The Hyperglycemia-Induced Inflammatory Response in Adipocytes:

The Role of Reactive Oxygen Species

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**Running title:** Reactive Oxygen Species in Adipocytes

**Abbreviations:**

Acrp30: adipocyte complement-related protein of 30 kDa; ROS: Reactive oxygen species;
Abstract

Hyperglycemia is a major independent risk factor for diabetic macrovascular disease. The consequences of exposure of endothelial cells to hyperglycemia are well established. However, little is known about how adipocytes respond to both acute as well as chronic exposure to physiological levels of hyperglycemia. Here, we analyze adipocytes exposed to hyperglycemia both \textit{in vitro} as well as \textit{in vivo}.

Comparing cells differentiated at 4mM to cells differentiated at 25mM glucose (the standard differentiation protocol) reveals severe insulin resistance in cells exposed to 25mM glucose. A global assessment of transcriptional changes shows an upregulation of a number of mitochondrial proteins. Exposure to hyperglycemia is associated with a significant induction of reactive oxygen species (ROS), both \textit{in vitro} as well as \textit{in vivo} in adipocytes isolated from streptozotocin-treated hyperglycemic mice. Furthermore, hyperglycemia for a few hours in a clamped setting will trigger the induction of a pro-inflammatory response in adipose tissue from rats that can effectively be reduced by co-infusion of N-Acetyl Cysteine (NAC). ROS levels in 3T3-L1 adipocytes can be reduced significantly with pharmacological agents that lower the mitochondrial membrane potential, or by overexpression of uncoupling protein 1 (UCP1) or superoxide dismutase (SOD). In parallel with ROS, IL-6 secretion from adipocytes is significantly reduced. On the other hand, treatments that lead to a hyperpolarization of the mitochondrial membrane, such as overexpression of the mitochondrial dicarboxylate carrier (mDIC) result in increased ROS formation and decreased insulin sensitivity, even under
normoglycemic conditions. Combined, these results highlight the importance ROS production in adipocytes and the associated insulin resistance and inflammatory response.
**Introduction**

Many genetic and environmental factors can lead to the development of insulin resistance. Once a degree of insulin resistance is established, decreased glucose tolerance arises and occasional bouts of hyperglycemia ensue. Hyperglycemia can in turn cause a further deterioration of insulin sensitivity in a number of tissues, such as the vascular endothelium, muscle and adipocytes (1).

In the vascular endothelium, hyperglycemia has been shown to activate protein kinase C isoforms, give rise to increased levels of glucose-derived advanced glycation end-products and to cause an increased glucose flux through the aldose reductase pathway. Normalization of mitochondrial reactive oxygen species by a number of different approaches prevents these phenomena (2).

In adipocytes, Tang and colleagues have shown that a combination of hyperglycemia and hyperinsulinemia results in reduced insulin-stimulated glucose uptake that was in part due to reduced insulin receptor dephosphorylation (3).

Gagnon and Sorisky have previously assessed the effects of low and high glucose levels on 3T3-L1 adipocytes and reported effects on insulin-mediated IRS-1 phosphorylation and associated PI3Kinase activity (4). Lu and colleagues (5) exposed primary rat adipocytes to hyperglycemic conditions and found reduced insulin sensitivity and increased ROS levels *in vitro* under those conditions. Similarly, Talior and colleagues (6) used in an *ex vivo* model of adipocytes isolated from animals with high fat diet-induced diabetes and demonstrated that *in vitro*, these adipocytes displayed significantly elevated ROS levels that could be normalized upon incubation of cells in
low glucose. These adipocytes also displayed increased basal glucose uptake and reduced insulin-mediated glucose uptake.

Here, we extend these observations to an in vivo setting and demonstrate increased oxidative damage in primary adipocytes. We use the 3T3-L1 cell line to further define the close link of nutrient excess, ROS production, insulin resistance and concomitant activation of the inflammatory response in adipocytes.
**Materials and Methods**

**Materials.** Thenoyltrifluoroacetone (TTFA) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were obtained from Sigma (St. Louis, MO); tetrakis (4-benzoic acid) porphyrin (TBAP) was from Calbiochem (San Diego, CA). 5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA) was from Molecular Probes (Eugene, OR). 8-OhdG-EIA Kit was from OXIS (Portland, OR). DMEM (Dulbecco's modified Eagle's Medium) was purchased from Cellgro Inc. Murine TNFα and IL-6 was purchased from Pharmingen. LPS (from *E.coli*) was purchased from SIGMA. [1-3H]-2-deoxyglucose was purchased from Amersham. Recombinant adenovirus vectors pAd5CMVK-NpA expressing UCP-1 or MnSOD were obtained as described in (2). All other chemicals were purchased from Fisher.

**Cell culture.** 3T3-L1 murine fibroblasts (a generous gift of Dr. Charles Rubin, Department of Molecular Pharmacology, Albert Einstein College of Medicine) were propagated and differentiated according to the protocol described in (7) with the exception that media contained either 4 or 25 mM glucose and media changes were performed daily. In brief, the cells were propagated in "FCS" (DMEM containing 10% fetal calf serum (JRH Biosciences) and Penicillin / Streptomycin (100U/ml each)) and allowed to reach confluence (Day -2). After two days (Day 0), the medium was changed to "DM1" (containing "FCS" and 160nM insulin, 250µM dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine). Two days later (Day 2), the medium was switched to "DM2" ("FCS" containing 160nM insulin). After another two days, the cells were switched
backed to "FCS". Cells were used between day 8 and 12 post induction of differentiation, were propagated in 5mM glucose and used only up to passage 12. Up to that passage number, no significant differences were observed with respect to the various read outs reported here.

