FALDH REVERSES THE DELETERIOUS ACTION OF OXIDATIVE STRESS INDUCED BY THE LIPID PEROXIDATION PRODUCT, HNE, ON INSULIN SIGNALING IN 3T3-L1 ADIPOCYTES.

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Running Title: FALDH reverses HNE-induced inhibition of insulin action.

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Received for publication 23 March 2007 and accepted in revised form 18 December 2007.
ABSTRACT

Objective: Oxidative stress is associated with insulin resistance and is thought to contribute to progression towards type 2 diabetes. Oxidation induces cellular damages through increased amounts of reactive aldehydes from lipid peroxidation. The aim of our study was to investigate (i) the effect of the major lipid peroxidation end-product, 4-hydroxynonenal (HNE), on insulin signaling in 3T3-L1 adipocytes, and (ii) whether Fatty Aldehyde Dehydrogenase (FALDH), which detoxifies HNE, protects cells and improves insulin action under oxidative stress conditions.

Research Design and Methods: 3T3-L1 adipocytes were exposed to HNE and/or infected with control adenovirus or adenovirus expressing FALDH.

Results: Treatment of 3T3-L1 adipocytes with HNE at nontoxic concentrations leads to a pronounced decrease in IRS-1/-2 proteins and in insulin-induced IRS and IRβ tyrosine-phosphorylation. Remarkably, we detect increased binding of HNE to IRS-1/-2 generating HNE-IRS adducts, which likely impair IRS function and favor their degradation. PI3-kinase and PKB activities are also downregulated upon HNE treatment resulting in blunted metabolic responses. Moreover, FALDH, by reducing adduct formation, partially restores HNE-generated decrease in insulin-induced IRS-1 tyrosine-phosphorylation and metabolic responses. Moreover, rosiglitazone could have an antioxidant effect as it blocks the noxious HNE action on IRS-1 by increasing FALDH gene expression. Collectively, our data show that FALDH improves insulin action in HNE-treated 3T3-L1 adipocytes.

Conclusion: Oxidative stress induced by reactive aldehydes, such as HNE, is implicated in the development of insulin resistance in 3T3-L1 adipocytes, which is alleviated by FALDH. Hence, detoxifying enzymes could play a crucial role in blocking progression of insulin resistance to diabetes.

ABBREVIATIONS. AGE, advanced glycated end product; AP-1, activator protein 1; FALDH, fatty aldehyde dehydrogenase; Grb2, growth factor receptor bound 2; HNE, 4-hydroxynonenal; IR, insulin receptor; IRS, insulin receptor substrate; JNK, cJun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen activated protein kinase; PBS, phosphate buffer saline; PKB, protein kinase B; PI3-kinase, phosphatidylinositol-3 kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; Shc, Src-homology-2-containing protein; XTT, sodium 3’-(1-phenylaminocarbonyl)-3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate.
Development of type 2 diabetes is associated with insulin resistance and insufficient insulin secretion by pancreatic beta cells (1). Hyperinsulinemia, hyperglycemia and impairment of insulin signaling have been implicated in disease situations such as diabetes and obesity. An association between insulin resistance and increased oxidative stress exists as markers of oxidative stress are higher in diabetes and obesity (2; 3). However, the mechanisms by which oxidative stress may play a role in a variety of diseases remain unclear. Insulin binding to its receptor leads to its rapid tyrosine phosphorylation which permits recruitment of adaptor proteins such as insulin receptor substrates (IRS) and Src-homology-2-containing proteins (Shc). These substrates transmit the insulin signal by activating two major pathways, the phosphatidylinositol-3 kinase (PI3-kinase) cascade for glucose, lipid and protein metabolism, and the mitogen activated protein kinase (MAPK) cascade for cell proliferation and differentiation (1; 4-6).

Oxidative stress induced by reactive oxygen species (ROS) is increased in diabetes suggesting their implication in the onset and/or progression of the disease process (7-9). H$_2$O$_2$ has been shown to impair glucose uptake in 3T3-L1 adipocytes, suggesting an implication of ROS accumulation in insulin resistance (10). Oxidative stress induces various cellular damages directly or indirectly through lipid peroxidation of reactive aldehydes such as 4-hydroxynonenal (HNE) (11; 12). Lipid peroxidation and therefore aldehydes represent markers of oxidative injury and may be more deleterious than the initial product, ROS, as they diffuse within the cell and thus propagate their noxious action. HNE is the major unsaturated aldehyde end-product and the most toxic one (13). It is produced during oxidative stress in relatively large amounts with intracellular concentrations reaching 0.1 μM to 5 mM and can increase itself ROS production (14; 15). At high concentrations, HNE exerts cytotoxic, mutagenic and genotoxic activities, and is largely responsible for cytopathological effects observed during oxidative stress (16). However, at micromolar concentrations, HNE induces nontoxic effects by reacting with amino acids such as cysteine, lysine or histidine, and forms stable adducts with proteins and DNA, leading to modulation of activities and/or expression of various proteins (16; 17). HNE also stimulates JNK and p38 MAPK resulting in activation of transcription factors such as cJUN, AP-1 (18-20). Long-term exposure to HNE induces apoptosis via increased caspase-3 activity (21). HNE accumulation in diabetic patients and in liver of diabetic rats has been reported (22; 23, Gopaul, 1995 #1290). Further, exposure of pancreatic islets to HNE decreases insulin secretion (24; 25). Collectively these data reveal HNE as a strong representative of oxidative agents, which plays a major role in the development of insulin resistance. However, the specific targets of aldehyde modification leading to insulin resistance and diabetes remain unknown.

