Obesity-induced protein carbonylation in murine adipose tissue regulates the DNA-binding domain of nuclear zinc finger proteins

In obesity-linked insulin resistance, oxidative stress in adipocytes leads to lipid peroxidation and subsequent carbonylation of proteins by diffusible lipid electrophiles. Reduction in oxidative stress attenuates protein carbonylation and insulin resistance, suggesting that lipid modification of proteins may play a role in metabolic disease, but the mechanisms remain incompletely understood. Herein, we show that in vivo, diet-induced obesity in mice surprisingly results in preferential carbonylation of nuclear proteins by 4-hydroxy-trans-2,3-nonenal (4-HNE) or 4-hydroxy-trans-2,3-hexenal (4-HHE). Proteomic and structural analyses revealed that residues in or around the sites of zinc coordination of zinc finger proteins, such as those containing the C2H2 or MATRN, RING, C3H1, or N4-type DNA-binding domains, are particularly susceptible to carbonylation by lipid aldehydes. These observations strongly suggest that carbonylation functionally disrupts protein secondary structure supported by metal coordination. Analysis of one such target, the nuclear protein estrogen-related receptor γ (ERR-γ), showed that ERR-γ is modified by 4-HHE in the obese state. In vitro carbonylation decreased the DNA-binding capacity of ERR-γ and correlated with the obesity-linked down-regulation of many key genes promoting mitochondrial bioenergetics. Taken together, these findings reveal a novel mechanistic connection between oxidative stress and metabolic dysfunction arising from carbonylation of nuclear zinc finger proteins, such as the transcriptional regulator ERR-γ.

Oxidative stress and the production of reactive oxygen species result in the direct side-chain oxidation of numerous amino acids, including proline, arginine, and lysine, resulting in a variety of carbonyl products (primary carbonylation) (1). In addition, reactive oxygen species attack double bonds of unsaturated fatty acids, producing lipid hydroperoxides, leading to the formation of a family of reactive lipid aldehydes (2). Secondary protein carbonylation refers to the post-translational modification of the side chains of lysine, cysteine, and histidine residues by reactive lipid aldehydes, such as 4-hydroxy-trans-2,3-nonenal (4-HNE)² or 4-hydroxy-trans-2,3-hexenal (4-HHE)³, ⁴. Such lipids are formed in response to increased oxidative stress or depletion of the cellular antioxidant capacity (5, 6). As such, primary and secondary protein carbonylation are largely recognized as a marker of pathological reactive oxygen species (ROS) production in a variety of cell types and tissues. Much work has focused on identifying protein targets of carbonylation with the goal of making specific mechanistic connections between oxidative stress and the subsequent development of metabolic disease (3, 7).

Previous work from our laboratory and others has demonstrated a striking decrease in the expression of major antioxidants in epididymal adipose depots from murine models of obesity as well as in cultured adipocytes in response to inflammatory stimuli (6, 8–10). This decrease in antioxidant capacity is coincident with elevated ROS and increased levels of reactive lipid aldehydes within the tissue (6, 9). This leads to a significant increase in secondary protein carbonylation of many different proteins within the adipocyte (11–13). Of the antioxidant enzymes, the down-regulation of GSH-S-transferase A4 (Gsta4) in response to tumor necrosis factor α is particularly noteworthy. This enzyme is a major route for detoxification of reactive lipid aldehydes, and depletion of Gsta4 in vivo leads to elevated protein carbonylation, oxidative stress, mitochondrial dysfunction, and impaired insulin signaling (8, 13). Consistent with glutathionylation of reactive aldehydes being linked to insulin action but not obesity per se, human GSTA4 is down-regulated in the obese and insulin-resistant states. This work was supported by National Institutes of Health Grant R01 DK084669 and the Minnesota Agricultural Experiment Station (to D. A. B.), National Institutes of Health Grant T32 GM008347 (to A. K. H.), and the CAPES Foundation, Ministry of Education of Brazil (BEX 13250/13-2) (to R. P.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Tables S1–S5 and Figs. S1–S3. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the data set identifier PXD008415.

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2 The abbreviations used are: 4-HNE, trans-4-hydroxy-2-nonenal; 4-HHE, trans-4-hydroxy-2-hexenal; ERR, estrogen-related receptor; ROS, reactive oxygen species; eWAT, epididymal adipose tissue; iWAT, inguinal white adipose tissue; HDF, high-fat diet; KRAB, Krüppel-associated box; ERRE, estrogen-related response element; DBD, DNA-binding domain; Hsp33, heat shock protein 33; DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay.

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resistant but not the obese and insulin-sensitive state (8). Moreover, in worm models of GSH-S-transferase deficiency (Caenorhabditis elegans gst10), life span was attenuated, and following rescue with murine Gsta4, life span was potentiated (14), suggesting that protein carbonylation plays a fundamental role in longevity. Together, these observations strengthen the hypothesis that oxidative stress and subsequent protein carbonylation play a causal role in metabolic disease.

Technical advances over the last decade have facilitated many proteomic studies aimed at identifying carbonylated proteins in a variety of disease models (13, 15, 16). Here, we expand upon these studies by performing site-specific analysis of in vivo protein carbonylation. We present the novel finding that secondary protein carbonylation endogenously accumulates in the nucleus of adipose tissue and modifies critical metabolic transcriptional regulators in the obese state. Furthermore, we have generated a method by which to identify these proteins and obtain site-specific and lipid-specific information. Importantly, this not only allows for more facile study of carbonylated targets but also allows for a broader analysis of carbonylation patterns and motifs.