**Measurement of [1-\(^{3}\text{H}\)]-2-deoxyglucose uptake.** Assay of \(^{\text{3}}\text{H}\)2-deoxy-glucose uptake was performed as described previously (8). Briefly, the cells were glucose starved for 30 minutes. Insulin was then added at the indicated concentrations for 5 minutes, followed by addition of the labeled glucose in the continued presence of the indicated amount of insulin. Glucose transport assays were carried out in a volume of 1 ml (Krebs-Ringer-HEPES buffer, pH 7.4) in a 3.5-cm dish for 25 min at 37°C. The concentration of 2-deoxyglucose was 50 \(\mu\)M with 0.33 \(\mu\)Ci of \(^{\text{3}}\text{H}\)2-deoxyglucose/ml. After terminating the transport assay with three washes of ice-cold assay buffer, each plate was solubilized in 1 ml of 0.1M NaOH and quenched with 50 \(\mu\)l of concentrated HCl. A 0.9-ml aliquot was removed for determination of radioactivity by liquid scintillation counting, and 50 \(\mu\)l aliquots were used for measurement of protein using a BCA assay. Glucose uptake remained linear within the first 30 minutes. Each measurement at the indicated insulin concentrations represents the average of 8 independent measurements, performed on two separate occasions with different cell preparations.

**Measurement of intracellular ROS generation.** Cells were washed with minimal essential medium (lacking phenol red) and then incubated in the dark with the fluorescent probe CM-H\(_2\)DCFDA (10 \(\mu\)M in Krebs–Ringer bicarbonate buffer) for 45 min at 37 °C.
The fluorescence of CM-DCF was analyzed in an HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer) at an excitation wavelength of 485 nm and emission at 530 nm by using the HTSoft program. ROS production was determined from an H$_2$O$_2$ standard curve (10–200 nmol/ml).

**Generation of adenoviruses.** Recombinant adenoviruses were generated as previously described (9,10), using the bacterial recombination method in BJ5183 *E. coli* cells (11). The adenovirus encoding caveolin-1 was a kind gift of Dr. Michael Lisanti, Albert Einstein College of Medicine.

**Adenovirus transduction.** 3T3-L1 adipocytes were infected by adenovirus at an MOI of 500 as described in (12). Adenovirus was incubated with serum-free medium containing 0.5 µg/ml of polylysine for 100 min prior to the addition to PBS-washed 3T3-L1 adipocytes. After 4 h, the medium was replaced with fresh medium and cells were cultured for an additional 2 days.

**Determination of 8-OHdG in DNA.** DNA from mouse adipose tissue was isolated as described in (13). High-purity distilled phenol was used to prevent artificial formation of 8-OHdG. After isopropanol precipitation, the DNA was reconstituted with 20 mM sodium acetate buffer containing 5 mM 2,2′-dipyridyl, which has been shown to inhibit free radical producing reactions. The samples were treated with nuclease P1 to digest DNA to nucleotides and then alkaline phosphatase. 100 µg of DNA was assayed for the levels of 8-OHdG using the 8-OHdG-EIA Kit.

**ELISA for IL-6.** IL-6 production in adipocytes was determined by enzyme linked immunosorbent assay (ELISA) with high-sensitivity ELISA kits from R&D Systems.
(Minneapolis, MN, USA). The measurement was carried out according to the manufacturer's instructions. Each sample was assayed in triplicate.

**Animals.** Male FVB or C56Bl6 mice were bred in house and used as indicated. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in individual cages and subjected to a standard light (6:00 AM to 6:00 PM) - dark (6:00 PM to 6:00 AM) cycle. All rats were fed *ad libitum* using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiological fuel value of 3.3 kcal/g chow. Rats were studied during young adulthood before (~ 300 g body weight, 3 months old, n=12). One week before the *in vivo* studies, rats were anesthetized by inhalation of methoxyflurane, and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery. This method of anesthesia allows fast recovery and normal food consumption after one day. The venous catheter extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Recovery was continued until body weight was within 3% of the pre-operative weight (~ 4-6 days). These chronically catheterized rats were studied after ~ 24 hours of fasting while awake, and unstressed.

**Hyperglycemic clamp studies.** Somatostatin (1.5 \( \mu \text{g/kg/min} \)) was infused to prevent endogenous insulin secretion and 25% glucose was infused intravenously to lean and obese rats to raise their plasma glucose concentration acutely to ~18 mM. Plasma glucose concentration was maintained at that level throughout the three hours of the study using a variable infusion of glucose, periodically adjusted according to plasma glucose levels. At the end of the clamp study, rats were sacrificed using 60mg pentobarbital-sodium/kg...
intravenously. The abdomen was quickly opened, and subcutaneous adipose tissue samples were freeze-clamped *in situ* with aluminum tongs pre-cooled in liquid nitrogen.

The study protocols were reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

**Streptozotocin treatment of mice.** Four week old male C57Bl6 mice were injected with a single i.p. load of STZ at 100mg x kg\(^{-1}\) BW. Blood collection was performed through tail bleeds. Blood glucose levels were measured with a Precision Q-I-D glucose meter (Medisence, Abbott Laboratories Inc.) on a daily basis. Tissues were harvested 7 days after blood glucose levels reached 300-400 mg/dl.

