Glutathionylated products of lipid peroxidation

A novel mechanism of adipocyte to macrophage signaling

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Obesity-associated insulin resistance has long been linked to both increased adipocyte oxidative stress as well as the presence of inflammatory changes in adipose tissue, including the infiltration and activation of tissue-resident macrophages. In order to investigate the connections between obesity-associated oxidative stress in adipocytes and increased inflammation in adipose tissue associated with the development of insulin resistance, our laboratory recently demonstrated that adipocytes form glutathionylated products of oxidative stress including glutathionyl-4-hydroxy-2-nonenal (GS-HNE) and glutathionyl-1,4-dihydroxynonene (GS-DHN). The abundance of both GS-HNE and GS-DHN were increased in the visceral adipose tissue of ob/ob mice and diet-induced obese, insulin-resistant mice. Further, these products of lipid peroxidation were shown to induce inflammatory changes in macrophages. Finally, in a mouse model, overproduction of GS-HNE was associated with increased fasting glucose levels and moderately impaired glucose tolerance. Together, these findings suggest a novel mechanism by which obesity-induced oxidative stress in adipocytes may lead to activation of tissue-resident macrophages. As adipose tissue inflammation has been shown to play an important role in the development of insulin resistance, further study of this pathway may lead to potential interventions to attenuate the metabolic consequences of obesity.

Obesity is widely acknowledged to be a critical global health problem. Obesity and overweight contribute significantly to major causes of death and disability, including cardiovascular disease and type 2 diabetes mellitus. The search for the pathogenic factors connecting obesity to human disease has led to the identification of "metainflammation", or chronic low-grade non-resolved inflammation, as a common thread.[1,2] The infiltration of macrophages into adipose tissue and the overexpression of inflammatory genes have been shown to be associated with obesity-related metabolic disease in humans[3] and are associated with adipocyte insulin resistance.[2] The resultant increased lipolysis and alterations in adipokine release have larger systemic effects, including overall insulin resistance and the development of other manifestations of metabolic disease.[4]

In addition to an increased inflammatory state, obese adipose tissue exhibits increased oxidative stress.[5,6] Further, adipose oxidative stress has been associated with insulin resistance in humans.[7] Conversely, caloric restriction can improve both inflammatory profile as well as decrease oxidative stress in adipose tissue[8] and obese female mice show parallel reduced insulin resistance as well as reduced adipose oxidative stress and inflammation, when compared with male mice.[9] While there are clearly associations between oxidative stress and inflammation in both obese adipose tissue as well as in other pathologic states, the directionality and mechanism of this relationship is yet to be fully elucidated.
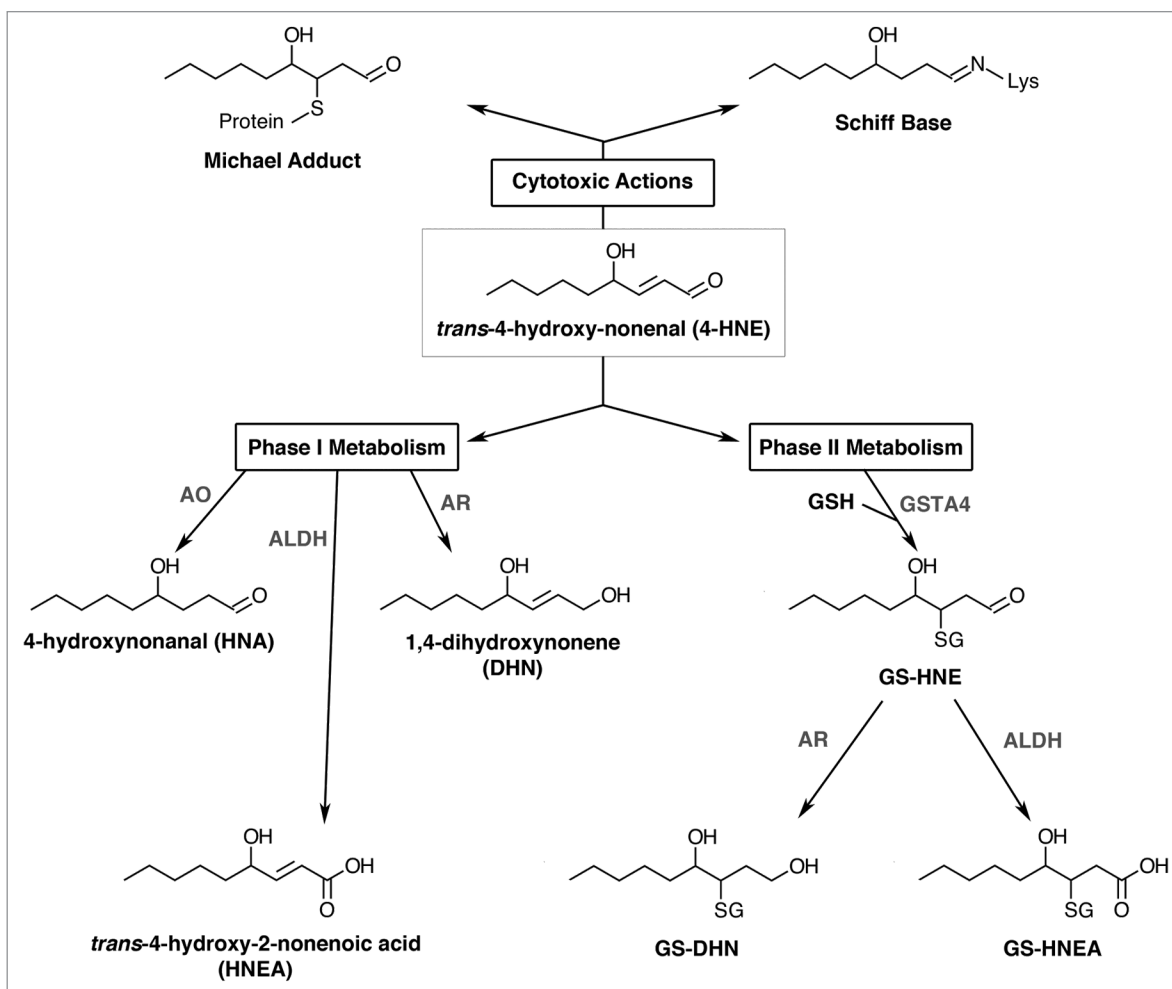
Figure 1. Metabolism of *trans*-4-hydroxy-2-nonenal (4-HNE). AR, aldose reductase; ALDH, aldehyde dehydrogenase; AO, aldehyde oxidase; DHN, 1,4-dihydroxynonene; GSTA4, glutathione-S-transferase A4; GS-DHN, glutathionyl-1,4-dihydroxynonene; GS-HNE, glutathionyl-4-hydroxy-2-nonenal; HNA, 4-hydroxynonanal; HNE, *trans*-4-hydroxy-2-nonenal; HNEA, trans-4-hydroxy-2-nonenoic acid.

