequent oxidative damage resulting from its deregulation. For this purpose, mice were fed either a control diet or a diet partially depleted for leucine as a means of inducing the GCN2 response. The diets used were enriched in sucrose and fat in order to increase the basal level of oxidative stress. Indeed, several studies have shown that a higher consumption of sugar and the substitution of unsaturated vegetable oil by lard increases oxidative damage [15–17]. GCN2+/+ and GCN2−/− mice were divided into two groups for each genotype and fed either on an experimental diet (ED) or a leucine-imbalanced diet (EDΔLeu diet). A detailed composition of these diets is provided in Supplementary data 1. ED and EDΔLeu were enriched with sucrose and lard as compared to the normal chow diet. In ED and EDΔLeu diets a part of the amino acids were supplied by two sources, casein (6%) and a mixture of free amino acids (14%) including or lacking leucine.

Animals were housed with a 12 h light-dark cycle and had free access to food during the dark period. Twenty-four weeks after the beginning of the experiment, animals were sacrificed, then oxidative damage and other biological parameters were measured.

3.2. Characterization of mice fed ED or EDΔLeu diets

We first measured the body weights of GCN2+/+ and GCN2−/− knock-out mice at the end of the experimental protocol. As a whole, Table 1 shows that body weights are not significantly affected by genotype and/or leucine imbalanced diet. Among the blood biochemical parameters, there is a basal increase in glucose in GCN2−/− mice as compared to wild type controls (see Table 1 for details). Furthermore, total cholesterol is lower in the GCN2−/− mice although this trend is not statistically significant.

In a different experimental model using a diet totally devoid of leucine, results demonstrated that GCN2 regulates fatty acid homeostasis [18]. Several blood parameters related to lipid metabolism were dramatically affected by the leucine deficient diet in both GCN2+/+ and GCN2−/− animals. In addition, hepatic steatosis was observed in GCN2−/− mice fed on a leucine devoid diet. In contrast, our experiemental diets do not affect lipid metabolism (Table 1), cause hepatic steatosis (data not shown) or affect overall animal health during the 24 weeks of the experiment.

To verify the effect of the diet diminished in leucine, we measured the blood leucine content and the activation of the GCN2/eIF2a/ATF4 pathway during the post-prandial period of the nycthemer. Blood was withdrawn from the tails of the mice 4 hours after

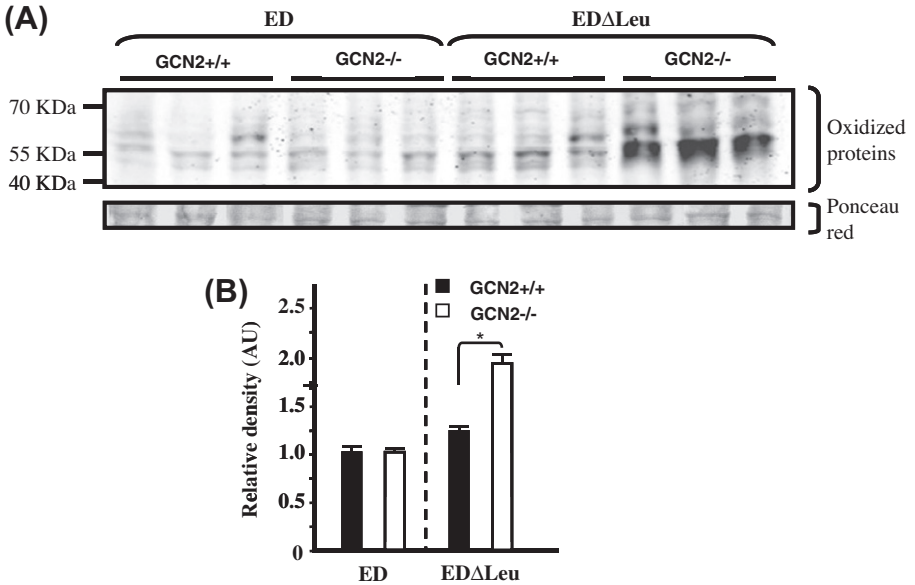

Fig. 2. GCN2−/− mice fed 24 weeks on EDΔLeu diet exhibit an accumulation of oxidized proteins in the liver. (A) The levels of oxidized proteins were estimated by western blot detection of carbonyl radicals as described in the Material and Methods section. The blot shown is representative of each group. (B) Quantitative intensity of each lane is expressed in arbitrary units. Quantification takes into account seven mice in each group (*$P < 0.05$).