**mRNA Isolation, Northern blot analysis and RT-PCR.** Isolation of mRNA from tissues and tissue culture cells was performed with Trizol (Life Technologies). Agarose gel electrophoresis of mRNA and its transfer to nylon membranes was described in (14). Hybridizations were performed overnight at 42°C in Ultrahybe (Ambion); the 32P-labeled DNA probes were used at concentrations of 2x10^6 cpm/ml. The filters were subsequently washed in 2x SSC/0.1% SDS and 0.1x SSC/0.1% SDS at 50°C before autoradiography. Samples from the TZD study were prepared as follows: total RNA was prepared from cells and tissue using the Ultraspec RNA isolation kit (Biotecx, Houston, TX) then DNAse treated using DNA-free® according to the manufacturer’s protocol (Ambion, Austin, TX). RNA concentrations were quantitated with Ribogreen® by following the supplier’s directions (Molecular Probes, Eugene, OR).

**Antibodies.** Antibodies to the Citrate Carrier were a kind gift of Dr. Ferdinando Palmieri, University of Bari. Antibodies to the amino-terminal domain of Adiponectin and DIC
were previously described (15). Phospho-specific antibodies against IRS-1 and perilipin antibodies are from Affinity BioReagents, Golden, CO., antibodies to the insulin receptor from Upstate, Lake Placid, NY, anti-caveolin and ant-phosphotyrosine antibodies from Transduction Labs/BD Biosciences, San Jose, CA, anti-GSK3β and Akt antibodies from Cell Signaling, Beverly, MA. Anti-GDI and anti-S3/12 antibodies were a kind gift from Dr. Perry Bickel, Washington University, and anti-resistin antibodies were previously described (16).

**Immunoblotting.** After SDS-PAGE, proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell). Nitrocellulose membranes were blocked in PBS or TBS with 0.1% Tween-20 and 5% non-fat dry milk. Primary and secondary antibodies were diluted in PBS or TBS with 0.1% Tween-20 and 1% BSA. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer’s instructions (DuPont-NEN).

**Other Methods.** Separation of proteins by SDS-PAGE, fluorography, and immunoblotting were performed as described previously (17). Automated DNA sequencing was performed by Genewhiz, New York, NY.
Results:

Conventional differentiation protocols cause insulin resistance in 3T3-L1 adipocytes. – The conventional differentiation protocol in use for *in vitro* differentiation of adipocytes calls for 25mM glucose to be present at all stages of differentiation. We wanted to test whether it is possible to differentiate cells at lower, more physiological glucose levels as well. We therefore tried to differentiate the cells in the presence of 4mM glucose. Lowering the glucose levels required a daily change of medium instead of a medium change every 48 hours. Cells differentiated under these conditions accumulated significantly fewer lipids as judged by the reduced level of oil red O staining (Fig. 1A). They did however induce differentiation to the same extent as control cells as judged by the induction of intracellular levels of a host of adipocyte-specific or adipocyte-enriched differentiation markers, such as adiponectin, the β chain of the insulin receptor, caveolin-1, GSK3β, Akt, and the lipid-binding protein S3/12. Interestingly, resistin levels were markedly higher if the cells were differentiated in high glucose (Fig. 1B). A closer examination by electron microscopy reveals an increased number of smaller lipid droplets upon differentiation in 4mM glucose (Fig. 1C, top). Consistent with the reduced size of lipid droplets, perilipin levels were markedly reduced in the cells differentiated at 4mM (Fig. 1C, bottom).

Hyperglycemia causes insulin resistance in 3T3-L1 adipocytes. – To determine whether there are functional differences between the adipocytes obtained under euglycemic or hyperglycemic conditions, we determined the degree of insulin sensitivity in cells

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obtained with the two differentiation protocols. With both protocols, a similar degree of
differentiation was routinely obtained as judged by the induction of several adipocyte-
specific marker proteins as previously demonstrated. A dose response to insulin was
performed, and the degree of insulin-induced phosphorylation of the Insulin receptor,
IRS-1 and Akt/PKB was assessed (Fig. 1D). Insulin-induced phosphorylation of the
insulin receptor and IRS-1 were markedly enhanced in cells differentiated at 4mM as
judged by an anti—phosphotyrosine Western blot of cell extracts. Similarly, Akt-
phosphorylation was significantly enhanced in cells differentiated in 4mM glucose. Total
Akt levels were comparable in all instances, and equal loading was also controlled for by
blotting for the constitutive marker GDI (GDP dissociation inhibitor; (18)). In an
independent experiment, the degree of phosphorylation of the insulin receptor as well as
total levels of insulin receptor were assessed with similar results (Fig. 1E). To further
expand these observations, we assessed the phosphorylation state of specific tyrosine and
serine residues on IRS-1 with a battery of recently generated phosphorylation-state
specific antibodies (Fig. 1F). Phosphorylation on tyrosine 608 was significantly elevated
in cells differentiated in 4mM glucose, even in the basal state, with an improved dose
response to insulin. No significant differences were observed on residue tyr 1172. With
respect to phosphorylation on specific serine residues (events associated with a decrease
in the affinity of IRS-1 with PI3-Kinase), striking differences were observed on residues
serine 307 and serine 636. Serine 307 phosphorylation is significantly increased at 25mM
glucose in the basal state and further stimulated upon insulin treatment. Phosphorylation
on residue serine 636 displays a very striking induction upon insulin treatment. In
summary, differentiation of cells under normal vs. hyperglycemic conditions results in highly significant differences in the phosphorylation state of key tyrosine and serine residues, with overall increases seen on tyrosine residues at 4mM and strong trends towards increased serine phosphorylation at 25mM glucose.