Reactive aldehydes are controlled by anti-oxidative systems in cells. One of the enzymes capable of metabolizing HNE is Fatty Aldehyde Dehydrogenase (FALDH). FALDH is a microsomal NAD/NADP-dependent enzyme, which belongs to the aldehyde dehydrogenase family and catalyses oxidation of HNE to the non-toxic 4-hydroxy-2-nonenioic acid (26; 27). We showed earlier that FALDH decreases ROS production induced by HNE and that its expression is decreased in white adipose tissue of diabetic mice (14). Therefore, we hypothesized that FALDH could play a key role in maintaining a physiological cellular milieu by detoxifying HNE.

The aims of our current study are to evaluate (i) the effect of oxidative stress on major insulin pathways, and (ii) the role of FALDH in HNE-induced modifications in insulin signaling. We report here that 3T3-L1 adipocytes treatment with HNE at non-toxic concentrations affects insulin signaling by decreasing IRS-1/2 proteins and their tyrosine phosphorylation. Further, increased FALDH expression by means of an adenoviral construct or after rosiglitazone-treatment reversed HNE deleterious effects, suggesting that FALDH protects cells against oxidative stress. In summary, detoxification enzymes, such as FALDH, appear to play a crucial role in maintaining insulin signaling during oxidative stress. Hence their dys-functioning could participate in development of insulin resistance.

**RESEARCH DESIGN AND METHODS**

**Materials.** Cell culture solutions, reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein A sepharose were from Life Technologies (Carlsbad, CA). Recombinant human insulin
was from Novo-Nordisk (Copenhagen, Denmark), 4-Hydroxynonenal (HNE) from Calbiochem (La Jolla, CA), L-α-Phosphatidylinositol from Sigma (St. Louis, MO), TLC silica plates from Merck (Darmstadt, Germany), Crostside from Upstate Biotechnology (Lake Placid, NY), polyvinylidiene difluoride (PVDF) membranes from Millipore (Bedford, MA), ECL reagents, 2-deoxy-D-[3H]glucose, [γ-32P]-ATP, D-[2-3H]-glucose from Amersham Pharmacia Biotech (Uppsala, Sweden). Rosiglitazone was from AG scientific (Paris, France).

**Antibodies.** Antibodies to PKBα/β, phospho-PKB (serine 473 and threonine 308), phospho-serine 307 and phospho-mitogen activated protein kinase (phospho-MAPK p42/p44) were from Cell Signaling Technologies (Beverly, MA), antibodies to IRS-1 and phosphotyrosine (clone 4G10) from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibody to IRS-1 and IRS-2, used for immunoprecipitation, was produced in our laboratory. Antibodies to IRβ, IRS-2, MAPK p42/p44 and Myc were from Santa Cruz Biotechnology (Santa Cruz, CA), antibody to HNE-Michael adduct from Calbiochem (La Jolla, CA) and R&D Systems (Minneapolis, MN), secondary anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase from Jackson Laboratories (Copenhagen, Denmark).

**Differentiation of 3T3-L1 cells.** 3T3-L1 fibroblasts, from the American Type Culture Collection (ATCC, Rockville, MD) were grown and differentiated to adipocytes as described (14). HNE cytotoxicity was determined measuring cell viability with XTT assay (Roche Applied Science, Mannheim, Germany) and lactate dehydrogenase activity (LDH) (28).

**Generation of recombinant adenoviruses.** Adenoviruses (Ad) expressing FALDH were generated as described (14; 29). Empty and FALDH adenoviruses titer of stocks was > 10⁸ plaques-forming units/ml.

**Protein isolation, immunoprecipitation and immunoblotting.** 3T3-L1 adipocytes were washed and solubilized as described (14). Lysates were kept on ice for 20 min and insoluble material was removed by centrifugation at 14,000 g for 20 min. Protein concentration was determined by colorimetric assay (BioRad). For immunoprecipitation and immunoblots, antibodies were used as described (33). Immunoreactive proteins were detected using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences, Inc.). Signal intensities were measured with ImageQuant.

**PI3-kinase assay.** 3T3-L1 adipocytes were incubated in serum-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.2 % (w/v) bovine serum albumin (BSA) for 12 h. Cells were incubated or not with HNE and with or without insulin for 10 min. Cells were solubilized and supernatants (300 μg) immunoprecipitated with antibody to IRS-1 coupled to protein A-sepharose beads for 3 h at 4°C. Immune pellets were washed and lipid kinase assays performed as described (30). Phosphorylated lipids were detected by autoradiography on Kodak X-Omat films.

**2-Deoxyglucose uptake and lipogenesis.** 3T3-L1 adipocytes, infected or not with adenoviruses, were incubated in serum-free DMEM containing 0.2 % (w/v) BSA for 12 hours and treated or not with HNE. For glucose uptake and lipogenesis, cells were incubated and treated as described (33). Radioactivity was counted and samples were normalized to protein concentration.