Results

Carbonylated proteins accumulate in the adipocyte nucleus

To assess secondary protein carbonylation in adipose tissue, we compared 4-HNE adducts formed in epididymal (eWAT) and inguinal (iWAT) depots of C57Bl/6J mice using an antibody directed toward 4-HNE Michael adducts. Western blotting of 4-HNE protein adducts from eWAT and iWAT adipose depots from 15-week-old mice fed a chow diet or HFD for 12 weeks. Ponceau stain of membrane. β-actin and histone H3 confirm equal loading. Western blotting of 4-HNE protein adducts from nuclear lysates from chow-fed and high fat–fed mice (Fig. 1C). Western blotting of 4-HNE protein adducts from nuclear lysates from chow-fed and high fat–fed mice (Fig. 1C).

Figure 1. Protein carbonylation accumulates in the nucleus of adipocytes in tissue and cultured models of oxidative stress. A (top), Western blotting of 4-HNE protein adducts of whole-cell lysates from eWAT and iWAT adipose depots from 15-week-old mice fed a chow diet or HFD for 12 weeks. Bottom, Ponceau stain of membrane. B, Western blotting of 4-HNE protein adducts from cytosolic and nuclear fractions of eWAT from mice fed a chow of HFD. C, Western blotting of 4-HNE protein adducts from nuclear lysates from chow-fed and high fat–fed mice. Error bars, S.E.; n = 3; p = 0.0005. Right, light microscope image (×60 magnification) of control and erastin-treated cells. *** p < 0.001. E, DAPI and Alexa Fluor 488 fluorescence (×60) in control and erastin-treated cells. 2° Only, incubated with Alexa Fluor 488 antibody but no primary antibody (α-4-HNE). Merge, DAPI and AF488 combined with the differential interference contrast image.
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accumulation of 4-HNE–modified proteins in nuclear extracts of HFD-fed mice compared with chow-fed animals (Fig. 1B). Furthermore, increased nuclear protein carbonylation was specific to the eWAT depot, as levels of carbonylated proteins in samples from iWAT between chow and HFD-fed animals were largely unchanged (Fig. 1C).

To evaluate the biology of carbonylated proteins in the nucleus, we utilized the cultured 3T3-L1 adipocyte cell system. Differentiated 3T3-L1 cells exhibited reduced GSH levels and robust protein carbonylation under conditions of oxidative stress (19, 20). To reduce cellular GSH levels and achieve a pro-oxidative environment in 3T3-L1 adipocytes, cells were treated with erastin. Erasin is an inhibitor of the cystine/glutamate antiporter (system Xc_−) (21), and treatment (10 μM) results in the depletion of GSH pools (Fig. 1D) without cytotoxicity or altered cell morphology (Fig. 1D and Fig. S1B). GSH is critical for maintenance of redox homeostasis, and its depletion represents a global cellular oxidative stress phenotype. In particular, a major mechanism by which reactive lipid aldehydes, such as 4-HNE and 4-HHE, are detoxified is through glutathionylation by GSH-S-transferase A4 (8, 22). Previous work from our laboratory and others has shown that GSH-S-transferase A4 mRNA and protein are down-regulated in the eWAT of obese, insulin-resistant C57Bl/6J mice (6) and in iWAT in obese, insulin-resistant humans (23). As such, erasin treatment of 3T3-L1 adipocytes phenocopies GSH depletion in vivo. Confocal immunofluorescence microscopy of erasin-treated cells showed a clear co-localization of 4-HNE–modified proteins with the DAPI-labeled region, suggesting that carbonylated proteins accumulate in the nucleus of 3T3-L1 adipocytes (Fig. 1E). Interestingly, the 4-HNE adducts appeared as distinct puncta but did not overlap with the DAPI staining, suggesting that there may be specific intranuclear sites that are preferentially modified.

Site-specific proteomic analysis of nuclear carbonylation

Several published studies of protein carbonylation have utilized MS-based methods and have led to the identification of numerous targets of protein carbonylation (13, 24–26). However, the majority of these studies lacked the ability to identify the sites of modification in a high-throughput manner. Furthermore, the hydrazide– or 2,4-dinitrophenylhydrazine–based derivatization method most commonly utilized is not specific for lipophilic carbonyl adducts and will recognize any free carbonyl, thereby making it difficult to determine whether a protein is subject to primary or secondary carbonylation. To address these issues, we utilized a label-free method by which to identify site-specific Michael adducts on nuclear proteins (Fig. 2A). This method relies on immune affinity purification with an antibody directed against 4-HNE Michael adducts and does not bind to the 6-carbon 4-HHE adducts (Fig. S1C), allowing for enrichment of proteins and peptides carrying specific linkages.