*Adipocytes differentiated in high glucose fail to downregulate basal glucose uptake and have a reduced insulin-mediated glucose uptake* – A hallmark of cells prone to hyperglycemic damage is the inability to downregulate glucose uptake in response to hyperglycemic conditions (1). Similar to endothelial cells, adipocytes fail to show a reduction in basal glucose uptake upon differentiation as judged by a significantly increased [1-^3^H]-2-deoxyglucose uptake in the absence of insulin (*Fig. 1G*). Furthermore, the reduced responsiveness to the insulin-mediated activation of insulin signal transduction cascade translates into a functional read out as well, i.e. insulin-stimulated glucose uptake is significantly reduced in cells differentiated in 25mM glucose (*Fig. 1H*).

*Hyperglycemia-induced transcriptional changes* – In order to gain a global overview of the differences at the transcriptional level between cells differentiated in normoglycemic versus hyperglycemic conditions, we performed a series of microarray studies in the basal, unstimulated state. The most striking differences are summarized in *Table I*. Not surprisingly, a vast number of genes are affected by the differential glucose concentrations. Notably, PKCδ is markedly induced. This was also seen in the progressive insulin resistance induced by FFA in liver which was associated with a
increase in hepatic PKCδ (19). Similar observations have been reported in the vascular endothelium (1). Among the many other changes, there is a trend towards increased transcriptional activities of mitochondrial genes. We have confirmed and expanded these observations by Northern blot analysis of a subset of these genes. We can detect a significant induction of UCP2 and the mitochondrial matrix chaperone Hsp60, while other mitochondrial genes, such as the ADP/ATP translocator and the dicarboxylate transporter DIC are not affected (Fig. 2). Message levels of the adipocyte-specific secretory protein adiponectin/Acrp30 are unaffected. Levels of Foxo1, which is a negative regulator of adipogenesis, are significantly upregulated in the hyperglycemic state. Insulin suppresses Foxo1 activity, and it has been suggested that sustained activation of Foxo1 due to insulin resistance may inhibit the formation of newly differentiated, lean adipocytes (20).

Hyperglycemia results in increased levels of reactive oxygen species (ROS) in vitro and in vivo, leading to an increased inflammatory response – Previous observations in endothelial cells and a number of other cell types have shown that hyperglycemia can trigger increased levels of reactive oxygen species. Very little is known about this phenomenon in mature adipocytes. We therefore tested whether differences in glucose levels can trigger differences in ROS levels within cells. Exposure to 30mM glucose indeed triggered a four-fold increase in ROS levels. This increase could be prevented with inhibitors of oxidative phosphorylation, such as the complex II inhibitor TTFA or complex III inhibitor TBAP. In addition, the mitochondrial uncoupler CCCP also
effectively prevented the build-up of ROS levels in 3T3-L1 adipocytes (Fig. 3A). Adenoviral gene delivery of superoxide dismutase (Mn-SOD) effectively lowered ROS levels. Similarly, overexpression of the mitochondrial uncoupling protein 1 (UCP1) also prevented ROS build-up compared to cells infected with a control virus (Fig. 3B). These observations are true whether cells are differentiated under hyperglycemic conditions or under euglycemic conditions and then shifted to hyperglycemic conditions after completion of differentiation. The increases to ROS are also not limited to tissue culture conditions, but can also be observed in vivo. Streptozotocin-treated mice offer a convenient model to assess the effects of hyperglycemia in a relatively acute setting. Mice were injected with streptozotocin, which promptly induced hyperglycemia at levels >400mg/dl. Seven days post conversion to hyperglycemia, adipose tissue was harvested. As an indicator of ROS-induced damage, we measured the levels of 8-OH deoxyguanidine (8-OHdG) normalized to total DNA levels. As shown in Fig. 3C, streptozotocin-treatment resulted in a significant increase in 8-OHdG, suggesting that ROS build-up is a phenomenon also occurring in vivo. Exposure to hyperglycemia can result in the induction of pro-inflammatory markers, such as IL-6. To test whether hyperglycemic conditions have an effect on 3T3-L1 adipocytes as well, we compared IL-6 production in adipocytes obtained with the two differentiation protocols. Cells differentiated at 25mM indeed produced higher levels of IL-6 (Fig. 3D). To test whether ROS play a critical role in the translation of excess nutrients to an increased inflammatory response, we measured IL-6 secretion in 3T3-L1 adipocytes differentiated under hyperglycemic conditions. IL-6 levels were measured either upon exposure to a
control adenovirus or upon adenoviral-mediated overexpression of UCP-1. A reduction of the ROS levels by overexpression of UCP1 indeed results in a reduction of IL-6 levels in the supernatant of these cells, suggesting that increased ROS levels are mediating, at least in part, the hyperglycemia-induced increase in the pro-inflammatory response in adipocytes (Fig. 3E).