the beginning of a meal. Fig. 1A shows that leucinemia is dramatically decreased after consumption of the leucine-imbalanced diet, regardless of the genotype. In a parallel experiment, two mice per group were sacrificed four hours after the beginning of a meal in order to measure the hepatic expression of ATF4, an indicator of the activation of the GCN2/eIF2a/ATF4 pathway. The liver represents an ideal model system for studying the response to amino acid-imbalanced diet on oxidative homeostasis. In particular, the liver is a key organ responsible for the metabolism of ingested alimentary amino acids. In addition, previous work demonstrated that the GCN2/ATF4 pathway is regulated in the liver by the alimentary amino acid content of the food [19,20]. Particularly, this pathway is strongly activated in response to amino acid imbalanced-diet [21]. Therefore, in our system of dietary amino acid imbalance, we confirmed that ATF4 expression is induced in the liver of the wild type animals whereas GCN2 ablation prevents this induction (Fig. 1B). Taken together, we established that the GCN2/ATF4 pathway is indeed active in our *in-vivo* model of amino-acid restriction.

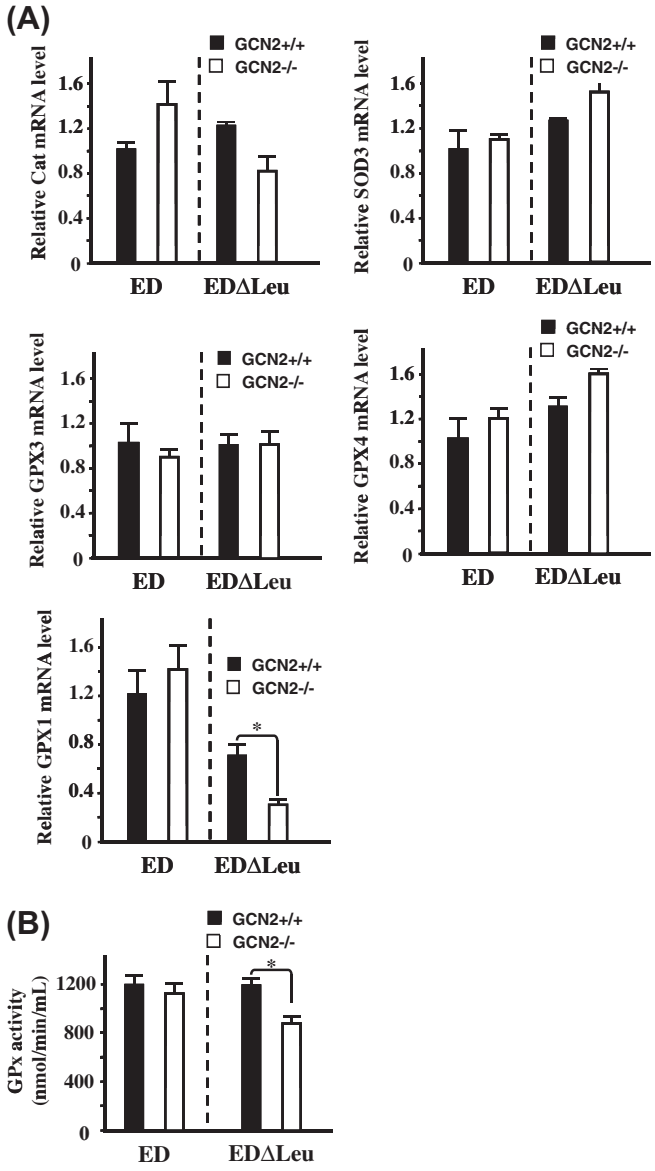

Fig. 3. GCN2−/− mice fed 24 weeks on EDΔLeu diet exhibit less expression of GPX1 associated with a decrease of total GPx activity in the liver. (A) mRNA levels were quantified by real-time qRT-PCR. β-Actin serves as a control gene. (B) Total GPx activity in the liver of GCN2+/+ and GCN2−/− mice fed with ED or EDΔLeu diets. Measurement of GPx activity was performed as described above. Values represent the mean of seven animals per group. (*) indicates statistically significant differences between genotypes as determined by student's *t*-test analysis ($P < 0.05$).