**Real Time Quantitative PCR.** Extracted total RNA was treated with Dnase (Ambion, Inc., Austin, TX), and 1 μg was reverse-transcribed using Reverse Transcription System kit (Promega, Charbonnieres, France) with random primers and oligo(dT)₁₅. Quantitative PCR was performed as described using oligonucleotides to quantify FALDH mRNA, and 36B4 mRNA as reference gene (14).

**Statistical analysis.** Results are presented as mean ± SE with experiments number indicated in the figure legend. Statistical significance was assessed using the Student’s t-test.

**RESULTS**

HNE decreases the protein levels of IRS-1 and IRS-2, but not of MAPK. Because insulin resistance has been associated with increased oxidative stress, we examined the HNE effect on insulin signaling in 3T3-L1 adipocytes. Exposure to HNE results in a dose- and time-dependent decrease in IRS-1 protein (figure 1A). Quantification of total IRS-1 indicates that the maximal decrease is 80 %. Western blotting reveals that HNE induces a decrease in IRS-2 protein (figure 1B), with a similar time- and dose-dependency compared to IRS-1. In contrast,
HNE has no detectable effect on p42/p44 MAPK proteins. Next, we searched for a possible action of HNE on molecules of the MAPK pathway. We found that the content of the three Shc isoforms and of Grb2 remained unchanged in cells exposed to HNE (figure 1C). Moreover, HNE has no effect on insulin-induced threonine and tyrosine phosphorylation of p42/p44 MAPK (figure 1D). Finally, we measured cell viability in HNE-treated cells using XTT reagent (figure 1E). Viability was only marginally decreased at the highest HNE concentrations used. Moreover, we quantified indirectly cell viability measuring Lactate Dehydrogenase (LDH) activity in the incubation medium, which reflects the presence of necrotic cells. We found no significant difference in cell viability after incubation with HNE. In summary, exogenous HNE at nontoxic concentrations does not appear to impinge on the Shc-MAPK signaling module in 3T3-L1 adipocytes, but leads to an important reduction in IRS-1/-2 proteins. This decrease is not due to a cytotoxic HNE effect on cell viability.

**HNE impairs insulin-induced tyrosine phosphorylation of IRβ and IRS.** To investigate whether HNE treatment impairs insulin-induced tyrosine phosphorylation of its receptor β subunit (IRβ) and its proximal substrates, IRS, we incubated 3T3-L1 adipocytes with increasing HNE concentrations. HNE addition to the medium before exposure to insulin inhibits IRβ and IRS tyrosine phosphorylation in a time- and dose-dependent manner compared to control cells (figure 2A-2B). The reduced IRS tyrosine phosphorylation can likely be accounted for by the reduction in IRS-1/-2 proteins and by reduced IRβ kinase activity. Importantly, the IRβ protein level remained virtually unchanged during HNE treatment. We next examined IRS-1 phosphorylation on serine 307 in presence of HNE (figure 2C). HNE transiently enhanced serine 307 phosphorylation between 1 and 4 hours HNE treatment.

**HNE generates HNE-Michael adducts with IRS-1 and IRS-2.** HNE is known to induce formation of HNE-Michael adducts by reaction of the HNE aldehyde moiety with cysteine, histidine or lysine residues in proteins (16). Indeed, HNE induced formation of several aldehyde-protein adducts in 3T3-L1 adipocytes as shown by western blot using antibody to HNE-Michael adducts (figure 3A). To determine whether IRS-1 and -2 could form adducts with HNE, we incubated 3T3-L1 adipocytes with HNE and analyzed immunoprecipitated IRS-1 and IRS-2 by western blot with antibody to HNE-Michael adducts (figure 3B). As expected, IRS-1 and -2 protein are decreased. Concomitantly, HNE-Michael adducts accumulate in a time-dependent manner. Thus, HNE is able to directly interact with IRS-1 and -2 leading to generation of HNE-IRS adducts. Taken together our results suggest that, under oxidative stress conditions, formation of HNE-modified IRS adducts is increased in 3T3-L1 adipocytes, and probably leads to IRS degradation. Moreover, pretreatment with the lysosomal inhibitor, chloroquine, partially prevents HNE-induced IRS-1 degradation (figure 3C). By contrast, pretreatment with MG132 or lactacystin, two proteasomal inhibitors, had no effect on IRS-1 level. To sum up, HNE-IRS-1 appears to be directed preferentially to the lysosomal pathway for degradation.

**Insulin-stimulated activation of PI3-kinase and PKB is altered upon HNE treatment.** We evaluated insulin’s ability to activate PI3-kinase and PKB after HNE exposure. As expected, insulin induces a 5-fold increase in PI3-kinase activity associated with IRS-1 in control cells. In contrast, HNE reduces basal PI3-kinase activity by 50 % and insulin-stimulated PI3-kinase activity by 72 % (figure 4A). It is likely that the decrease in PI3-kinase activity is linked to decreased IRS-1 tyrosine phosphorylation in HNE-treated cells. To assess whether this defect in PI3-kinase activity is associated with blunted PKB activation, we evaluated insulin-induced PKB phosphorylation following HNE treatment. Firstly, we observed a dose-dependent decrease in PKB protein in presence of HNE (figure 4B). Secondly, we found that HNE treatment leads to a time-dependent decrease in insulin-stimulated PKB phosphorylation on serine 473 and threonine 308 (figure 4C). The reduction in PKB phosphorylation is correlated with the decrease in protein suggesting that reduced PKB activation can be accounted for by a decrease in both decreased PKB protein and insulin receptor signaling.

