Dysregulated glutathione metabolism links to impaired insulin action in adipocytes

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Kobayashi H, Matsuda M, Fukuhara A, Komuro R, Shimomura I. Dysregulated glutathione metabolism links to impaired insulin action in adipocytes. Am J Physiol Endocrinol Metab 296: E1326–E1334, 2009. First published April 14, 2009; doi:10.1152/ajpendo.90921.2008.—Oxidative stress plays an important role in obesity-related metabolic diseases. Glutathione peroxidase (GPX) is an antioxidant enzyme downregulated in adipose tissue of obese mice. However, the role of GPX in adipocytes remains elusive. The objective of this study was to clarify the pathophysiological changes in GPX activity and glutathione (GSH) metabolism and their roles in the pathogenesis of insulin resistance in adipocytes. To achieve this goal, we measured cellular GPX activity, glutathione (GSH) contents, GSH/GSSG ratio, and mRNA expression of γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme for de novo GSH synthesis, in adipose tissue of control and ob/ob mice and in 3T3-L1 adipocytes treated with insulin, H2O2, free fatty acid (FFA), or TNFα. Furthermore, we investigated the effects of GPX inhibition with a specific GPX inhibitor or RNA interference against GPX, H2O2, and reduced GSH on insulin signaling in 3T3-L1 adipocytes. ob/ob Mice showed not only a decrease in cellular activity of GPXs (GPX1, -4, and -7) but also an increase in γ-GCS expression, resulting in increased GSH contents in adipose tissue. These alterations in glutathione metabolism were also observed during differentiation of 3T3-L1 cells and their exposure to insulin, FFA, or H2O2. Inhibition of GPX activity, addition of GSH, and H2O2 resulted in impaired insulin signaling in 3T3-L1 adipocytes. These results suggest that decreased GPX activity and increased γ-GCS expression lead to overaccumulation of GSH, which might be involved in the pathogenesis of insulin resistance in obesity.

glutathione peroxidase; γ-glutamylcysteine synthetase; oxidative stress; insulin resistance

Obesity is a state of chronic inflammation of adipose tissue, with increased production of proinflammatory cytokines and chemokines such as tumor necrosis factor (TNFα), interleukin (IL)-6, and monocyte chemoattractant protein-1 (41), which is the causative factor of insulin resistance (7, 27). Recent reports have suggested that such chronic inflammation of adipose tissue is partially mediated by various cellular stresses, including endoplasmic reticulum stress (26), hypoxia (13), and oxidative stress (10). Oxidative stress, mediated by the intracellular accumulation of reactive oxygen species (ROS), has been implicated in obesity-associated metabolic diseases (9, 10) as well as in atherosclerosis, microvascular complications of diabetes, and pancreatic β-cell failure in type 2 diabetes (1, 34). We demonstrated previously that the levels of various markers of systemic oxidative stress correlated with the extent of fat accumulation and inversely correlated with plasma adiponectin levels in nondiabetic human subjects (9, 10). Obese mice also showed increased oxidative stress in adipose tissue (10). In cultured adipocytes, the addition of ROS suppressed mRNA expression and secretion of adiponectin and also increased IL-6 and monocyte chemoattractant protein-1 mRNA expression levels (10). Oxidative stress induced by TNFα or glucocorticoid is involved in insulin resistance in 3T3-L1 adipocytes (14). In addition, treatment with apocynin, an NADPH oxidase inhibitor, or MnTBAP, a ROS scavenger, improved glucose metabolism in obese mice (10, 14), indicating that oxidative stress is a potentially suitable therapeutic target for obesity-associated metabolic disease.

The increased ROS in hypertrophied adipose tissue is accompanied by not only an increase in mRNA expression levels of subunits of NADPH oxidase, an enzyme complex that generates ROS, but also a decrease in mRNA expression levels and activities of antioxidant enzymes such as glutathione peroxidase (GPX), Cu/Zn superoxide dismutase (Cu/Zn SOD), and catalase (10), which are essential for homeostasis of the redox state (23) and are induced to detoxify ROS when cells are exposed to oxidative stress in other organs (33). Thus, dysregulation of antioxidant enzymes should lead to dysfunction of adipocytes. However, the precise regulatory mechanisms and roles of each antioxidant enzyme in adipocytes remain to be elucidated.