To identify carbonylated proteins that accumulate in the nucleus, we isolated nuclei from eWAT depots from obese C57Bl/6J mice. We analyzed unenriched samples and samples enriched for adducts at both the protein and the peptide level before proteomic analysis to identify peptides carrying 4-HNE adducts (Fig. 2A). Shown in Fig. 2A are the linear structures of the Michael adduct formed between 4-HNE and target amino acids. It should be noted that linear lipid aldehydes adducted onto proteins typically cyclize to form the more stable hemiacetal (27). However, in the workflow, we reduced all adducts with sodium borohydride to allow for efficient immunoaffinity capture. Through this workflow, we identified 65 proteins and 90 sites of 4-HNE modification (Fig. 2B and Table S2). 4-HHE is a reactive lipid that is closely related to 4-HNE and has been studied at length for its role in oxidative stress–related outcomes (28–31). As such, we also searched for 4-HHE modifications and identified 115 sites on 57 distinct proteins (Fig. 2B and Table S3). Representative spectra for 4-HNE and 4-HHE modifications are shown in Fig. S1 (D and E, respectively). Because the antibody we used for enrichment is specific for the 9-carbon adduct, this suggests that carbonylated proteins in the nucleus may be organized in a complex or physically associate with each other. Interestingly, we did not identify any overlapping proteins or sites for both 4-HNE and 4-HHE modification, suggesting that despite their chemical similarity, these lipids display distinct molecular protein targets. Analysis of the sites of modifications revealed a roughly equal distribution between lysine, histidine, and cysteine modifications, suggesting that neither 4-HNE nor 4-HHE exhibits a side chain preference (Fig. 2C). It should be noted that in this workflow analysis, histones were excluded. We have found that histones are a major nuclear carbonylation target, and specifics as to their sites, specificity, and biological impact will be reported separately.

Molecular function analysis (PANTHER) of all sites identified revealed that roughly one-third of proteins identified exhibit enzymatic activity (Fig. 2D, top). The remaining proteins cluster into a variety of categories, including a large number of nucleic acid–binding proteins (Fig. 2D, bottom). To further assess the physiological significance of carbonylation in the nucleus, we performed pathway gene ontology analysis of the combined (4-HNE and 4-HHE) protein data set. Interestingly, there was no significant enrichment for biological processes or KEGG pathways (Fig. 2D [top] and Table S4). In molecular function enrichment analysis, we found that carbonylation substrates were highly enriched for metal-binding proteins (adjusted p = 1.2 × 10−5), nucleic acid–binding proteins (adjusted p = 9.7 × 10−5), and zinc ion–binding proteins (adjusted p = 0.005). In addition, keyword enrichment analysis and sequence feature analysis revealed that zinc finger–containing proteins were the most highly enriched class of proteins (adjusted p = 2.6 × 10−12) (Fig. 2D [bottom] and Table S4).

Sequence preference analysis for carbonylation sites

Using our data set containing over 100 carbonylated proteins, we evaluated the amino acid sequence flanking each carbonylation site to determine whether there are sequence motifs that are enriched at 4-HNE or 4-HHE sites. First, we calculated the occurrence of each amino acid surrounding the modification from the −7 to +7 positions (Fig. 3, A–C). This analysis revealed a strong preference for cysteine at the +3 position and
a cysteine or proline at the –3 position for both 4-HNE and 4-HHE cysteine modifications (Fig. 3A).

Whereas the 4-HNE and 4-HHE cysteine modifications displayed clear similarities in their flanking sequences, the lysine and histidine modifications did not. 4-HNE lysine modifications showed a strong preference for polar charged residues in the –2, –1, +1, and +2 positions (Fig. 3B). In contrast, the 4-HHE lysine adduct was more commonly surrounded by hydrophobic residues on both sides (Fig. 3B). 4-HNE histidine adducts were flanked by polar uncharged residues on the N terminus, whereas the 4-HHE modification was surrounded primarily by hydrophobic residues on both sides (Fig. 3C). Overall, lysine enrichment in the flanking sequences for all modifications was particularly notable. Analysis of combined lysine content for all modifications (Fig. 3D), all 4-HNE modifications (Fig. 3E), and all 4-HHE modifications (Fig. 3F) shows high lysine content for all substrates.

Motif analysis using Motif-X revealed three highly enriched motifs for cysteine carbonylation and one for lysine modification, with no enriched motifs for histidine modification (Fig. 3G). The identified motifs are all based upon the location of neighboring cysteine residues; for cysteine modification, there was a statistically significant enrichment for sites with a cysteine in the +5 position (Fig. S2A), the +3 position (Fig. 2B), or the –3 position (Fig. 2C) and for lysine modification at the +1 position. Interestingly, ontology analysis of the aligned peptides for each motif indicates that both C$_X$C$_C$ motifs and motif -KC- (Fig. S2D) are driven by zinc finger substrates (Fig. 2, F–H), whereas the motif C$_X$C is not (Fig. S2E).

Zinc-coordinating residues are hot spots for protein carbonylation

Due to the strong enrichment of zinc-coordinating proteins in our proteomic and motif analysis, we explored these proteins further in detail. 93% of 4-HNE–modified and 87% of 4-HHE–modified zinc-coordinating proteins were zinc finger–containing proteins (Fig. 4A). Importantly, 70% of the zinc-binding proteins with 4-HNE adducts were modified within the zinc-binding domain, suggesting that zinc coordination renders residues particularly susceptible to modification (Fig. 4B). Roughly a third of 4-HHE adducts on zinc-binding proteins were within zinc-binding domains (Fig. 4B).
Of the zinc fingers identified, 50% (19 proteins) were C2H2 zinc fingers (Fig. 4C). The C2H2 zinc finger is a common DNA-binding domain that mostly exhibits the motif $C_{x3}C_{x12}H_{x3}H$ (32), where the two cysteines and two histidines coordinate a single zinc ion (Fig. 4D). 11 C2H2 zinc fingers had carbonylation sites within the zinc-binding domain. Alignment of these domains showed that the two cysteine residues involved in zinc coordination are common sites for modification (Fig. 4E). Interestingly, seven of these C2H2 zinc fingers were found in proteins that also contain an N-terminal Krüppel-associated box (KRAB domain) (Fig. 4F). An additional three KRAB-containing proteins were also identified (2810021J22RIK, ZFP68, and 2610021A01RIK) with modifications outside of the zinc-coordinating sites. The KRAB domain is generally associated with transcriptional repression through the binding of KAP1 (KRAB-associated protein 1) and subsequent recruitment of chromatin-modifying enzymes (33). The enrichment of KRAB-containing proteins is intriguing and suggests a potential role for protein carbonylation in mediating the effects of these transcriptional regulators in adipose tissue.