Mitochondrial hyperpolarization per se is sufficient to trigger insulin resistance – The experiments above have shown that elevated glucose levels trigger insulin resistance. They also show that elevated glucose levels trigger increased ROS levels. We wanted to test whether mitochondrial hyperpolarization per se – even in the absence of elevated glucose levels, is sufficient to induce insulin resistance. We have previously identified and cloned the murine mitochondrial dicarboxylate transporter DIC and demonstrated that overexpression of this transporter (which is normally expressed predominantly in white adipose tissue and to a lesser extent in liver and kidney) leads to hyperpolarization of mitochondria (15). Here, we have used adenoviral-mediated gene transfer to further overexpress this transporter in adipocytes and assess the effects on ROS production and insulin sensitivity. Overexpression of DIC leads to a marked increase in ROS levels (Fig. 4A). Interestingly, this increase in ROS levels is primarily driven by overproduction of DIC and not by elevated glucose levels, since ROS levels are increased to the same level at both 5mM and 25mM glucose in the presence of excess DIC. This cannot be observed upon infection with an “empty” adenovirus or infection with adenoviruses expressing β-galactosidase (lacZ) or caveolin-1. This offers a unique opportunity to distinguish unrelated effects of high vs. low glucose from effects directly related to the

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mitochondrial membrane potential without the toxic effects observed upon prolonged treatment with uncoupling agents. Increasing the mitochondrial membrane potential and the concomitant increase in ROS in the presence of 5mM glucose causes a reduction in insulin sensitivity as judged by reduced phosphorylation state of the insulin receptor (Fig. 4B), IRS-1 (Fig. 4C) and Akt (Fig. 4D). This is the first demonstration that links mitochondrial hyperpolarization directly to decreased sensitivity to insulin action.

In order to determine whether the DIC-induced effects are specific for this transporter, or whether similar effects can be obtained by overexpression of other mitochondrial transporters, we used adenoviral expression constructs for the oxoglutarate carrier, the citrate carrier as well as the ADP/ATP translocator. None of these transporters had an effect comparable to DIC with respect to mitochondrial ROS production (Fig. 4E), even though several-fold overexpression was effectively achieved, as demonstrated by western blot analysis of citrate transporter and DIC overexpressing cells (Fig. 4F).

*The hyperglycemia-induced insulin resistance not readily reversible* - In order to assess whether the increased levels of ROS accumulated in cells differentiated under hyperglycemic conditions impose a longer term imprinting effect on the cells, 3T3-L1 adipocytes were differentiated in 25mM glucose. Upon full differentiation (8 days), cells were either infected with a control virus or an adenovirus expressing UCP1 or SOD. 48 hours later, cells were treated for 10 minutes with various concentrations of insulin and total levels of Akt and phospho-Akt were measured. As shown in Fig. 4G, insulin-mediated activation of Akt was not affected within 48 hours of lowering ROS levels. This suggests that the differentiation of cells under hyperglycemic conditions results in
permanent changes at the DNA or protein level that are not readily reversible over a period of two days.

*Hyperglycemia induces a pro-inflammatory response that is attenuated by quenching of ROS.* – We wanted to determine whether these results obtained in a tissue culture system are relevant *in vivo*. As previously shown, hyperglycemic conditions lead to the induction of a host of acute phase reactants in adipocytes. Here, we extend the analysis to pentraxin 3 (ptx-3), a close relative of C-reactive protein (CRP) and 24p3 and PAI-1 (Fig. 5A and B). In an independent clamp study (Study B), we tested whether the hyperglycemia-induced upregulation of acute phase reactants in white adipose tissue can be prevented by lowering ROS levels, we co-infused the anti-oxidant N-Acetyl-cysteine (NAc) during the hyperglycemic clamp and probed for the induction of PAI-1. Co-infusion of NAc during these hyperglycemic clamp studies prevented the induction of PAI-1 completely (Fig. 5C), indicating that ROS production is responsible for the induction of this pro-inflammatory transcriptional program in adipose tissue *in vivo* as well.
Discussion

We find that changing the glucose levels for 3T3-L1 adipocytes from the conventional 25mM glucose (corresponding to physiologically severe hyperglycemic conditions) to the more physiologically relevant 4mM does not affect the extent of differentiation of the adipocytes. Markers, such as adiponectin, the insulin receptor as well as downstream mediators of the insulin signal transduction cascade are induced to the same extent. 3T3-L1 adipocytes differentiated under normoglycemic conditions display a significant improvement with respect to insulin-mediated signaling events as well as insulin-mediated glucose uptake. In particular, we have focused on a battery of phosphorylation state-specific antibodies against IRS-1 and demonstrated a significant increase in insulin-induced serine phosphorylation on residues 307 and 636 under hyperglycemic conditions, both of which have been implicated in a reduced insulin-sensitive state (21,22). Tyrosine phosphorylation on residue 608 that plays an important inducing role for the interactions of IRS-1 with PI3-K and PTP2C (23) was significantly enhanced under normoglycemic conditions.