Adipose tissue exposed to chronic over nutrition shows increased endoplasmic reticulum (ER) stress and mitochondrial dysfunction.[10] The resultant deterioration in efficiency of oxygen consumption and electron transport leads to increased production of superoxide anion.[11,12] Superoxide anion can be converted by superoxide dismutase to hydrogen peroxide (H_2O_2) that in turn can be metabolized in various pathways. Both catalase and glutathione peroxidases decompose hydrogen peroxide into oxygen and water.[13-16] An alternate pathway is the non-enzymatic degradation of hydrogen peroxide via iron-mediated Fenton chemistry that produces the hydroxyl radical.[17] The hydroxyl radical has many potential deleterious effects and it is the reactive oxygen species (ROS) which

acts upon polyunsaturated fatty acids (PUFA) in the cell membrane resulting in formation of lipid peroxidation products.[18] These products include reactive α,β-unsaturated aldehydes such as *trans*-4-hydroxy-2-nonenal (4-HNE) and *trans*-4-oxo-2-nonenal (4-ONE).[18] 4-HNE is able to react with cysteine, histidine and lysine residues of proteins via Michael addition in a process termed protein carbonylation.[19] These same amino acids also are capable of covalent modification via Schiff base formation but due to the lack of a free carbonyl group are typically not included in discussions of protein carbonylation. Protein carbonylation can affect both protein function and abundance[20,21] and has been associated with alterations in the insulin signaling pathway[21] and mitochondrial function,[10] and may represent a

mechanism by which oxidative stress leads to metabolic changes in the adipocyte and the organism at large.

The cell is able to protect against damage by reactive lipid aldehydes via a variety of phase I and phase II enzymes. As shown in **Figure 1**, these enzymes catalyze oxidation, reduction or glutathionylation of 4-HNE, 4-ONE and other reactive aldehydes. For the sake of illustration, the metabolism of 4-HNE will be primarily discussed but it should be noted that a variety of reactive aldehydes are metabolized via parallel phase II pathways. Of these enzymes, glutathione-S-transferase A4 (GSTA4) mediates glutathionylation of 4-HNE and has been proposed as playing an important role the metabolic consequences of obesity as increased adipose tissue oxidative stress and inflammatory

markers correlate strongly with down-regulation of GSTA4.[5] Glutathionylated 4-HNE (GS-HNE) can be further reduced by aldose reductase (AR, also known as aldoketoreductase) to glutathionyl-1,4-dihydroxynonene (GS-DHN). Both GS-HNE[22,23] and GS-DHN[24] are exported rapidly by the ATP-dependent transporter RLIP76.