In addition to the C2H2 zinc fingers, several other types of zinc fingers were identified with modifications, including (among others) MATRIN, RING, C3H1, and N4-type domains. Analysis of the sites of modification within the non-C2H2 zinc fingers revealed an additional 12 carbonylated zinc-coordinating sites (Fig. 4F–I and Table S5).

Functional analysis of estrogen-related receptor $\beta$- and $\gamma$-carbonylation

Due to the central role that zinc coordination has in stabilizing the DNA-binding capacity of proteins (34, 35), we hypothesized that carbonylation of zinc-coordinating residues would disrupt DNA binding. To directly test this hypothesis, we selected estrogen-related receptor $\beta$/$\gamma$ (ERR-$\beta$ and ERR-$\gamma$) to investigate the functional consequences of the identified modification. The estrogen-related receptors are a family of three orphan nuclear receptors that orchestrate transcriptional responses by binding to estrogen-related response elements (ERRE) to activate transcription (36). Expressed in a variety of tissues, these receptors are critical for maintaining metabolic homeostasis through the regulation of diverse processes, including browning, thermogenesis, adipogenesis, oxidative metabolism, and lipid metabolism (37–41). Importantly, the
ERR family receptors are critical for transcriptional activation of mitochondrial genes encoded in the nucleus (36).

Two 4-HHE modifications were identified in the proteomic screen that were located in the DNA-binding domain (DBD) of ERR-α/β (Fig. 5A). Cys-155 is the third zinc-coordinating cysteine of the second finger within the DBD, whereas Lys-162 is located immediately adjacent to the C terminus of the second finger (Fig. 5, A and B). Because the DNA-binding domains of ERR-α and ERR-β are identical, it was not possible to distinguish which of these two proteins resulted in the tryptic peptide carrying the two modifications (Fig. 5C). However, because ERR-γ is expressed ubiquitously whereas ERR-α is expressed primarily in embryonic tissues (36), it is likely that the parent protein is ERR-γ (Fig. 5C). Western blots of nuclear lysates from eWAT showed robust protein expression of ERR-γ that is significantly decreased in HFD-fed mice (Fig. 5, D and E). Furthermore, treatment of 3T3-L1 adipocytes with a specific ERR-γ inhibitor, GSK5182, resulted in decreased expression of known ERR-γ targets Cpt2, Ttx2, Atp2a2, and Ndufs2 (Fig. S3A).

To confirm that ERR-γ is carbonylated, we isolated nuclei from eWAT depots and used biotin-hydrazide to biotinylate free protein carbonyls. Affinity purification of the biotinylated substrates followed by Western blotting for ERR-γ in both the enriched and supernatant fractions revealed that ERR-γ is detectable in the biotin-labeled fraction, indicating that ERR-γ is carbonylated in obese eWAT (Fig. 5F). Because ERR-α is also expressed in white adipose tissue and the sites of modification in ERR-γ are conserved between all three ERRs, we used the same strategy to test whether ERR-β is carbonylated in white adipose tissue (Fig. 5G).

For example, treatment of 3T3-L1 adipocytes with a specific ERR-γ inhibitor, GSK5182, resulted in decreased expression of known ERR-γ targets Cpt2, Ttx2, Atp2a2, and Ndufs2 (Fig. S3A).
mutated Lys-162 to alanine or arginine and performed electrophoretic mobility shift assays (EMSAs). Mutation of Lys-162 to alanine results in a nearly complete loss of DNA binding capacity (Fig. 5H). Similarly, a lysine-to-arginine mutation to control for electrostatic differences also led to a significant decrease in DNA binding, suggesting that Lys-162 not only plays a structural role but also may play a direct role in sequence-specific DNA recognition (Fig. 5H). Next, we in vitro-modified ERR2-DBD with 4-HHE, resulting in a robust time-dependent increase in protein carbonylation (Fig. 5I). EMSA analysis of the carbonylated and noncarbonylated ERR2-DBD showed a decrease in DNA-binding capacity that depended on the relative level of 4-HHE modification (Fig. 5, I and J). Collectively, these data demonstrate the critical nature of these residues for DNA binding and support a model in which carbonylation of ERR-γ at these residues inhibits binding to ERREs in the genome, thereby preventing transcriptional activation of target genes.