**HNE impairs metabolic responses induced by insulin.** As signaling through PI3-kinase was
altered upon HNE treatment, we investigated the HNE effect on insulin-induced metabolic responses. As expected, insulin induces a 3-fold increase in glucose uptake in control cells, whereas within 4 hours both basal (2-fold relative to control) and insulin-stimulated glucose transport (4-fold relative to control) are decreased in cells incubated with HNE (figure 5A). Similarly we found in cells incubated with HNE a time-dependent decrease in basal and insulin-stimulated lipogenesis as compared to non-treated cells (8-fold decrease within 4 hours) (figure 5B).

**FALDH partially blocks HNE inhibitory action on insulin-induced IRS-1 tyrosine-phosphorylation by decreasing formation of adducts.** We previously showed that FALDH plays an important role in the decrease in reactive oxygen species production in 3T3-L1 adipocytes exposed to HNE (14). Therefore, we hypothesized that FALDH could prevent the deleterious HNE action on insulin signaling. As expected, in control cells infected with empty adenovirus, HNE induces a robust decrease (70 %) in insulin-induced IRS-1 tyrosine phosphorylation (lane 4-5) as compared to non-treated cells (lane 2-3) (figure 6A). In contrast, in cells infected with FALDH adenovirus, we observed only a slight decrease (25 %) in IRS-1 tyrosine phosphorylation in presence of HNE (lane 9-10) compared to non-treated cells (lane 2-3) (figure 6A). In contrast, in cells infected with FALDH adenovirus, we observed only a slight decrease (25 %) in IRS-1 tyrosine phosphorylation in presence of HNE (lane 9-10) compared to non-treated cells (lane 7-8) (figure 6B). Looking at IRS-1 levels, we found that ectopic FALDH expression partially restores IRS-1 levels in presence of HNE (lane 9-10) in contrast to control cells (lane 4-5), which explains the improvement of IRS-1 tyrosine phosphorylation. Moreover, cells infected with FALDH adenovirus show a decrease in formation of HNE-adducts compared to control cells (figure 6C). Finally, we found a reduction in HNE-IRS-1 adducts in cells expressing ectopic FALDH compared to control cells (figure 6D). Interestingly, these results indicate that by reducing the generation of HNE-IRS-1 adducts, increased FALDH expression allows partial restoration of the decrease in insulin-induced IRS tyrosine phosphorylation caused by HNE. To summarize, FALDH protects 3T3-L1 adipocytes against HNE action, and hence permits to maintain an unperturbed insulin-induced activation of IRS.

**FALDH partially restores insulin-induced metabolic responses in presence of HNE.** To strengthen the idea that increased FALDH expression prevents HNE damage on signaling events induced by insulin, we investigated the consequence of ectopic FALDH expression on glucose uptake and lipogenesis in 3T3-L1 adipocytes incubated with HNE. As expected, HNE treatment of control cells induces a robust decrease (60 %) in insulin-induced glucose transport (figure 7A, compare lane 1 and 2). In contrast, in cells expressing ectopically FALDH, HNE reduces insulin-stimulated glucose transport only by 30 % (compare lane 3 and 4). Moreover, infection with empty or FALDH adenovirus had no effect on basal glucose transport (data not shown). Concerning lipogenesis, the impairment in triglycerides synthesis observed when control cells were treated with HNE (figure 7B, compare lane 1 and 2) is more important than in cells ectopically expressing FALDH (compare lane 3 and 4). Indeed, in control cells exposed to HNE, lipogenesis is decreased by 78 %, whereas in FALDH-infected cells, the HNE-induced decrease amounts to 44 %. Moreover, infection with empty or FALDH adenovirus had no effect on basal lipogenesis (data not shown). Note that insulin-stimulation of glucose uptake or of lipogenesis is slightly lower in infected cells compared to non-infected cells (figure 5), probably due to some noxious effect of adenovirus infection.

To sum up, expression of FALDH can partially prevent the deleterious HNE action on metabolic responses induced by insulin, demonstrating the importance of detoxifying enzymes to preserve insulin signaling under oxidative stress.

**Rosiglitazone inhibits downregulation of IRS-1 proteins induced by HNE.** As we previously showed that FALDH gene expression is increased by insulin, we hypothesized that rosiglitazone may protect against oxidative damage by increasing FALDH gene expression. Indeed, activation of PPARs, which are implicated in regulation of gene expression in response to insulin or to the anti-diabetic drugs glitazones, has been reported to reduce cellular damage due to AGE or oxidative stress (31-33). To determine whether rosiglitazone induces FALDH gene expression, we measured the amount of FALDH mRNA after rosiglitazone treatment (figure 8A). Thus, rosiglitazone induces a 3-fold increase in FALDH gene expression compared to non-treated cells. Next, we looked at the effect of pre-incubation of 3T3-L1 adipocytes with rosiglitazone on HNE-induced IRS-1 downregulation (Figure 8B). As
expected, IRS-1 protein is decreased about 50 % in control cells, whereas cells incubated with rosiglitazone showed only a 10 % decrease indicating that rosiglitazone protects cells against HNE-induced oxidative stress.