GPX is one of the antioxidant enzymes downregulated in hypertrophied adipose tissue. Seven members of the GPX family have been identified so far. We reported previously that GPX1 (10) and GPX3 (22) are expressed in adipose tissue. GPX1 is the most characterized cytosolic and mitochondrial GPX (3), whereas GPX3 is the extracellular GPX recognized as serum GPX and is secreted mainly by the kidney (21). However, there is little or no information on the role of other GPXs in the homeostasis of redox state in adipocytes.

GPXs scavenge and inactivate hydrogen and lipid peroxides to water or lipid hydroxyls in a glutathione (GSH)-dependent reduction reaction in mammalian cells (5, 6). Thus, GSH is an essential factor for the enzymatic function of GPXs on the reduction reaction and also an antioxidant itself to scavenge ROS. The intracellular content of GSH and the ratio of reduced GSH to oxidized GSH (GSH/GSSG ratio) are regulated through several steps to maintain homeostasis. GPXs facilitate the oxidation of GSH to GSSG upon the catalytic reaction to reduce H2O2. GSSG is recycled to GSH by GSH reductase (GSH-R). The de novo synthesis of GSH is accomplished through two consecutive steps catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase.
synthetase (γ-GCS) and GSH synthetase (GS) (8, 25, 31). To our knowledge, these regulatory systems of intracellular GSH content and GSH/GSSG ratio have not been investigated in adipose tissue.

The present study identified abundant expression of GPX1, -4, and -7, as cellular GPXs, in adipocytes. Furthermore, ob/ob mice showed not only low activity levels of tissue GPX but also overexpression of γ-GCS, resulting in high GSH content in adipose tissue. Inhibition of GPX activity using a chemical GPX inhibitor or RNA interference of GPX1, -4, and -7 resulted in impaired insulin signaling. Interestingly, treatment of 3T3-L1 adipocytes with GSH led to insulin resistance, similar to that noted upon GPX inhibition. Taken together, the results suggest that overaccumulation of intracellular GSH, possibly through downregulation of GPX activity and/or up-regulation of γ-GCS expression, might be involved in the impaired insulin signaling in adipose tissue of obesity.

MATERIALS AND METHODS

Reagents and antibodies. Mercaptosuccinate (MS), GSH-reduced ethyl ester, H$_2$O$_2$, insulin, sodium selenite, buthionine sulfoximine (BSO), and palmitate were purchased from Sigma (St. Louis, MO). The reduced form of GSH was obtained from Nacalai Tesque (Kyoto, Japan). Human TNFα was from Peprotech (Rocky Hill, NJ). 5,6-Carboxymethyl-2',7'-dichlorofluorescein diacetate (DCFDA) kit was purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies (pAb) to insulin receptor substrate (IRS)-1 and rabbit antiserum to phosphatidylinositol 3-kinase (PI3K) p85 subunit were from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt and anti-phospho-Akt (Ser473) pAbs were from Cell Signaling Technology (Beverly, MA). Anti-phospho-IRS-1 (Tyr608) pAb was from Calbiochem (San Diego, CA).

Cell culture and treatment conditions. 3T3-L1 cells were maintained and differentiated into adipocytes, as described previously (16).

Animals. Seven-week-old C57BL/6J and ob/ob mice were purchased from CLEA Japan, All mice were housed in a room maintained at 23°C with a fixed light-dark cycle. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University, Graduate School of Medicine.

RNA isolation and Northern blot analysis. Total RNA from 3T3-L1 adipocytes and tissues were extracted using Sepasol-RNA I Super and incubated overnight at 68°C with a$^{32}$P-radiolabeled gene-specific probe. The activity was normalized to protein content.

Measurement of intracellular GSH content and GSH/GSSG ratio. Intracellular GSH content and the GSH/GSSG ratio in adipose tissue and 3T3-L1 adipocytes were measured using Bioxytech GSH/GSSG-412 kit (Oxis Research, Portland, OR). Cells or tissue sample were washed with ice-cold PBS and 5% metaphosphoric acid with or without 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate, a GSH-specific scavenger. After sonication, they were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was used to measure the concentrations of GSH and GSSG. The assays were performed at 412 nm optical density for 3 min with a Vient Spectrophotometer (Dainippon Sumitomo Pharma, Tokyo, Japan).