**Discussion**

Protein carbonylation by reactive lipid aldehydes has long been appreciated as a direct consequence of elevated reactive oxygen species (42, 43) and/or depletion of GSH stores. As such, carbonylation targets have been studied at length in tissues and disease models in which oxidative stress is elevated (2). In obesity-linked insulin resistance, a variety of studies have shown that oxidative stress plays a causal role in metabolic dysfunction, but the mechanism(s) remains unclear (44, 45). In the majority of animal models and in human biology, accumulation of adipose tissue in the visceral depot is a positive risk factor for

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*Figure 5. 4-HHE modification of estrogen-related receptor β/γ disrupts DNA binding.* A, ribbon diagram of the DNA-binding domain (DBD) of ERR-β/γ (Protein Data Bank entry 1LO1). Blue spheres represent zinc ions that are each coordinated by four cysteine residues in blue. Residues in red are carbonylation sites. Amino acid designations represent the location in ERR-β. B, cartoon model of the DBD of ERR-β/γ. C, alignment of the DBD of the ERR family members. Blue residues coordinate zinc, and yellow residues differ between ERR-α and the other family members. D, Western blotting of whole-cell lysates from chow- and HFD-fed mice concurrently blotted for ERR-γ and β-actin and detected using IR-680 – or IR-800 – conjugated secondary antibodies, respectively. The ladder shown is duplicated for both top and bottom panels to show the position of the molecular weight markers as detected using the IR-680 secondary antibody. E, quantitation of D; error bars, S.E.; n = 4 for chow; n = 6 for HFD. F and G, Western blotting of immunoprecipitated biotin-hydrazide–labeled proteins from eWAT of HFD-fed mice. IP: BH, total immunoprecipitated fraction; Supernatant, all protein remaining post-IP. Biotin label is detected with IR-Streptavidin. H, representative EMSA from three experiments of WT ERR2-DBD, K162A, and K162R mutants. Probe-only control contains no ERR2-DBD. I, Western blotting of in vitro-modified ERR2-DBD that has been in vitro-modified with 0.5 mM 4-HHE for 0, 2, 4, 24, or 48 h.
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Figure 6. Summary. Depletion of GSH and increased ROS production from a variety of sources, including the mitochondria, endoplasmic reticulum, and NADPH oxidase (NOX), leads to elevated levels of reactive lipid aldehydes and accumulation of nuclear protein carbonylation in the obese state. Among the many zinc finger proteins (ZFP) modified, ERR-γ and ERR-α are targets of this modification, leading to decreased binding capacity to target genes.

metabolic disease, whereas subcutaneous adipose tissue is often considered to be protective in the context of obesity (46). Whereas oxidative stress and GSH depletion in the visceral depot are linked to insulin resistance, the molecular mechanisms connecting such changes to metabolic dysfunction remain poorly characterized. Herein, we show that protein carbonylation is selectively up-regulated in the nucleus of obese eWAT but not in the nuclear fraction of inguinal adipose tissue (Fig. 1). Moreover, using 3T3-L1 adipocytes, GSH depletion using erastin resulted in increased protein carbonylation that localized to the nucleus as well (Fig. 1E). Prior work from the laboratory has shown that the expression of key antioxidants such as peroxiredoxin 3, GSH peroxidase 4, and GSH-S-transferase A4 are selectively down-regulated in the visceral, but not subcutaneous, depot of high-fat–fed mice and that the decreased expression is correlated with increased levels of reactive aldehydes, including 4-HNE (6). Whereas plasma membrane NADPH oxidase, endoplasmic reticulum Erp1, xanthine oxidase/dehydrogenase, and the mitochondrial electron transport chain have been considered to be the major sites for ROS synthesis in adipose tissue, the specific sites for protein carbonylation have been less well-characterized. The results of this study are surprising because they point toward the nuclear localization of carbonylated proteins and the potential for transcriptional control by oxidative stress–dependent mechanisms (Fig. 6).

Analysis of the adipose carbonylome resulted in the identification of over 100 novel, endogenous targets of protein carbonylation as well as the type of aldehyde (9-carbon 4-HNE or 6-carbon 4-HHE) carrying out the modification. Contrary to previous work on mitochondrial carbonylation that delimited highly enriched pathways (13), the nuclear carbonylome did not exhibit any such bias. This is not unusual for chemically driven modifications where enzymatic regulation is not a driving factor. Previous studies defining cytoplasmic or mitochondrial protein targets of carbonylation have been limited in depth due to the use of nonspecific derivatization methods that do not enable one to distinguish between sites of modification or the many possible types of carbonylation events (13, 47). Our proteomic workflow (Fig. 2A) herein differs in several important ways from that utilized previously by our group (13) in that we used an antibody specific for 4-HNE to enrich for carbonylated proteins. This allowed for enrichment of 4-HNE–modified substrates and specific identification of 4-HNE adduction sites. In addition, we enriched at both the protein and the peptide levels. This ensures that even low-abundance peptides with the modification will be detected. Importantly, we found that reduction of nuclear extracts using sodium borohydride dramatically increased the reproducibility of analysis and the yield of carbonylated proteins and peptides.

The major protein group we identified as carbonylation targets were zinc-coordinating proteins, and the site of modification was typically a residue on or adjacent to the zinc-coordinating amino acids (Fig. 2). From this analysis, we also defined the protein landscape surrounding carbonylation sites, providing for the first time insight into the chemical environment that makes modification favorable by endogenous lipid aldehydes. In general, we found that for both 4-HNE and 4-HHE, carbonylation occurred approximately equally on cysteine, histidine, and lysine side chains. For cysteine carbonylation, the most striking motif defined by the proteomic analysis was the \( CX_2CX_2C \) motif in which the central modified cysteine residue (boldface type) was flanked at the +3 or the −3 position by another cysteine residue for both 4-HNE and 4-HHE modification. Although there was no general motif evident from the modification of histidine or lysine side chains, the region surrounding the carbonylated amino acid had a relatively high content of basic lysine residues. Interestingly, analysis of each of the six modifications (Fig. 3) revealed the presence of a proline residue within the +7 to −7 region, suggesting the potential for a unique structural determinant for carbonylation.