While the metabolism and export of these reactive lipids would be presumed to be metabolically beneficial, it is interesting to note that RLIP76 knockout mice, which have significantly attenuated export of these metabolites, are in fact protected against obesity-induced inflammation and, notably, from oxidative stress-induced insulin resistance.[25,26] This observation, together with studies showing injection of GS-HNE into murine peritoneal cavities induced leukocyte infiltration and pro-inflammatory leukotriene production,[27] raised the possibility that glutathionylated products of lipid peroxidation were not merely inert metabolites destined for excretion, but rather had potential as inflammatory mediators.

In order to explore potential signaling roles for glutathionylated products of lipid peroxidation, we examined factors influencing GS-HNE and GS-DHN production in tissue culture and in mouse models as well as the potential activity of these metabolites on macrophages in culture.[28] We reported that both GS-HNE and GS-DHN were increased in the adipose tissue of ob/ob mice. GS-HNE was also noted to be increased in high fat fed obese mice relative to lean controls, while GS-DHN showed only a non-significant trend, possibly related to higher variability among individual mice.[28] Of note, both the ob/ob and high fat diet models are known to have increased oxidative stress and inflammation in adipose tissue as well as reduced glucose tolerance overall.[5,29] We further examined the production of GS-HNE and GS-DHN in cell culture systems, showing that 3T3-L1 adipocytes, but not RAW264.7 macrophages, produced both GS-HNE and GS-DHN under basal conditions and in increased levels in response to treatment with hydrogen peroxide to induce oxidative stress.

As there is a known association between elevated serum glucose levels and increased oxidative stress in a variety of tissues,[30] the effect of elevated exposure to glucose has been previously examined in 3T3-L1 adipocytes. 3T3-L1 adipocytes chronically exposed to high-glucose culture medium (25 mM) show increased oxidative stress and insulin resistance when compared with those grown in low glucose culture medium (5.5 mM).[31] As we also showed, high glucose exposure was also associated with greater lipid accumulation, as seen by increased number and size of lipid droplets. Interestingly, GS-HNE and GS-DHN production showed only a small but non-significant increase high glucose cells relative to controls; however, when paired with hydrogen peroxide treatment, the response of the high glucose cells to hydrogen peroxide was significantly increased for both GS-HNE and GS-DHN relative to the response of cells grown in low glucose. This potentiation of the response to oxidative stress after chronic high glucose exposure could represent increased lipid peroxidation in these cells or perhaps increased expression of GSTA4, the enzyme responsible for glutathionylation of 4-HNE.

As GSTA4 has been implicated in the development of obesity-induced insulin resistance and mitochondrial dysfunction,[10] we sought to better characterize the role of GSTA4 expression in production of GS-HNE and GS-DHN. Both GSTA4 overexpressing and GSTA4 knockdown 3T3-L1 adipocytes were assessed for production of glutathionylated lipid aldehydes at baseline and in response to hydrogen peroxide. Under basal conditions, the GSTA4 knockdown cells produced less GS-HNE than the control cells, but GS-DHN did not significantly differ. The GSTA4 overexpressing cells showed no basal difference from control for either GS-HNE or GS-DHN production. When challenged with hydrogen peroxide, however, the induction of GS-HNE and GS-DHN production were significantly less in the GSTA4 knockdown cells relative to the controls. Conversely, the GSTA4 overexpressing cells had significantly higher induction of both GS-HNE and GS-DHN production in response to hydrogen peroxide. Together, these results indicate that at baseline, GSTA4 expression does not limit production of glutathionylated lipid aldehydes; however, when subjected to oxidative stress, GSTA4 expression significantly impacts total production of GS-HNE and GS-DHN.