DISCUSSION

Insulin is a master regulator of several key functions in metabolism control. Defects in these control points due to inhibition of insulin signaling contribute decisively to the development of insulin resistance and type 2 diabetes. Oxidative stress has emerged as a causative factor for insulin resistance and as participating in the disease process leading to diabetes and its complications (8; 9; 34). During oxidative stress, several reactive products are generated which include ROS, and aldehydes such as HNE through a lipid peroxidation process (11; 17). Elevated ROS or HNE levels are correlated with hyperglycemia and type 2 diabetes, whereas antioxidants are known to reverse, at least in part, insulin resistance in rodents (35; 36). HNE is the major peroxidation product of polyunsaturated fatty acids and is the most reactive one. HNE can be produced in relatively large amounts reaching up to milliM concentrations both in vitro and in vivo in response to oxidative insults (16; 37; 38). Moreover, increased lipid peroxidation and HNE concentrations are found in diabetic subjects and the HNE concentrations used in our study are within the range of those reported in pathophysiological conditions including diabetes (24). HNE accumulation has been implicated in several deleterious processes such as inhibition of enzymes by chemical modification at low non-toxic concentrations, and by doing so hampers their functions (16; 17; 39). In addition, HNE affects insulin secretion and signaling. Indeed, HNE restrains glucose-induced insulin secretion and in beta cells of Goto-Kakizaki rats, a model of non-obese type 2 diabetes, hyperglycemia might be responsible for the increase in HNE-modified proteins (25). Both increased lipid peroxidation and altered removal by detoxifying enzymes occur in diabetic rats and are possible causes of HNE accumulation (22; 23). To sum up, growing evidence implicates HNE in functional alterations in diverse disease processes including alterations in glucose homeostasis. As insulin resistance inevitably precedes development of type 2 diabetes, we hypothesized that an increased level of toxic aldehydes, such as HNE, could be involved in dysfunctioning of signaling molecules, and hence could hamper insulin action. We chose 3T3-L1 adipocytes as model system for insulin action to analyze the effect of oxidative stress induced by HNE on insulin signaling. Indeed, augmented adipose mass due to nutrient excess is associated with increased lipid-derived metabolites that impair insulin signaling and generate insulin resistance (40; 41).

We show a time- and dose-dependent decrease in IRS-1/-2 proteins in 3T3-L1 adipocytes exposed to nontoxic HNE concentrations, which is associated with a reduction in IRS tyrosine phosphorylation and an increase in IRS-1 phosphorylation on serine 307. In addition, we found increased JNK activation by HNE which could explain this IRS-1 serine phosphorylation (data not shown). In addition, HNE reduces IRβ activation by insulin without decreasing significantly IRβ protein. We favor the idea that HNE acts by generating structural modifications in IRβ leading to its impaired function. Our findings agree with a report showing a decrease in PDGFRβ activation in presence of HNE without reducing total PDGFRβ number (42). Moreover, in our experiments HNE seems to have no effect on MAPK signaling. Indeed, Shc, Grb2 and MAPK protein levels were not affected by HNE. The small decrease in IRβ does not perturb MAPK activation in our experimental conditions, very likely due to the intact Shc/Grb2 module.

HNE interacts spontaneously with amino acids of proteins leading to generation of Michael adducts (11; 38). Therefore, we investigated whether the decrease in IRS proteins is associated with formation of HNE-modified IRS-1 and -2 adducts. Remarkably, we found in cells exposed to HNE a time-dependent accumulation of IRS-1 and -2 tagged with HNE. Importantly, no increase in HNE-modified MAPK protein adduct was detected, which could explain the absence of decrease in MAPK protein in presence of HNE (data not shown). While it is known that proteins tagged with HNE are more sensitive to degradation than native proteins, the mechanisms underlying degradation of HNE adducts are not well-defined (43-45). We show that IRS-1 protein level in presence of HNE is partially restored by pre-treatment of cells with the lysosomal inhibitor chloroquine. However, inhibition of lysosomes is not sufficient to totally prevent...
Because IRS proteins play a crucial role in insulin signaling and because HNE leads to their downregulation, we looked at the HNE effect on PI3-kinase and PKB. As expected, we found that HNE treatment of 3T3-L1 adipocytes results in decreased insulin-stimulated PI3-kinase activity. Moreover, when cells are exposed to HNE, PKB phosphorylation on the two sites necessary for optimal activity is reduced, reflecting decreased PKB activation. Next, we were interested in possible consequences of oxidative stress on key insulin metabolic responses. We show that nontoxic HNE concentrations decrease glucose transport and lipogenesis. The decrease in glucose transport agrees with other studies reporting altered glucose transport in oxidative stress conditions induced by H$_2$O$_2$ (10; 46).