In light of the well-established effects of hyperglycemia on the production of reactive oxygen species in endothelial cells, we embarked on a series of experiments that demonstrate the close link of nutrient excess in the form of hyperglycemia, and the concomitant increase in ROS in adipocytes. All of the basic principles established in the endothelium (2) hold up in adipocytes as well. Importantly, we demonstrate for the first time increases in ROS levels in freshly isolated adipocytes from hyperglycemic animals. In additional in vivo experiments during which we subject animals to euinsulinemic
hyperglycemic clamp studies, we demonstrate the induction of a strong pro-inflammatory
program in adipose tissue that include the lipocalin 24p3 (24,25) and the acute phase
reactant pentraxin-3 (26), a close homolog to C-reactive protein, as well as PAI-1
(27,28). With respect to several recent reports highlighting the contributions of adipose
tissue borne macrophages (29-31), we do not know whether these proteins primarily
originate from adipocytes or from macrophages within the fat pad that induce these
proteins in response to adipocyte-derived factors. We had previously demonstrated
induction of the acute phase reactant SAA3 under these conditions, and show that
production of SAA3 was exclusively at the level of the adipocytes (32). However, while
the relative contribution of each individual cell types is difficult to gauge, mounting
evidence suggests that there is a high degree of paracrine interchange between adipocytes
and adipose tissue macrophages, with both cell types required for the full inflammatory
response (33). Independent of the origin of these factors within adipose tissue,
simultaneous treatment during the hyperglycemic clamp with N-acetylcysteine (NAC)
that efficiently quenches ROS (34) abolishes the induction of acute phase reactants,
consistent with an important role of ROS in adipose tissue in vivo under those conditions
as well.

In tissue culture, the increased ROS levels can efficiently be lowered by
uncoupling mitochondria, inhibiting the electron transport chain or by simply scavenging
the ROS by overexpression of SOD (2). While we establish a firm connection between
hyperglycemia and increased ROS levels, there may be other hyperglycemia-induced
effects that lead to decreased insulin responsiveness. We therefore sought an
experimental approach that would allow us to increase ROS production intracellularly at the level of mitochondria under normoglycemic conditions. We had previously identified and cloned the gene for the mitochondrial dicarboxylate transporter mDIC (15). In our original report we noted that overexpression of this transporter in a heterologous cell line resulted in a significant degree of hyperpolarization of the mitochondrial membrane. The underlying mechanism for this phenomenon has not been explored in great detail. However, it is likely that mDIC which promotes increased uptake of succinate by the mitochondria, feeds directly into complex-2 of the electron transport chain via succinate dehydrogenase, thereby bypassing the TCA cycle, unlike the bulk of the other substrates that enter via other carriers that critically depend on the TCA cycle. We took advantage of this observation and further overexpressed the transporter in 3T3-L1 adipocytes and demonstrated a significantly increased formation of ROS, even under normoglycemic conditions. Even at 4mM glucose, these cells display significantly reduced insulin sensitivity, thereby functionally linking mitochondrial ROS production to insulin resistance. Interestingly, mDIC is already expressed at very high levels in adipocytes (more than in any other cell type) (15), yet it still represents a rate limiting factor for the mitochondrial respiratory chain. Importantly, this feature is characteristic of mDIC, since overexpression of a number of other, closely related carriers failed to have an effect on ROS levels. Future experiments will have to address whether lowering levels of DIC in adipocytes may have beneficial effects, both with respect to its role as a rate limiting step for ROS production as well as an integral part of the biochemical cascade that leads to
glyceroneogenesis, thereby locally supplying the backbone for fatty acid esterification into triglycerides (35).

We and others have used 3T3-L1 cells extensively for the characterization of inflammatory read outs and repeatedly demonstrated the highly potent pro-inflammatory potential of these cells (18,36,37). We were very surprised to see that upon altering the differentiation conditions to euglycemic levels, the basal inflammatory state (as judged by IL-6 production) are dramatically reduced in these cells despite the fact that these adipocytes have fully completed their differentiation program. This further underscores the importance of the differentiation conditions and highlights the expanded potential of 3T3-L1 cell line that can be used as a model cell for a healthy, insulin sensitive adipocyte with a smaller lipid load upon differentiation under euglycemic conditions as well as a model for an insulin-resistant, lipid – laden adipocyte upon differentiation under hyperglycemic conditions. While the effects on ROS levels and inflammation are relatively readily reversible, impairments in the insulin signal transduction cascade are not as judged by the observation that overexpression of UCP1 or SOD in cells differentiated at 25mM does not enhance insulin-mediated activation of Akt. This hints at an interesting long term effect that may be caused by posttranslational modification of some of the key signal transduction components. Future studies will have to show which proteins are most prone to these modifications.

Tu and Weissman (38) have recently reviewed the evidence that suggests that the secretory pathway may represent a significant source of cellular ROS production (estimated to be up to 25% of total cellular ROS produced). Molecular oxygen is used as
a terminal acceptor for electrons generated during oxidation of disulfide bonds. The rather small cytoplasmic volume of adipocytes that is not taken up by lipid droplets contains a very active secretory pathway that could significantly contribute to increased oxidative stress. Adipocytes are very long-lived cells. In fact, there is little evidence to suggest at this stage that adipocytes turn over under normal physiological conditions. ROS-induced damage, integrated over the very long life span of an adipocyte, could therefore represent a major contributor towards the pathological changes observed in hypermetabolic states.
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References