Our results did reveal a strong enrichment of zinc-binding proteins with the majority of defined carbonylation sites on zinc-coordinating residues (Fig. 4). Zinc coordination is generally achieved via a tetrahedral geometry of four residues that typically include 2–3 cysteine residues (35). Importantly, the ability of a cysteine to participate in zinc binding depends on a completely reduced, unmodified thiol. Signaling mechanisms that couple redox reactions to zinc binding are not uncommon; indeed, there are many proteins that undergo a redox switch whereby oxidation of a zinc-coordinating residue results in the release of zinc and a conformational change in the protein (34). For example, heat shock protein 33 (Hsp33) is activated in response to oxidative stress and acts to prevent protein unfolding and aggregation (48). Structural studies of the Hsp33 mechanism revealed that under normal conditions, Hsp33 exists in a monomeric state with a tight zinc-coordinating domain on the C terminus. In response to oxidative stress, one of the four
zinc-coordinating cysteines is oxidized, leading to the unfolding of the C-terminal domain and subsequent dimerization of Hsp33 into its active state (48). Importantly, this is most often a reversible process, as cysteine thiols can readily be re-reduced, resulting in restored zinc-binding capability (34). As such, it is possible that carbonylation of the zinc-coordinating residues identified in this study results in the release of zinc and loss of structural integrity of the domain. However, because there are no known enzymes that can remove 4-HNE and 4-HHE, Michael adducts from their substrates, these modifications result in the permanent disruption of secondary structure in those proteins.

Finally, we explored the functional significance of the carbonylation of zinc fingers and showed that the ERR family of nuclear receptors are carbonylated in vivo, and this leads to a severe impairment in DNA-binding capacity. Zinc fingers are most commonly observed in DNA-binding proteins and are critical for sequence-specific DNA binding of a wide variety of proteins involved in transcription, replication, and repair (49, 50). It is appreciated that these proteins are sensitive to oxidative stress, although the mechanisms by which this occurs remain elusive (34, 51). Our data strongly support a model by which oxidative stress results in carbonylation of zinc finger proteins, disrupting their ability to bind DNA, resulting in a loss of transcriptional regulation (Fig. 6). 4-HHE modification of ERR-γ is an example of one such event that is of critical importance for energy metabolism. The site of modification of ERR-α/γ was within or adjacent to the second zinc finger that resides on the helix that caps and is perpendicular to the DNA-binding helix. Immunoprecipitation experiments confirm that ERR-α/γ are carbonylated in obese adipose tissue. Consistent with this, many transcriptional targets for ERREs are down-regulated in the obese state relative to lean controls, including Cpt2, Txn2, Atp2a2, and Ndufs2 (52), leading to the consideration that ERR carbonylation may be functional and selectively reduce gene expression. ERR-α and ERR-γ are broadly expressed in tissues that have high bioenergetics demands. Although their specific effects and gene targets are broad, they are most well-known for their role in promoting mitochondrial bioenergetics pathways, including oxidative phosphorylation, fatty acid oxidation, tricarboxylic acid cycle, and electron transport chain proteins (36). Our data are consistent with a model in which oxidative stress leads to the carbonylation of ERR-α/γ, leading to impairment in the expression of target genes (Fig. 6).

A major unknown revealed by this study is the source of ROS and aldehydes that lead to nuclear carbonylation. When considering either high-fat feeding in vivo or GSH depletion in 3T3-L1 adipocytes, there are numerous sources of ROS that can lead to 4-HNE or 4-HHE production. These include, but are not limited to, NADPH oxidase, xanthine oxidase, and the mitochondrial electron transport system. Whereas superoxide anion is highly labile and has a short half-life, hydrogen peroxide is relatively stable and able to diffuse across membranes to various sites within the cell. The hydroxyl radical, which is the critical species of ROS that leads to the production of reactive lipid aldehydes, is also highly labile and, as such, defining the site of ROS synthesis is critical. In parallel, medium-chain lipid aldehydes are also capable of rapid diffusion across biological membranes; therefore, whereas the nucleus may be the site of protein carbonylation, it is not clear what is the origin of the aldehydes. The results presented herein suggest either reactive oxygen species and aldehyde synthesis in the nucleus or retrograde movement to the nucleus from their site of origin.

Materials and methods

Animal protocol

WT C57Bl/6j mice were weaned at 3 weeks of age and subsequently fed either a high-fat diet (20% protein, 35.5% fat, 36.3% carbohydrate; BioServ, F3282) or a chow diet for 12 weeks. Animals were sacrificed by cervical dislocation, and epididymal and inguinal white adipose depots were removed for analysis. The University of Minnesota institutional animal care and use committee approved all experiments with animals.

Cell culture

3T3-L1 fibroblasts were grown to confluence and differentiated as described previously (53), using the standard differentiation mixture consisting of dexamethasone, methylisobutyloxanthine, and insulin. For some experiments, on day 7 of differentiation, cells were treated with 10 μM erastin (Cayman Chemical 177754) or vehicle (DMSO) in Dulbecco’s modified Eagle’s medium + 5% fetal bovine serum for 24 h before harvesting or for use in microscopy experiments. For ERR-γ inhibitor experiments, day 8 cells were treated with 10 μM GSK5182 (Aobios AOB1629) or vehicle (DMSO) for 6 or 20 h.