Measurement of ROS. 3T3-L1 cells were transfected with control siRNA oligonucleotides or GPX siRNA cocktail specific to GPX-1, -4, and -7, using DeliverX transfection reagent according to the protocol recommended by the manufacturer. Forty-eight hours later, 3T3-L1 adipocytes were washed with PBS twice and incubated with 1 μM DCFDA in PBS for 30 min at 37°C. Subsequently, 3T3-L1 adipocytes were washed with PBS, and then cells were trypsinized and analyzed by fluorescence with FACs Aria flow cytometer (BD Biosciences, San Diego, CA).

Statistical analysis. Values were expressed as means ± SE. Differences between two groups were examined for statistical significance using Student’s t-test, whereas differences among several groups were examined by one-way analysis of variance followed by post hoc analysis. A P value <0.05 was considered statistically significant.

RESULTS

Intracellular GSH content, redox state, and expression of associated enzymes in adipose tissues of obese mice. The mRNA levels of cellular GPXs (GPX1, -2, -4, -5, -6, and -7) and a secretory GPX (GPX3) were estimated by quantitative real-time RT-PCR in adipose tissues (data not shown). We were able to measure the mRNA levels of GPX1, -3, -4, and -7, but the expression levels of GPX2, -5, and -6 in adipose tissues were below the detection limit. The mRNA expression levels
of the above four cellular and extracellular GPX isoforms in various tissues and 3T3-L1 adipocytes were confirmed by Northern blotting (Fig. 1A). The expression of these GPX isoforms was detected in epididymal and brown fat, whereas only a weak expression of GPX3 was detected in 3T3-L1 adipocytes. Next, we compared total cellular GPX activity, possibly the combined activity of GPX1, -4, and -7, in adipose tissue between control C57BL/6 and ob/ob mice. Total cellular GPX activity in adipose tissue was significantly decreased in either 8- or 21-wk-old ob/ob mice compared with the respective control (Fig. 1B). Furthermore, in ob/ob mice, the mRNA levels of the two components of γ-GCS, the rate-limiting enzyme in the de novo synthesis of GSH, were significantly elevated, whereas that of GSH-R, by which GSSG is recycled to GSH, was also increased, albeit insignificantly (Fig. 1C). In addition, we measured intracellular GSH content and GSH/GSSG ratio in adipose tissue. GSH content was significantly higher in adipose tissues of 8- and 21-wk-old ob/ob mice compared with the control mice (Fig. 1D). On the other hand, the GSH/GSSG ratio was low, i.e., a tilt toward more oxidation, in ob/ob mice compared with the control mice (Fig. 1E).

The pathological outcome of an inhibition of GPX activity in 3T3-L1 adipocytes. To investigate whether the downregulation of GPX activity can be involved in the pathogenesis of adipocyte dysfunction, we conducted the experiments of GPX inhibition in 3T3-L1 adipocytes using MS, a specific GPX inhibitor (4), or a siRNA cocktail specific to GPX1, -4, and -7.

Figure 2A shows the potency of MS, indicating that it significantly suppressed the cellular GPX activity in 3T3-L1 adipocytes. Interestingly, pretreatment of 3T3-L1 adipocytes with MS inhibited insulin-induced tyrosine phosphorylation of IRS-1 and protein interaction of PI3K to IRS-1, leading to a decrease in the insulin-induced serine phosphorylation of Akt (Fig. 2B).