Our demonstration that HNE impairs insulin signaling would indicate that this aldehyde plays an important role in insulin resistance development and therefore could foster progression to type 2 diabetes. We hypothesized that an increased anti-oxidant potential of cells and tissues is likely to improve insulin resistance. Indeed, anti-oxidant treatment, based on $\alpha$-lipoic acid addition or anti-oxidant enzyme overexpression, improves insulin sensitivity (34; 35). Concerning anti-oxidation machineries, we previously reported that FALDH decreases ROS production induced by HNE treatment (14). Moreover, we found that FALDH mRNA expression is increased by insulin and decreased in insulin-resistant animal models. Therefore, to test whether an enzyme involved in detoxification can improve insulin signaling in cells exposed to oxidative stress, FALDH appeared to us as an appropriate candidate. Remarkably, 3T3-L1 adipocytes infected with an adenoviral construct encoding FALDH present stronger IRS-1 tyrosine phosphorylation in response to insulin than control cells, and this is associated with partial restoration of IRS-1 protein. Consistent with this, FALDH partially prevents formation of HNE adducts with several proteins including IRS-1, which confirms that HNE binding to IRS favors their degradation. Finally, increased FALDH expression improves insulin metabolic responses such as glucose transport and lipogenesis in HNE-treated cells.

To further document the potential role of FALDH protection against oxidative stress of diabetes, we looked at the effect of the anti-diabetic drug, rosiglitazone. PPAR agonists are known to modulate expression of anti-oxidant genes such as NADP(H) oxidase or Cu$^{2+}$/Zn$^{2+}$ superoxide dismutase (33; 47). Interestingly, we show that FALDH gene expression is increased by rosiglitazone. At the concentration used, rosiglitazone is expected to activate both PPAR$\alpha$ and $\gamma$. Therefore, our result agrees with studies showing an increase in FALDH gene expression in response to clofibrate, an activator of PPAR$\alpha$, or a decrease in PPAR$\alpha$ null mice (48; 49). Moreover, we found that IRS-1 protein is virtually unchanged by HNE in 3T3-L1 adipocytes pre-treated by rosiglitazone, compared to non-treated cells. The anti-oxidant effect of rosiglitazone is likely to be due, at least in part, to increased endogenous FALDH which detoxifies HNE. However, because rosiglitazone modulates several cellular functions, we cannot exclude participation of molecules other than FALDH. Our finding showing an anti-oxidative action of rosiglitazone through increased FALDH gene expression fits with studies showing that rosiglitazone and troglitazone improve atherosclerosis by decreasing ROS generation and lipid peroxidation (31; 50). The control exerted by the anti diabetic drug, rosiglitazone, on expression of genes, such as FALDH, involved in protection against oxidative stress, might reflect the existence of cellular defense mechanisms against damage of oxidative stress on insulin action.

To conclude, the key findings of our study are (i) 3T3-L1 adipocytes treatment with HNE impairs insulin action, and (ii) increased FALDH expression protects 3T3-L1 adipocytes against HNE-induced oxidative stress. As we previously showed that in insulin-resistant animals the stimulatory action of insulin on FALDH gene expression is lost, and because HNE induces insulin resistance, we would like to suggest the occurrence of a vicious circle between HNE accumulation and insulin resistance. Considering our findings on the ability of FALDH to reverse the harmful impact of HNE on insulin action, it is likely that means leading to increased detoxification of aldehydes are potential novel candidates to combat insulin resistance.

**ACKNOWLEDGMENTS**

We thank Sophie Grillo for assistance in the initial part of the study and Jean Giudicelli for LDH activity assay. Our research was supported by INSERM, PN RD of INSERM (2004), Conseil Général des Alpes-Maritimes et Conseil.
Régional PACA, University of Nice Sophia-Antipolis, and by a grant from the European Community (FP6 Eugene2 (LSHM-CT-2004-512013)). Damien Demozay was supported by ARC (Association pour la Recherche contre le Cancer).
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FIGURE LEGENDS

Figure 1: HNE decreases the protein level of IRS-1 and IRS-2, but not of MAPK.
3T3-L1 adipocytes were incubated with increasing concentrations of HNE (25 to 100 μM) for 4 hours (left), or at 75 μM for 1 to 6 hours (right). Cells were solubilized and total lysates were separated by SDS-PAGE. Finally, western blot analysis was performed with antibody to IRS-1 (A), IRS-2 (B), Shc (C), Grb2 (C) or MAPK. The results concerning IRS-1 were quantified and correspond to four individual experiments, of which one representative immunoblot is shown. For MAPK activation (D), 3T3-L1 adipocytes were serum-deprived overnight and incubated for 5 min with insulin (100 nM) or buffer after different times of HNE treatment. Lysates were separated by SDS-PAGE and a western blot was performed with antibodies to phospho-MAPK and to MAPK. Cell viability was analyzed by a XTT assay (left) and measuring LDH activity (right) (E).