Nuclear lysate preparation

Fresh tissue was minced on ice in hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) with protease and phosphatase inhibitors. Tissue was homogenized with a glass-Teflon electric homogenizer (8 strokes, 1600 rpm), centrifuged at 1000 rpm at 4 °C for 10 min, and the fat cake was discarded. Samples were briefly mixed and centrifuged at 3700 rpm at 4 °C for 10 min to pellet and collect nuclei. For some experiments, the post-nuclear extract was stored for Western blot analysis. Nuclei were washed three times with PBS and resuspended in reduction buffer (15 mM NaBH₄ in PBS) and incubated for 30 min at 4 °C. Samples were dialyzed overnight into PBS, sonicated, and centrifuged (16,000 rpm at 4 °C for 10 min) to remove nuclear debris. Protein content was determined with the bicinchoninic acid protein quantitation assay (Sigma-Aldrich). For Western blotting, Nonidet P-40 and SDS were added to the samples to a final concentration of 1 and 0.1%, respectively.

GSH content assay

GSH analysis was carried out using the GSH/GSSG ratio detection kit (Abcam 65393) according to the manufacturer’s instructions.

Western blot analysis

Samples were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, blocked for 1 h with blocking buffer (LI-COR 927-4000), and incubated with primary antibodies overnight. Membranes were then washed and incubated
for 1 h with fluorescently labeled secondary antibodies (LI-COR) and imaged using the LI-COR Odyssey imager. Antibodies used include 4-HNE (Millipore, 393207), 4-HHE (31) (a generous gift from Dr. Matthew Picklo, Grand Forks Human Nutrition Research Center, Grand Forks, ND), β-actin (Sigma-Aldrich, A5441), ERRγ (Abcam, ab128930), ERRα (Thermo Fisher Scientific, 28390) histone H3 (Abcam, 10799), and histone H4 (Cell Signaling, 29355).

Immunofluorescence and microscopy

3T3-L1 fibroblasts were grown on glass coverslips in 6-well plates, differentiated, and treated with erastin or DMSO for 24 h on day 7. On day 8, cells were fixed with 4% paraformaldehyde (EMS15714). Cells were blocked and permeabilized with blocking buffer (PBS containing 0.1% Triton X-100, 0.2% gelatin, and 1% BSA). Cells were incubated with primary antibody (α-4-HNE; Abcam, 46545) overnight and washed with PBS + 0.1% Triton X-100. Cells were then incubated with secondary antibody to antibody 1:1000 dilution (donkey anti-rabbit IgG Alexa Fluor 488, Abcam 150073) and 500 nM DAPI for 30 min. Following washing, cells were mounted on slides with mounting solution (Invitrogen, P10144) and stored at 4 °C until imaging. Slides were imaged at the University of Minnesota Imaging Centers using the Olympus FV1000 Bx2 upright confocal microscope at ×60 with an oil immersion lens.

Immunoprecipitation of carbonylated proteins and in-gel digestion

For immunoprecipitation of proteins, 70–100 µg of reduced nuclear extract (in PBS) was diluted to 500 µl with a PBS containing 1% Nonidet P-40 and precleared with protein A/G–agarose resin (Santa Cruz Biotechnology, sc-2004). The 4-HNE primary antibody (Millipore, 393207) was cross-linked to protein A/G–agarose resin using dimethyl pimelimidate imidoester cross-linking reagent (Thermo Scientific) according to the manufacturer’s instructions. Cross-linked antibody/resin was incubated with nuclear lysates overnight, washed three times with radioimmunoprecipitation assay buffer (10 mM Tris-Cl, pH 8, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), and eluted by boiling in 4X Laemmli buffer. Samples were resolved by SDS-PAGE and stained with Imperial Protein Stain (Thermo Scientific) before in-gel digestion.

SDS-polyacrylamide gel bands were washed using 50% EtOH for 12 h and then in water for 1 h. Each gel band was minced into small pieces and dehydrated using acetonitrile, and the proteins were reduced and alkylated at room temperature in the dark using 15 mM tris(2-carboxyethyl)phosphine hydrochloride and 45 mM iodoacetamide in PBS. The gel pieces were washed sequentially using 50 mM ammonium bicarbonate and then 100% acetonitrile and vacuum-dried. Trypsin (Promega) (10 ng/µl in 50 mM ammonium bicarbonate) was added for in-gel digestion overnight at 37 °C. Peptides were extracted sequentially using extraction buffer (5% TFA and 50% acetonitrile in water (v/v)) and 100% acetonitrile. The pooled peptide extracts were dried and desalted using C18 StageTips (54).

In-solution protein digestion and immunoprecipitation of peptides

Proteins were resuspended in urea lysis buffer (9 M urea in PBS (pH 7.2) with HALT protease inhibitor mixture (Thermo Fisher Scientific) and then subjected to reduction and alkylation at room temperature in the dark using 10 mM tris(2-carboxyethyl)phosphine hydrochloride and 30 mM iodoacetamide in PBS. Excess iodoacetamide was quenched using 30 mM cysteine. After 6-fold dilution with PBS, proteins were digested using 10 ng/µl sequencing-grade trypsin (Promega) at room temperature for 12 h followed by a second tryptic digestion (5 ng/µl) for 3 h. Peptides were desalted with Sep-Pak C18 cartridges (Waters) following the manufacturer’s instructions. Following digestion, samples were incubated with 4-HNE-agarose cross-linked resin overnight and eluted with 0.1% TFA.