Figure Legends

Figure 1: 3T3-L1 can fully differentiate into mature adipocytes in medium with a more physiologic 4 mM concentration of glucose and are more insulin sensitive. (A) 3T3-L1 adipocytes differentiated in 4 mM or 25 mM glucose medium were stained with Oil Red O to identify lipid droplet formation (magnification, x40). (B) Western blot analysis of adiponectin, insulin receptor β chain, caveolin-1, GDI, GSK3β, Akt, resistin and S3/12 expression in 3T3-L1 adipocytes differentiated in 4 mM or 25 mM glucose medium. (C) Electron micrographs of 3T3-L1 adipocytes differentiated in 4 mM or 25 mM glucose medium to assess the size of lipid droplets. Bar indicates 2 nanometers. (D) Effects of high vs. low glucose on insulin-induced phosphorylation events in 3T3-L1 adipocytes. 3T3-L1 adipocytes differentiated in 4 mM or 25 mM glucose medium were stimulated with various concentrations of insulin as indicated for 10 minutes. Proteins isolated from the cells were subjected to SDS-PAGE and then Western blot analysis. Antibodies against phosphotyrosine and phosphoAkt were used to assess the insulin response, while pan Akt and GDI antibodies were used to normalize for loading. (E) Effects of high glucose on insulin-induced phosphorylation of IR. Anti-phosphotyrosine antibody was used for the western blot analysis of cell extracts prepared under similar conditions as in (D). Immunoblot with anti-IR and GDI antibodies served as control for equal loading amount. The experiments in (D) and (E) were performed at least 4 times independently. Representative blots are shown. (F) Western blot analysis was carried out by using phosphospecific antibodies against Tyr608, Tyr608, Ser636 or Ser307 residues in IRS-1 after 10 minutes of insulin treatment at the indicated concentrations. The total amount
of IRS-1 was detected by probing the membranes with anti-IRS-1 antibody. (G) Basal glucose uptake in the absence of insulin is increased (n=8); *Statistically significant (p≤0.001) differences between 4mM and 25mM differentiated cells. (H) Insulin-stimulated glucose uptake in the presence of the indicated amount of insulin. Glucose uptake in the absence of insulin for the same amount of time has been set as 100%. (n=8); *Statistically significant (p≤0.01) differences between 4mM and 25mM differentiated cells.

**Figure 2: High glucose induces gene expression changes in 3T3-L1 adipocytes.** Fragments comprising the open reading frame of mouse uncoupling protein-2 (UCP2), dicarboxylate carrier (DIC), adiponectin, Forkhead transcription factor FKHR (Foxo1), heat shock protein 60 (Hsp60), ADP/ATP translocator and β-actin (as an equal loading control) were used as probes for Northern blot analysis of RNA isolated from 3T3-L1 adipocytes differentiated in 4 mM or 25 mM glucose medium.

**Figure 3: Effects of high glucose on intracellular ROS generation in adipocytes.** (A) 3T3-L1 adipocytes differentiated in 25 mM glucose medium were incubated in 5 mM, 30 mM glucose alone or 30 mM glucose plus either 10 mM tenoyltrifluoroacetone (TTFA), 0.1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP), or 100 M tetrakis (4-benzoic acid) porphyrin (TBAP) for 18 h. Intracellular ROS was determined as described in “Materials and methods.” (Data are means ± SD, n = 24). * indicates significant differences over 30mM glucose treatment alone (p≤0.001). (B) 3T3-L1 adipocytes
differentiated in 4 mM glucose medium were infected with either control, UCP1- or MnSOD-expressing recombinant adenovirus. 48 h post-transduction, the cells were incubated in 4 mM or 25 mM glucose medium for 18 h and then assessed for intracellular ROS production. (Data are means ± SD, n = 12). * indicates significant differences over ctrl infection (p≤0.001). (C) Hyperglycemia induced by streptozotocin treatment resulted in increased levels of 8-OHdG in mouse adipose tissue due to oxidative damage to DNA. (Data are means ± SD, n = 9). * Statistically significant (p≤0.01) differences between treatment group and controls. (D) Exposure to hyperglycemic conditions results in increased IL-6 secretion. 3T3-L1 adipocytes were differentiated at either 4mM or 25mM glucose. Day 8 adipocytes were allowed to secrete proteins for 24 hours into serum free medium and IL-6 levels were determined. n=6; * indicates significant differences between 4mM and 25mM (p≤0.001). (E) Dissipation of ROS results in decreased IL-6 secretion. 3T3-L1 adipocytes differentiated at 25 mM glucose were infected with either control virus or UCP1-expressing recombinant adenovirus for 24 hours. UCP1 expression results in a decreased of IL-6 secretion. n=6; * indicates significant differences over ctrl infection (p≤0.01).

Figure 4: Effect of mitochondrial hyperpolarization on insulin sensitivities in 3T3-L1 adipocytes. 3T3-L1 adipocytes differentiated in 4 mM glucose medium were infected with either control adenovirus, or recombinant adenovirus expressing various genes as indicated. Analysis was done at 48 h after virus infection. (A and E) Cells were incubated in 4 mM or 25 mM glucose medium for another 18 h and then assessed for intracellular...
ROS production. (Data are means ± SD, n = 12). (B, C and D) Cells were stimulated with various concentrations of insulin as indicated for 10 minutes. Proteins isolated from the cells were separated by SDS-PAGE, and identified by Western blotting with anti-phosphotyrosine (panels B and C) or anti- phosphorylated Akt (Ser 308) (panel D) antibodies. The quantified data from multiple experiments for the insulin receptor (panel A), IRS-1 (panel C) and Akt (panel D) are shown, normalized for the total levels of the respective proteins. Data were expressed as percent of value of 100 nM insulin-stimulated control cell samples (*P < 0.05). (F) Overexpression of mitochondrial proteins mediated by adenovirus transduction was confirmed by Western blot analysis. Anti-dicarboxylate transporter and anti-citrate carrier antibodies were used for the immunoblots. (G) Dissipation of ROS levels does not immediately translate into improvements in insulin-stimulated Akt phosphorylation. Cells were differentiated in 25mM glucose. On day 8 of differentiation, they were infected with control virus or adenovirus preparations either expressing UCP1 or SOD. Cells were stimulated for 10 min with insulin.