Figure 2C shows the effect of a cocktailed siRNA specific to GPX1, -4, and -7, indicating that it significantly reduced the mRNA expressions of GPX1, -4, and -7 but not that of Cu/Zn SOD or catalase. Similar to the effect of MS, this cocktailed siRNA suppressed insulin-stimulated phosphorylation of IRS-1 and Akt (Fig. 2D). These results indicate that inhibition of cellular GPX by either MS or siRNA of GPXs led to insulin resistance in adipocytes. On the other hand, MS treatment did not alter the

Fig. 1. Altered glutathione (GSH) peroxidase (GPX) activity and GSH metabolism in adipose tissue of obese mice. A: total RNA was extracted from the indicated tissues of control C57BL/6 mice and subjected to Northern blot analysis with the probes specific for GPX1, GPX3, GPX4, and GPX7. B: cellular GPX activity in epididymal white adipose tissue (WAT) from C57BL/6 mice (control) and ob/ob mice at 8 and 21 wk of age. Values are expressed as means ± SE (n = 5–7). C: the mRNA levels of adiponectin, 2 components of γ-glutamylcysteine synthetase (γ-GCS; γ-GCS-h and γ-GCS-l), and GSH reductase (GSH-R) were quantified by real-time RT-PCR from C57BL/6 mice (control) and ob/ob mice at 13 wk of age. Values are normalized to the level of cyclophilin mRNA and expressed as means ± SE (n = 6). D: intracellular GSH content. E: GSH/oxidized GSH (GSSG) ratio in epididymal WAT of C57BL/6 mice (control) and ob/ob mice at 8 and 21 wk of age. Data are means ± SE (n = 5–7). *P < 0.05; **P < 0.01; ***P < 0.001. BAT, brown adipose tissue; NS, not significant.
mRNA levels of adiponectin, plasminogen activator inhibitor-1, and IL-6 (data not shown), which was somehow different from the mRNA changes by the treatment with ROS, as we reported previously (10).

Possible involvement of GSH and ROS in insulin resistance resulting from GPX inhibition. To understand the effect of GPX inhibition on cellular homeostasis of GSH and redox state, we assessed the dynamic changes of cellular GSH content and GSH/GSSG ratio upon GPX inhibition in 3T3-L1 adipocytes. MS treatment significantly elevated intracellular GSH content and alters GSH/GSSG ratio (Supplementary Fig. S1) (36, 39, 40). In addition, as another possible mediator for the effect of GPX inhibition, we examined the effect of H$_2$O$_2$ on insulin signaling because GPX is a scavenger of H$_2$O$_2$. H$_2$O$_2$ treatment also resulted in the suppression of insulin-induced tyrosine phosphorylation of IRS-1, the subsequent protein interaction of PI3K to IRS-1, and serine phosphorylation of Akt inhibited by MS (Fig. 1C). These results suggest that both ROS production and GSH accumulation contribute to impaired insulin signaling by GPX inhibition.

Regulation of GSH content, redox state, and expression of associated enzymes. Next, the regulation of GPX activity, intracellular GSH content, GSH/GSSG ratio, and mRNA expression levels of γ-GCS and GSH-R were investigated in 3T3-L1 adipocytes. During their differentiation, 3T3-L1 adipocytes exhibited less GPX activity, more intracellular GSH content, and more GSH/GSSG ratio than preadipocytes (Fig. 4, A–C). The mRNA expression levels of γ-GCS and GSH-R were higher in differentiated 3T3-L1 adipocytes than in undifferentiated ones (Fig. 4D). To identify the regulatory mechanism(s) underlying the alterations in GSH metabolism in adipose tissue of obese mice, 3T3-L1 adipocytes were treated with insulin, H$_2$O$_2$, free fatty acid (FFA; palmitate), or TNFα,
which are circulating factors elevated in obese mice. Treatment with insulin, H$_2$O$_2$, or FFA reduced GPX activity and increased intracellular GSH content (Fig. 5, A and B). Insulin treatment also augmented GSH/GSSG ratio (Fig. 5C). On the other hand, TNF$\alpha/H9251$ increased GPX activity but did not alter intracellular GSH content or redox state. H$_2$O$_2$ increased the mRNA expression level of $\gamma$-GCS, whereas TNF$\alpha/H9251$ and insulin decreased it, and TNF$\alpha$ suppressed that of GSH-R (Fig. 5D). These results indicate that insulin and H$_2$O$_2$ inhibit GPX activity, leading to intracellular GSH accumulation, and that H$_2$O$_2$ increased the expression of $\gamma$-GCS.

**DISCUSSION**

In the present study, we found abundant expression of cellular GPX1, -4, and -7 in adipose tissues and reduced cellular GPX activity in adipose tissue in ob/ob mice. Inhibition of GPX activity using a chemical GPX inhibitor or RNA interference of GPX1, -4, and -7 resulted in amelioration of insulin-induced phosphorylation of IRS-1 and Akt in 3T3-L1 adipocytes.