Previous studies had shown that a cell-permeable ethyl ester of GS-DHN induced NFκB signaling in RAW 264.7 macrophages.[32,33] However, as the endogenously produced non-esterified GS-HNE and GS-DHN are poorly membrane soluble,[34] the previous studies did not fully address the potential effect of glutathionylated lipid aldehydes produced by a paracrine source on macrophage inflammatory state. We showed that both GS-HNE and GS-DHN induced production of TNF-α by both RAW264.7 and mouse peritoneal macrophages. In both cell types, GS-DHN appeared to be a more potent stimulus. Production of leukotriene C_4, also a macrophage inflammatory mediator, was similarly stimulated by both GS-HNE and GS-DHN. Neither treatment affected production of MCP-1 or IL-6 by macrophages. To broaden the analysis of gene expression regulated by glutathionylated lipids, the expression of genes involved in the inflammation was evaluated by microarray analysis using cDNA generated from primary peritoneal macrophages treated with either GS-HNE or GS-DHN. Both GS-HNE and GS-DHN significantly increased the expression of Nos2 and NFκB1 implicated in increasing pro-inflammatory gene expression[35,36] as well as Fos, which interacts with toll-like receptor signaling pathways, contributing to inflammatory response.[37] Both glutathionylated lipid aldehydes also induced genes that contribute to the innate immune response: C3 and C4b, proteins that play a role in the complement cascade[38] and Igtb2 found in myeloid cells.[39] GS-HNE further induced expression of MCSF (macrophage colony stimulating factor) which plays a role in differentiation of peripheral monocytes into macrophages and is important in the pro-inflammatory M1 polarization of macrophages;[40] IL23R, the interleukin 23 receptor;[41] and TLR6 and TLR9, both involved in pathogen recognition and the innate immune response.[42] GS-DHN alone induced expression of CD40, a member of the TNF receptor superfamily that binds interferon γ.[43] This data

supports the conclusion that GS-HNE and GS-DHN, while differing somewhat, both induce an inflammatory response via genes that promote polarization of macrophages to a pro-inflammatory phenotype and potentiation of the innate immune response.

While there are similarities in the production of GS-HNE and GS-DHN, it is important to note that the enzyme responsible for conversion from GS-HNE to GS-DHN, AR, is upregulated by a variety of factors including oxidative stress, 4-HNE itself,[44] as well as by fibroblast growth factor (FGF)-1, FGF-2 and epidermal growth factor (EGF).[45] This implies that the abundance of GS-DHN to GS-HNE could increase with increasing severity of oxidative stress. This could explain the greater variability in GS-DHN levels in high fat diet-fed mice. By extension, the relative abundance of GS-DHN to GS-HNE could impact the magnitude and type of inflammatory response generated in the macrophage. Studies in other tissues have shown differences in their signaling function. Peritoneal leukocyte infiltration and pro-inflammatory lipid and cytokine production is induced by GS-HNE, but not GS-DHN.[25,27] GS-DHN and not GS-HNE, in contrast, has been shown to induce mitogenic signaling in smooth muscle.[34] This is further reflected in the differences in greater potency of GS-DHN relative to GS-HNE in induction of TNF-α by macrophages.

In order to examine the role of glutathionylated products of lipid peroxidation in the whole mouse, we generated transgenic mice overexpressing GSTA4 in adipose tissue under the FABP4 promoter. The mice were maintained on a high fat diet, and the transgenic mice did not differ in weight from wild-type mice on high fat diet. While GSTA4 expression and enzymatic activity were higher in the fat from the transgenic mice relative to controls, there was no difference in total GS-HNE and GS-DHN per gram of visceral fat. The discrepancy between increased GSTA4 capacity and unchanged levels of GS-HNE and GS-DHN in transgenic relative to wild-type mice may be explained by the active transport and excretion of these metabolites, making the content of the tissue at any given time representative

of steady-state levels in the extracellular matrix and local tissue vasculature. It is also possible that production of GS-HNE and GS-DHN in the high fat fed mice is limited by production of the precursor 4-HNE, which could be equally elevated in wild-type and transgenic mice. Despite this, the GSTA4-overexpressing mice had significantly higher fasting blood glucose level and they were mildly glucose intolerant and had higher blood glucose on average at all time points than the wild-type mice. Given the results with the GSTA4 transgenic mice, it is intriguing to consider that the metabolic improvement measured in the RLIP76 null animals may be linked, at least in part, to reduced export of glutathionylated aldehydes and concomitant loss of inflammatory signaling by macrophages.

The importance of GSTA4 in adipose immune regulation via production of GS-HNE and GS-DHN is further supported by the observation that GSTA4 null mice show a greater susceptibility to bacterial infection.[25,46] It is also interesting to note that while GSTA4 null mice showed increased steady-state level of 4-HNE in their tissues,[25] they were noted to have an extended life span.[26] Our studies suggest that decreased levels of chronic inflammation may explain this improvement.