**Figure 5: High glucose upregulates the pro-inflammatory responses in adipocytes.**

(A, B) Hyperglycemia induces acute phase reactants in adipose tissue. Hyperglycemic / euinsulinemic clamp studies were performed as indicated in “Materials and Methods” with glucose levels that were kept around 300 mg/dl, whereas the insulin levels were kept at basal levels. At the end of the 3 h study, total RNA from different adipose depots was isolated and analyzed by Northern blot analysis with lipocalin 24p3, long pentraxin Ptx3,
PAI-1 and β-actin (for normalization) probes. Acute hyperglycemia induces 24p3, Ptx3 and PAI-1 mRNA compared with saline infused animals. (C) Hyperglycemia-induced upregulation of acute phase reactant mRNA expression can be prevented by free-radical scavenger. Rats were infused with saline, glucose to achieve a concentration of 15 mM (HG), or glucose plus antioxidant N-acetylcysteine (NAC) for 6 hours as previously described (39), after which total RNA from different adipose depots was isolated and analyzed by Northern blot analysis and quantitative PCR for the levels of PAI-1 and β-actin. This analysis was performed independently on four separate rats, and data for all of the rats are shown. The quantification is based on RT-PCR results (*, # indicates significant increase over ctrl or hyperglycemic sample, p<0.05)
### Lin et al., Table I

<table>
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Lin et al., Fig. 1

A  Oil-red O staining

4mM Glucose  25mM Glucose

B

<table>
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<tr>
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Lin et al., Fig. 1

Differentiated in 4mM 25mM Glucose
Lin et al., Fig. 1

D

- 0.1 1 5 20

- 0.1 1 5 20 nM insulin

phospho-IRS1
phospho-IR
phospho-Akt
Akt
GDI

4mM glucose  25mM glucose

E

- 0.1 1 5 20

- 0.1 1 5 20 nM insulin

pIR
IR
GDI
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<th>TNFα</th>
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</tr>
<tr>
<td>25mM</td>
<td>0 0.1 1 10 100</td>
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</table>

- **4mM Glucose**
  - αP-Tyr 608 IRS-1
  - αP-Tyr 1172 IRS-1
  - αP-Ser 636 IRS-1
  - αP-Ser 307 IRS-1
  - αRS-1

- **25mM Glucose**
  - αP-Tyr 608 IRS-1
  - αP-Tyr 1172 IRS-1
  - αP-Ser 636 IRS-1
  - αP-Ser 307 IRS-1
  - αRS-1
Lin et al., Fig. 2

4mM 25mM Glucose

UCP2

DIC

Adiponectin

Foxo1

Hsp60

β Actin
ADP/ATP Translocator
Lin et al., Fig. 3

A

ROS (nmol/ml)

5mM glucose
30mM glucose
30mM glucose + TTFA
30mM glucose + CCCP
30mM glucose + TBAP

B

ROS (nmol/ml)

Ctrl Infection
Mn-SOD
UCP1

4mM Glucose
25mM Glucose

C

8-OHdG (ng/ml/µg DNA)

Ctrl
STZ

Adipose Tissue
Lin et al., Fig. 3

D

![Graph showing IL-6 concentration in response to glucose levels.]

E

![Graph showing relative IL-6 concentration in response to viral infection and UCP1 expression.]
Lin et al., Fig. 4

A

![Graph showing ROS levels with different conditions and glucose concentrations.](http://www.jbc.org)

- **X-axis**: Ctrl, Infection, DIC, LacZ, Cav-1
- **Y-axis**: ROS (nmol/ml)

- **Legend**:
  - 4mM Glucose
  - 25mM Glucose

* indicates a significant difference compared to other conditions.
Lin et al., Fig. 4

**D**

Phospho-Ser in AKT (arbitrary units) vs. Insulin (nM), 10min

- **CTRL**
- **DIC**

**E**

ROS Production (% of basal) vs. Treatment

- 5mM Glucose
- 25mM Glucose

- No Infection
- Oxoglutarate Carrier
- Citrate Carrier
- DIC
- ADP/ATP Translocator
Lin et al., Fig. 5

C

Epididymal Saline High Glucose High Glucose + NAC

PAI-1

β-Actin

PAI-1/β-actin Ratio in Epididymal WAT

Saline High Glucose High Glucose + NAC

* #
The hyperglycemia-induced inflammatory response in adipocytes: The role of reactive oxygen species

Ying Lin, Anders H. Berg, Puneeth Iyengar, Tony K.T. Lam, Adria Giacca, Terry P. Combs, Michael W. Rajala, Xueliang Du, Brent Rollman, Weijie Li, Meredith Hawkins, Nir Barzilai, Christopher J. Rhodes, I. George Fantus, Michael Brownlee and Philipp E. Scherer

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