3.3. GCN2 protects hepatic proteins from leucine restriction-induced oxidation

Based on published data [14,9,8] which shows that GCN2 plays a role in redox homeostasis, we hypothesized that, in animals fed a leucine imbalanced diet during the post-prandial period, activation of the GCN2/eIF2a/ATF4 pathway is involved in this regulation *in-vivo*. To determine if oxidation levels are modulated in our experimental conditions, we measured the amount of oxidized proteins in the liver (Fig. 2). We found a dramatic increase in the level of protein carbonylation only in GCN2−/− mice fed a leucine imbalanced diet demonstrating that GCN2 activity is necessary to allow cells to resist leucine starvation-induced oxidative stress.

Since oxidative stress results from an imbalance between ROS production and metabolism, we measured the expression level of several genes encoding antioxidant enzymes. Fig. 3A shows that the expression of catalase, super oxide dismutase 3, glutathione peroxidase 3 and 4 (GPX3 and GPX4) is not significantly affected by either genotype or an amino acid imbalanced diet. However, in the liver, GPX1 mRNA levels are decreased in all mice fed an EDΔLeu as compared to mice fed with ED diet but the trend is not statically significance. The decrease of GPX1 mRNA is exacerbated upon GCN2 ablation. We then measured total GPX activity in the liver. As GPX1 is the major isoform of Glutathione Peroxidase expressed in the liver, total GPX activity predominantly reflects GPX1 activity [22,23]. Fig. 3B illustrates that GPX activity only decreases in response to a leucine imbalanced diet in GCN2−/− mice. This suggests that GCN2 is involved in the maintenance of GPX1 expression in response to amino acid starvation and participates in the control of redox homeostasis. Taken together, these results indicate a central role of GCN2 activity and the eIF2a/ATF4 pathway in the control of redox state as a function of nutritional status.

In cultured cells, it has previously been shown that the basal level of eIF2a phosphorylation determines cellular antioxidant status [8]. Our results clearly demonstrate that the GCN2/eIF2a/ATF4 pathway can serve a similar function in animals. This study reveals that a leucine imbalanced diet can (1) modulate redox homeostasis in the liver and (2) activate the GCN2 pathway which, in turn, contributes to the maintenance of the equilibrium between pro and antioxidant compounds. The molecular mechanisms involved in these two processes are not yet understood. We can speculate that amino acid deficiency affects the intracellular amounts of antioxidants (amino acids, glutathione, etc.) leading to oxidative stress. Contrastingly, amino acid starvation activates GCN2, which controls the expression of genes involved in the resistance to oxidative stress (enzymes involved in ROS scavenging, amino acid transporters, etc.). For example, GPX1 is dramatically downregulated in GCN2−/− mice fed an amino acid-imbalanced diet. The underlying molecular mechanisms through which GCN2 regulates GPX1 expression remain to be investigated.

EIF2a phosphorylation is at the intersection of several stress response pathways and, therefore, can be initiated not only in response to amino acid limitation but also in response to various other stresses. The roles of the signaling pathways downstream of eIF2a phosphorylation are complex and numerous. Depending on the context, two fundamentally different outcomes can ensure: adaptation to stress or initiation of programmed cell death. The outcome appears to be determined by the duration and the intensity of the stimulation of the pathway and also by the interplay with other signaling pathways. Regulation of the antioxidant status by the eIF2a/ATF4-signaling pathway is one aspect of the multiple roles of this pathway but appears to be of paramount importance,

as it appears deregulated in several diseases. For example, nerve cell death which occurs in Alzheimer's disease is thought to be linked to oxidative stress [7]. Lewerenz et al. [8] have shown that, in the human brain, ATF4 and phospho-eIF2a levels are tightly correlated and up-regulated in Alzheimer's disease. Taken together, our data combined to results from the literature point to a central role of eIF2a/ATF4 in the control of oxidative stress, suggesting that the eIF2a/ATF4-signaling pathway could be a possible target for manipulating the oxidative status of cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.10.027.

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