Figure 2: HNE impairs insulin-induced tyrosine phosphorylation of IRβ and IRS.
3T3-L1 adipocytes were serum-deprived overnight and incubated or not with HNE at different concentrations (25 to 100 μM) for 4 hours (A) or at 75 μM for different lengths of time as indicated (B,C), and then exposed to insulin (100 nM) for 5 min or left untreated. Cells were solubilized, total lysates were separated by SDS-PAGE and transferred proteins blotted with antibody to phosphotyrosine. Immunoblots were stripped and reprobed with antibodies to IRS-1, IRS-2, IRβ or MAPK. The bands corresponding to tyrosine-phosphorylated IRS-1 in the dose-response experiments were quantified and correspond to three independent experiments; the results are expressed as a percentage of basal condition. Cell lysates were prepared and immunoprecipitated with antibody to IRS-1 (C). After washes, immune pellets were suspended in in 3% (wt/vol) SDS sample buffer, separated by SDS-PAGE and immunoblotted with antibody to IRS-1 phospho-serine 307. Immunoblots were stripped and reprobed with antibodies to IRS-1. The bands corresponding to phospho-serine 307 were quantified and the values were corrected for differences in the total amount of IRS-1 recovered and expressed as a percentage of the basal condition. Means ± SE from three individual experiments are shown. *P<0.07 vs. no HNE, **P<0.005 vs. no HNE.

Figure 3: HNE generates HNE-Michael adducts with IRS-1 and IRS-2.
3T3-L1 adipocytes were serum-deprived overnight and incubated with 75 μM HNE for different lengths of time as indicated. Samples from total lysates were separated by SDS-PAGE and transferred proteins blotted with antibody to HNE-adduct (A). Cells lysates were prepared and immunoprecipitated with antibody to IRS-1 or to IRS-2 (B). After washes, immune pellets were suspended in 3% (wt/vol) SDS sample buffer, separated by SDS-PAGE and immunoblotted with antibody to HNE-adduct. Immunoblots were stripped and reprobed with antibody to IRS-1 or to IRS-2. Cells were incubated for 3 hours with or without 50 μM MG132, 10 μM lactasystin (Lact) or 100 μM chloroquine (Chlq), and then with 75 μM HNE for 4 hours (C). Samples from total lysates were separated by SDS-PAGE and transferred proteins blotted with antibodies to IRS-1 and MAPK. The bands corresponding to IRS-1 were quantified and expressed as a percentage of the basal condition.

Figure 4: Insulin-stimulated activation of PI3-kinase and PKB is altered upon HNE treatment.
For PI3-kinase assay (A), 3T3-L1 adipocytes were serum-deprived overnight and incubated with HNE (100 μM) for 3 hours. Cell lysates were extracted and immunoprecipitated with antibody to IRS-1 and incubated after washes with phosphoinositides (10 μg/ml), [γ-32P]-ATP for 15 minutes as described in Research Design and Methods. Samples were analyzed by chromatography. 32P incorporated in phosphoinositides was quantified and expressed as a percentage of activity measured in absence of insulin. 3T3-L1 adipocytes were serum-deprived overnight and incubated with HNE at different concentrations as indicated for 4 hours (B). Cell lysates were extracted and lysates were separated by SDS-PAGE. PKB expression level was evaluated by immunoblotting with antibody to PKB. For PKB activation, 3T3-L1 adipocytes were serum-deprived overnight, incubated or not with HNE at different times (1 to 4 hours) at 75 μM (C) as indicated. Cells were then exposed to insulin (100 nM) for 5 min or left untreated. Cells were solubilized, lysates were separated by SDS-PAGE and immunoblotted with antibody to phospho-PKB (Thr 308 or Ser 473). Immunoblots were stripped and reprobed with...
antibodies to PKB or MAPK. Representative immunoblots are presented from experiments repeated at least four times.

**Figure 5: HNE impairs metabolic responses induced by insulin.**
3T3-L1 adipocytes were serum-deprived overnight and incubated or not with HNE at 75 μM for different times as indicated. For glucose uptake (A) cells were exposed or not for 10 min to insulin (100 nM) and 2-deoxy-D-[3H]glucose uptake was measured as described in Research Design and Methods. 3H incorporated in DOG was counted, corrected for protein concentration and glucose uptake was expressed relative to cells not exposed to insulin nor HNE. For lipogenesis (B), cells were exposed for 15 min to insulin (100 nM) or buffer, and the assay was performed as described in Materials and Methods. 3H incorporated in lipids was counted, corrected for protein concentration and was expressed as a percentage of lipogenesis in cells not exposed to insulin or HNE.

**Figure 6: FALDH partially blocks the inhibitory action of HNE on insulin-induced IRS-1 tyrosine phosphorylation by decreasing formation of adducts.**
3T3-L1 adipocytes were infected with control (empty) adenovirus (Ad) or adenovirus expressing FALDH for 3 hours. 24 hours after infection, cells were serum-deprived for 12 h and incubated or not with HNE at 75 μM for 4 hours. Cells were then exposed or not for 5 min to insulin (100 nM). Cell lysates were extracted and lysates were separated by SDS-PAGE and western blot analysis was performed using antibody to phosphotyrosine (A). Lysates extracted from insulin and insulin/HNE conditions were deposited in duplicate (lane 2-3, 4-5, 7-8 and 9-10). Immunoblots were stripped and reprobed with antibodies to IRS-1 or MAPK. As control of FALDH expression, immunoblots were probed with antibody to Myc. Quantification of three different experiments was performed and results are expressed as a percentage of IRS-1 tyrosine phosphorylation in cells infected with empty adenovirus and not exposed to insulin nor HNE (B). Duplicate samples from total lysates were separated by SDS-PAGE and transferred proteins blotted with antibody to HNE-adduct (C). Cell lysates were prepared and immunoprecipitated with antibody to IRS-1 (D). After washes, immune pellets were suspended in 3% (wt/vol) SDS sample buffer, separated by SDS-PAGE and immunoblotted with antibody to HNE-adduct. Immunoblots were stripped and reprobed with antibody to IRS-1.