Taken together, these recent studies suggest that GS-HNE and GS-DHN may be a novel class of signaling molecules that are increased in times of increased oxidative stress and serve to translate this into increased local macrophage inflammation. Overnutrition not only causes oxidative stress in the adipocyte but also result in the adipocyte exceeding its lipid storage capacity. This is associated with defects in lipogenesis, increased adipocyte insulin resistance, and increased lipolysis. The recruitment and activation of macrophages could serve to scavenge the detritus of adipocytes driven to apoptosis or the local effects of excess free fatty acids from increased lipolysis, and thus a signaling mechanism that communicates the level of local adipocyte oxidative stress could serve a useful purpose for the health of the tissue.

The mechanism by which GS-HNE and GS-DHN transmit their message to the macrophages is as yet unknown, but

a cell surface receptor would seem to be most likely. The most obvious possibility for a GS-HNE and/or GS-DHN receptor is one of the toll-like receptors (TLRs), potentially TLR6 or TLR9, both of which show upregulated expression in response to GS-HNE and GS-DHN. However, given the structural similarity between the glutathionylated lipid aldehydes and the cysteinyl leukotrienes, a receptor similar to the cysteinyl leukotriene receptors, or the receptors themselves may be another potential candidate. To date two such receptors CysLTR1 and CysLTR2 have been identified.[47]

The model depicted in **Figure 2** outlines the generation of 4-HNE as a result of the action of ROS on membrane polyunsaturated lipids. 4-HNE and other reactive aldehydes can go on to impact cell function and metabolism via protein carbonylation. Our recent studies demonstrate that the further metabolism of 4-HNE into GS-HNE and then GS-DHN result in metabolites that are actively exported by RLIP76 and induce macrophage inflammatory changes via an unknown receptor and signaling pathway. This in turn results in the production of the inflammatory mediators, TNF-α and LTC$_4$. Macrophage inflammation can then feed forward and cause further oxidative stress and metabolic changes in the adipocyte including insulin resistance and changes in adipokine secretion. This model implies that the regulation of GSTA4 function (and the entire antioxidant pathway) plays an important role in determining the poise of the metainflammatory state. While both protein carbonylation and increased macrophage inflammation have the potential to negatively affect adipocyte function, the subtleties and importance of this balance have yet to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Author Contributions

B.I.F. wrote the manuscript. D.A.B. reviewed/edited manuscript. D.A.B. is the guarantor of this work, had full access to all data, and takes full responsibility for the integrity of the data and accuracy of data analysis.
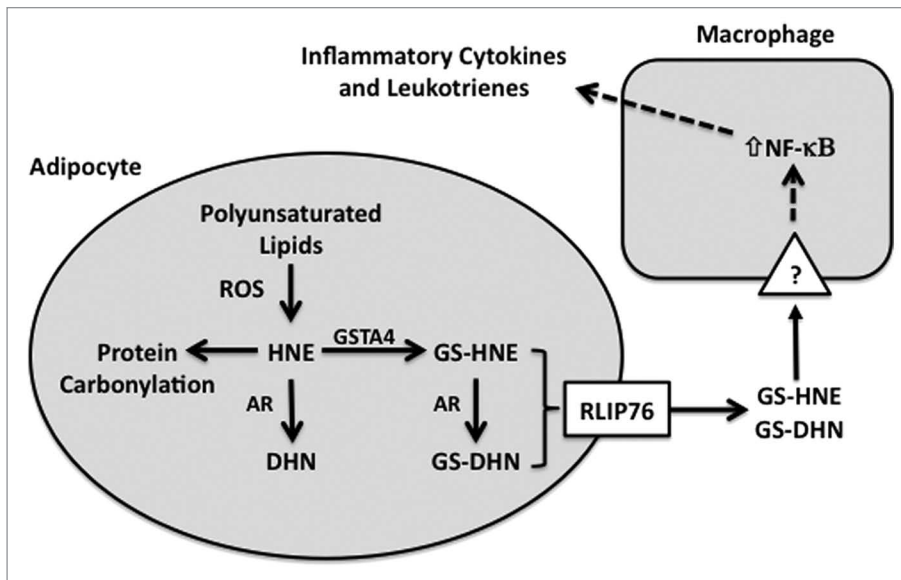
Figure 2. Model for lipid peroxidation and signaling by glutathionylated products of lipid peroxidation. AR, aldose reductase; GSTA4, glutathione-S-transferase A4; GS-DHN, glutathionyl-1,4-dihydroxynonene; GS-HNE, glutathionyl-4-hydroxy-2-nonenal; HNE, *trans*-4-hydroxy-2-nonenal; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species.

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