LC-MS/MS

Peptides were reconstituted in HPLC buffer A (0.1% formic acid in water (v/v)) and injected into a Proxeon Easy nLC 1000 HPLC system (Thermo Fisher Scientific). Peptides were separated using a C18 column (15 cm × 75 µm, ReproSil-Pur Basic C18, 2.5 µm, Dr. Maisch GmbH) with a linear gradient of 5–35% HPLC buffer B (0.1% formic acid in acetonitrile (v/v)) at a flow rate of 200 nl/min. Eluted peptides were directly electrosprayed into the Fusion Orbitrap MS (Thermo Fisher Scientific). The instrument was operated in a data-dependent mode. Full mass spectra were acquired with a resolution of 120,000 FWHM (full-width half-maximum) at 200 m/z, and MS/MS spectra were acquired using collision-induced dissociation with 35% collision energy for detection in the ion trap.

MS data processing

Raw MS data were processed using MaxQuant software (version 1.5.3.12) for protein identification (55). Variable modifications included carboxyamidomethylation on cysteine and lysine; methionine oxidation; protein N-terminal acetylation; 4-HNE and reduced 4-HNE on cysteine, histidine, and lysine; and 4-HHE and reduced 4-HHE on cysteine, histidine, and lysine. Semi-trypsin was selected as the proteolytic enzyme, and a maximum of two missing cleavages were allowed. The precursor ion mass tolerance was set to ±4.5 ppm, and the fragment ion mass tolerance was set to ±0.6 Da. MS data were searched against the Uniprot mouse database (released on September 27, 2013 and containing 43,310 sequences). Peptides were filtered with a 1% false discovery rate at the peptide, protein, and modification site levels. A minimum Andromeda score of 40 was required for the identification of modified peptides. The results reported were compiled from four separate experiments whose data sets were merged and analyzed together to present the summary figures and tables. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (56) partner repository with the data set identifier PXD008415.

Bioinformatic analysis

Pathway and functional annotation enrichment was performed using DAVID bioinformatics resources (57, 58). All statistics reported are Benjamini–Hochberg corrected p values.
Nuclear carbonylation of zinc finger proteins

Molecular function categorization was performed using PANTHER (59). Statistical analysis of enriched motifs was performed using Motif-X (60, 61). Network analysis was performed using ingenuity pathway analysis.

Cloning and expression of GST-ERR2

The ERR2 DNA-binding domain (ERR2-DBD) in pET24a vector was kindly provided by Peter Wright (Scripps Institute, La Jolla, CA). ERR2-DBD was subcloned into pGEX2T using the following primers: ERR2 pGEX Fwd, CACCGGATCCGC-TATCCGAAGCGCCTGTG; ERR2 pGEX Rev, CGATGAA-TTCTTAAGAGGTTTGCATCCA. The GST-ERR2-DBD fusion was expressed in BL21 LysS E. coli, absorbed onto GSH-Sepharose, and eluted with 50 mM Tris, pH 8.0, containing 100 μM ZnCl2 and 10 mM GSH. The purity of ERR2-DBD was evaluated by SDS-PAGE.

In vitro modification with 4-HHE

Purified protein was dialyzed into 100 mM potassium phosphate pH 7.4, 150 mM NaCl and treated for 1–48 h with 0.5 mM 4-HHE (Cayman Chemical, 32060). The reaction was quenched with 5 mM DTT and verified by Western blotting.

Quantitative RT-PCR

RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and real-time amplification was performed using a Bio-Rad iCycler thermocycler with iQ SYBR Green Supermix. Gene expression assays were performed for Atp2a2, Cpt2, Ndufs2, Pdk4, and Tnx2. Primer pairs are listed in Table S1. Relative gene expression was calculated using transcription factor 2E as an endogenous control.

EMSA

Purified GST-DBD in 10 μl was combined with 2 μl of 10× binding buffer (100 mM Tris, pH 7.5, 500 mM KCl, 10 mM DTT), 1 μg of poly(dI-dC), 2 μl of 25 mM DTT, 2.5% Tween 20, 1 μl of DNA probe, and water to final volume of 20 μl. Reactions were incubated for 20–30 min in the dark and terminated with 10 μl of loading dye. Electrophoresis was carried out using 5% TBE Criterion Precast gels (catalog no. 3450048) in TBE running buffer. Gels were imaged using the LI-COR Odyssey imager. The following IR700 probes were used: 5IRD700, TCGAGCA-TTGGATCAAGGTCAGTGATGC (forward) and TCGAGCATTCACTGACCTTGATCAATGC (reverse).

Biotin-hydrazide derivatization and immunoprecipitation

Nuclear extracts (from 1 g of starting tissue) were diluted to 1 ml with biotin hydrazide coupling buffer (100 mM sodium acetate, pH 5.5, 20 mM NaCl, 0.1 mM EDTA, 2% SDS, protease inhibitors) and biotin hydrazide (Pierce, 21339) added to a final concentration of 0.5 mM for 2 h. Samples were dialyzed into PBS and incubated overnight with streptavidin-conjugated agarose (Thermo Scientific, 20357). The resin was washed three times with PBS + 0.01% SDS, resuspended in 2× Laemmli buffer, and boiled to elute proteins. The supernatant was retained and pooled with the washes to assess the noncarbonylated fraction.

Author contributions—Experiments were designed by D. A. B., A. K. H., and Y. C., A. K. H., T. Z., and W. H. carried out the experiments. A. K. H., D. A. B., and Y. C. evaluated the experiments and carried out data analysis. Computational evaluation of the data was carried out by A. K. H., R. P., and R. K. The manuscript was written by A. K. H. and D. A. B. and was edited by all of the authors.

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