**Figure 7: FALDH partially restores insulin-induced metabolic responses in presence of HNE.**
3T3-L1 adipocytes were infected with control (empty) adenovirus or adenovirus expressing FALDH for 3 hours. 24 hours after infection, cells were serum-deprived for 12 h and incubated or not with HNE at 75 μM for 4 hours. Then, 2-deoxy-D-[3H]glucose uptake (A) and lipogenesis assay (B) were performed as described in Materials and Methods. 3H incorporated in DOG or 3H incorporated in lipids were counted, corrected for protein concentration and respectively expressed as a percentage of glucose uptake or lipogenesis in cells infected with empty adenovirus, and exposed to insulin only (control). As control of FALDH expression, Western blots were performed and probed with antibody to Myc. Means ± SE from five individual experiments for A and four individual experiments for B, each performed in triplicate, are shown. **P<0.005 and *** P<0.0005.

**Figure 8: Rosiglitazone inhibits downregulation of IRS-1 proteins induced by HNE.**
3T3-L1 adipocytes were serum-deprived and then pretreated or not with rosiglitazone (1μM). After 6 or 10 hours treatment, total RNA was extracted and analyzed by real time quantitative PCR using FALDH primers (A). RNA expression was normalized using 36B4 RNA levels. Results are expressed in comparison to the basal condition. 3T3-L1 adipocytes were serum-deprived and then pretreated or not with rosiglitazone (1μM) for 10 hours. Cells were solubilized and total lysates were separated by SDS-PAGE. Finally, Western blot analysis was performed using antibody to IRS-1 or MAPK (B). The results were quantified and expressed using basal condition as 100 %, the results correspond to three individual experiments, of which one representative immunoblot is shown. *P<0.07.
Figure 1: HNE decreases the protein level of IRS-1 and IRS-2, but not of MAPK.

A) HNE (μM): 0 25 50 75 100
   HNE (h): 0 1 2 4 6
   IRS-1
   MAPK
   IRS-1 levels (% of basal):
   0 25 50 75 100

B) HNE (μM): 0 50 75 100
   HNE (h): 0 2 4 6
   IRS-2
   MAPK

C) HNE (h): 0 2 4 6
   p66
   p52
   Shc
   p46
   Grb2

D) HNE (h): 0 2 4 6
   ins: - + + +
   Phospho-MAPK
   MAPK

E) % of cell viability:
   HNE (4h, μM): - 75 100
   HNE (75μM, h): - 4 6
Figure 2: HNE impairs insulin-induced tyrosine-phosphorylation of IRβ and IRS.

**A)**
- **Insulin:** +
- **HNE (μM):** 0, 25, 50, 75, 100
- IRS
  - pY
  - IRS-1
  - MAPK

**B)**
- **Insulin:** +
- **HNE (h):** 0, 2, 4, 6
- IRS
  - pY
- IRS-1
- IRS-2
- IRβ
- MAPK

**C)**
- **HNE (h):** 0, 0.5, 1, 2, 4
- IP: IRS-1
- IB: p-Ser307
- IB: IRS-1

Graphs showing tyrosine phosphorylation levels of IRS and MAPK with varying HNE and insulin concentrations.
Figure 3: HNE generates formation of HNE-Michael adducts with IRS-1 and IRS-2.

A) HNE: - +  

B) HNE (h): 0 4 6 IP: IRS-1  
IB: HNE-adduct  
IB: IRS-1

C) Inhibitors: None MG132 Lact Chlo  
HNE: - + - + - +  
IB: IRS-1  
IB: MAPK

IRS-1 levels (% of basal)
Figure 4: Insulin-stimulated activation of PI3-kinase and PKB is altered upon HNE treatment.

A) HNE (h): 0 3
Insulin: - + - +

B) HNE (μM): 0 25 50 75
PKB MAPK

C) Insulin: - +
HNE (h): 0 1 2 4
pThr308-PKB pSer473-PKB PKB MAPK
FALDH reverses HNE-induced inhibition of insulin action.

Figure 5: HNE impairs metabolic responses induced by insulin.
FALDH reverses HNE-induced inhibition of insulin action.

Figure 6: FALDH partially blocks the inhibitory action of HNE on insulin-induced IRS-1 tyrosine phosphorylation by decreasing formation of adducts.
Figure 7: FALDH partially restores insulin-induced metabolic responses in presence of HNE.
FALDH reverses HNE-induced inhibition of insulin action.

Figure 8: Rosiglitazone inhibits downregulation of IRS-1 proteins induced by HNE.