To investigate the underlying mechanism, we first examined the effect of H$_2$O$_2$ on insulin signaling in 3T3-L1 adipocytes (Fig. 3D) because GPXs are antioxidant enzymes that detoxify H$_2$O$_2$, and DCFDA assay revealed the induction of H$_2$O$_2$ in 3T3-L1 adipocytes treated with siRNA cocktail specific to GPX1, -4, and -7 (Supplementary Fig. S2). 3T3-L1 adipocytes chronically treated with H$_2$O$_2$ exhibited a decrease in insulin-induced phosphorylation of Akt and IRS-1, as described previously (12, 29, 37), similar to those upon inhibition of GPXs. However, in a series of preliminary experiments, knockdown
of catalase, another important antioxidant enzyme for H2O2, did not result in insulin resistance (data not shown). This finding tempted us to hypothesize that there might be another GPX-dependent mechanism leading to insulin resistance resulting from inhibition of GPXs. GPXs facilitate the oxidative reaction from GSH to GSSG upon the catalytic reaction to reduce H2O2. Therefore, inhibition of GPXs should lead to accumulation of GSH content with increased GSH/GSSG ratio, as we demonstrated in vitro (Fig. 3, A and B). Therefore, we investigated the effect of GSH on insulin signaling. Interestingly, our data demonstrated that treatment of adipocytes with GSH (36, 39, 40) resulted in the suppression of insulin-mediated phosphorylation of IRS-1 and Akt. GSH has been reported to enhance lipopolysaccharide-induced activation of p38 MAPK (39), although the mechanism of this action remains elusive. Since activation of p38 MAPK has been reported to ameliorate insulin signaling (20), this property of GSH might be involved in GSH-induced insulin resistance. Our in vitro findings indicated that intracellular overaccumulation of GSH might be involved in insulin resistance in 3T3-L1 adipocytes. Furthermore, we demonstrated elevated GSH content in the adipose tissue of ob/ob mice (Fig. 1D), which was consistent with the previous report of high GSH content in adipose tissues of 11-wk-old obese Zucker rats (11). Taken together, our findings suggest that overaccumulation of GSH in adipose tissue might be associated with amelioration of insulin signaling. However, since GSH itself has antioxidative properties, GSH protects cells from ROS-induced cell damage in the presence of ROS (18). Further studies are required to define the role of GSH in adipose tissues of obesity.

GSH content is regulated not only by GPXs but also other pathways; GPXs facilitate the oxidation of GSH to GSSG, which is recycled to GSH by GSH-R, and GSH is synthesized de novo through two consecutive steps catalyzed by γ-GCS and GS (8, 25, 31). In the present study, we found that the mRNA expression of γ-GCS, but not GSH-R, was elevated in adipose tissue of ob/ob mice, thus providing another mechanism for the accumulation of GSH. Furthermore, in our in vitro study, treatment of adipocytes with H2O2, but not insulin or TNFα, increased the mRNA expression of γ-GCS (30), suggesting the involvement of oxidative stress in the upregulation of γ-GCS expression in obese adipose tissue.

Our results also showed lower activity of cellular GPX and overexpression of γ-GCS, with high GSH content and GSH/GSSG ratio in mature adipocytes, compared with preadipocytes. We also demonstrated that inhibition of GPX or accumulation of GSH impaired insulin signaling. On the other hand, it is well known that insulin signaling is enhanced after differentiation of adipocytes with the induction of insulin receptor, IRS-1, IRS-2, and PKB (28). These inductions should dominantly contribute to the upregulation of insulin signaling in mature adipocytes.

Incubation of mature adipocytes in the presence of insulin, H2O2, or FFA resulted in further suppression of cellular GPX activity and further accumulation of GSH. Among these factors, insulin reduced GPX activity, elevated GSH content, and induced mRNA levels of γ-GCS, all of which were observed in adipose tissues of ob/ob mice. Taken together, in obese mice, chronic hyperinsulinemia might explain, at least in part, the mechanism of insulin resistance through the dysregulated metabolism of GSH. Collectively, the results indicate that GSH content and GSH/GSSG ratio in adipocytes are actively regulated in a complex fashion cell type dependently or by circulating factors, suggesting that GSH metabolism is probably regulated not only in a compensatory fashion by oxidative stress but also actively by physiological status. Our in vivo data indicated that, in adipose tissue, GSH content was consistently higher, but GSH/GSSG ratio varied according to age of ob/ob mice compared with control mice (Fig. 1, D and E). Further studies are required to determine the precise in vivo mechanism involved in the regulation of GSH metabolism.

In our results, incubation of mature adipocytes with TNFα resulted in augmentation of cellular GPX activity. Lee et al. (19) showed that plasma GPX3 activity was increased by rosiglitazone, an agonist of peroxisome proliferator-activated receptor-γ, and was decreased by TNFα. Consistent with their report, TNFα treatment inhibited mRNA expression of GPX3.
(data not shown); however, mRNA levels of GPX1 and GPX4 and activity of cellular GPX were increased by this treatment (data not shown). Taken together, induction of GPX1 and GPX4 may be involved in the augmentation of GPX activity in TNFα-treated 3T3-L1 adipocyte.

Among the seven GPXs known so far, we found that GPX4, and -7, in addition to GPX1 and -3, were abundantly expressed in the adipose tissue. GPX4 is a membrane-associated GPX and catalyzes a wide range of lipid hydroperoxides, including those derived from cholesterol and cholesterol esters (17). GPX7 is a cytoplasmic protein with a structure domain homologous to that of GPX4, and overexpression of GPX7 increases the resistance to the toxic effects of H2O2 and polyunsaturated fatty acids (38). In this regard, Imai and Nakagawa (15) reported that phospholipid hydroperoxidases such as GPX4 and GPX7 play an important role in protecting cells from lipid peroxidation. We previously showed that lipid peroxidation was increased in adipose tissue of obese mice (10). Upregulation of GPX4 and GPX7 in adipose tissue likely indicates their important role in protecting adipocytes from lipid peroxidation. In this regard, a number of GPXs are selenoproteins, and their expression levels are upregulated by selenium at both transcriptional and posttranscriptional levels (2, 24). Recently, Lee et al. (19) reported that overexpression of GPX3, a secreted type of GPX isoform in adipocytes, improved high glucose-induced insulin resistance and inflammatory gene expression. MS is an inhibitor of selenium containing GPXs, including GPX3; therefore, we confirmed that siRNA cocktail specific to the cytosolic GPXs, GPX1, -4, and -7, was sufficient to suppress insulin signaling (Fig. 2D). Taken together, we con-

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Fig. 5. Regulation of cellular GPX activity, intracellular GSH content, and redox state in 3T3-L1 adipocytes. A–C: 3T3-L1 adipocytes cultured in medium containing 100 nM sodium selenite were treated with 100 nM insulin, 500 μM H2O2, 400 μM palmitate, or 30 ng/ml TNFα for 24 h. Changes in cellular GPX activity (A), intracellular GSH content (B), and GSH/GSSG ratio (C) were measured. Values are expressed as means ± SE (n = 3). D: the mRNA levels of adiponectin, γ-GCS, and GSH-R were quantified by real-time RT-PCR. Values are normalized to the level of 36B4 mRNA and expressed as means ± SE (n = 3). *P < 0.05; **P < 0.001.

Fig. 6. Schematic diagram of the proposed roles of GPX activity and GSH metabolism in insulin resistance in adipose tissue. ROS, reactive oxygen species.
sider that serum and cytosolic GPXs should regulate insulin signaling cooperatively, and further analyses of contribution of each GPX isoform on metabolic homeostasis might provide novel approaches to suppress oxidative stress and improve insulin resistance.

Figure 6 illustrates our working hypothesis regarding the roles of GPX and GSH metabolism in insulin resistance. Our findings suggest that increased ROS and hyperinsulinemia may lead to a suppression of GPX activity and induction of γ-GCS mRNA expression, resulting in impaired detoxification of \( \text{H}_2\text{O}_2 \) and overaccumulation of GSH, which might be involved in the pathogenesis of insulin resistance in obesity.

GRANTS

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