Tissue-specific differences in the development of insulin resistance in a mouse model for type 1 diabetes

Running title:
Insulin resistance in type 1 diabetes

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Abstract

While insulin resistance is known to underlie type 2 diabetes, its role in the development of type 1 diabetes is gaining increasing interest more recently. In a model of type 1 diabetes, the non-obese diabetic (NOD) mouse, we found that insulin resistance driven by lipid- and glucose-independent mechanisms is already present in liver of pre-diabetic mice. Hepatic insulin resistance is associated with a transient rise in mitochondrial respiration, followed by increased production of lipid peroxides and c-jun N-terminal kinase (JNK) activity. At the onset of diabetes, increased adipose tissue lipolysis promotes myocellular diacylglycerol accumulation. This is paralleled by increased myocellular protein kinase C theta (PKCθ) activity and serum fetuin A levels. Muscle mitochondrial oxidative capacity is unchanged at the onset, but decreases at later stages of diabetes. In conclusion, hepatic and muscle insulin resistance manifest at different stages and involve distinct cellular mechanisms during the development of diabetes in the NOD mouse.
Insulin resistance is not only a feature of obesity and type 2 diabetes mellitus (T2DM), but can also be present in patients with type 1 diabetes mellitus (T1DM) (1; 2), resulting in a condition termed ‘double diabetes’. T1DM is characterized by absolute insulin deficiency due to autoimmune-mediated β-cell destruction (3), leading to uncontrolled glucose homeostasis. Although insulin resistance in T1DM may occur independently, the accelerator hypothesis postulates that it can be triggered by autoimmune diabetes itself (4). The causes and mechanisms linking T1DM and insulin resistance remain still unknown.

Prolonged hyperglycemia may induce insulin resistance by mechanisms summarized as glucotoxicity (5). On the other hand, insulin deficiency also decreases the suppression of lipolysis in adipose tissue and liver, thereby raising circulating triglycerides (TG) and fatty acids (FA). This can in turn impair insulin-stimulated glucose transport by various mechanisms summarized as lipotoxicity (6; 7). Both the diacylglycerol (DAG)/protein kinase C isoforms (nPKC) pathway (6) as well as toll-like receptor 4 (TLR4)/fetuin A-mediated ceramide synthesis (8) have been shown to inhibit insulin signaling.

Furthermore, glucotoxicity and lipotoxicity strongly associate with lower mitochondrial function. In muscle, lower mitochondrial activity frequently associates with insulin resistance in humans with or at risk of T2DM (9; 10). Insulin deprivation can also reduce muscle mitochondrial ATP production by altered expression of mitochondrial genes (11), and insulin resistant patients with long-lasting T1DM may have lower flux to ATP synthase (1). Recent studies show that reduced hepatic energy metabolism also correlates with insulin resistance, hyperglycemia and altered hepatic lipid content in T2DM (12; 13). However, others questioned the role of mitochondrial function for insulin resistance (14). The contribution of mitochondrial function to insulin resistance has not yet been clarified in T1DM.

We hypothesized that the onset of T1DM is associated with impaired insulin sensitivity and lower mitochondrial function. While glucolipotoxicity may cause insulin resistance in the liver, muscle insulin resistance may develop independent of ambient glycemia. To test this
hypothesis, we examined diabetes-related effects in female non-obese diabetic (NOD) mice independently of other confounding factors such as gender, obesity, ageing and inherited mitochondrial abnormalities.
Research Design and Methods

Animals

NOD and wild-type C57BL6 (WT) mice were maintained under specific pathogen-free conditions on a 12-h light-dark cycle and received a standard rodent diet (Ssniff M-Z Extrudat, 4.5% fat; SSNIFF Spezialdiäten GmbH, Soest, Germany) and water ad libitum. We studied only female mice, because 80% of female but only 30% of male NOD develop diabetes within 30 weeks of age (15). Experiments were performed three days (acute diabetic NOD, A-DM) or 8 weeks after onset of diabetes (insulin-treated chronic diabetic NOD, C-DM) in randomly assigned mice. Non-diabetic NOD (N-DM) mice were matched for age and body weight. Mice were studied in the fed state and after 6 hours of fasting. Insulin-treated C-DM were only examined in the fed state to avoid hypoglycemia. After decapitation, trunk blood and tissues were collected, weighed and used for high-resolution respirometry or snap-frozen in liquid nitrogen. All experiments were performed according to the guidelines for care and use of animals (GV-SOLAS, Society for Laboratory Animal Science) and approved by the local council of animal care in line with the requirements of the German animal protection act.

Detection and treatment of hyperglycemic NOD mice

Diabetes was detected by testing for glycosuria (Diabur-test 5000, Roche Diagnostics, Mannheim, Germany) and confirmed by tail blood glucose measurements >250 mg/dl on two consecutive days (Precision Xtra Plus, Abbott, Wiesbaden, Germany). Insulin was administered subcutaneously in LinBit pellets (LinShin Canada, Toronto, Canada) for 8 weeks aiming to maintain blood glucose between 250 and 500 mg/dl. At glucose >500 mg/dl for more than one week, an additional insulin pellet was administered.
Indirect calorimetry

Mice were individually housed and placed in an 8-chamber indirect calorimetry system (PhenoMaster; TSE Systems, Bad Homburg, Germany). After 24 hours of acclimatization, energy expenditure, respiratory quotient (RQ), physical activity, food and water intake were simultaneously analyzed for 48 hours.

Hyperinsulinemic-euglycemic clamp test

A silicon catheter (Silastic laboratory tubing, Dow Corning, Midland, MI) was placed into the right jugular vein under Isofluran (CP Pharma, Burgdorf, Germany) anesthesia. Mice were allowed to recover for 4-5 days and fasted for 6 h on the day of the experiment (03.00–09.00a.m.). To assess basal whole-body glucose disposal, D-[6,6-²H₂]glucose (98% enriched; Cambridge Isotope Laboratories, Andover, MA) was infused at a rate of 4 µmol/kg/min for 120 min. The hyperinsulinemic-euglycemic clamp was performed with a primed (40 mU/kg)-continuous infusion (4 mU/kg/min; Huminsulin, Lilly, Giessen, Germany) for 180 min. Euglycemia was maintained by periodically adjusting a variable 20% glucose infusion. D-[6,6-²H₂]glucose was co-infused together with insulin solution (0.4 µmol/kg/min) and variable glucose infusion to obtain stable tracer concentrations during varying glucose infusion rates. Blood samples were taken at 10-min intervals during the last 30 min of basal, and hyperinsulinemic-euglycemic clamps. Additional clamps were performed under identical conditions except for measuring FA levels at time points -15, 0, 5, 15, 30, 60, 90, 120 and 150 min to assess FA suppression and injecting 10 µCi of 2-deoxy-D-[¹⁴C]glucose at the end of the clamp to calculate rates of insulin-stimulated glucose uptake by gastrocnemius and soleus muscles (16). After the clamps, mice were exsanguinated through cervical incision, sacrificed by cervical dislocation and serum and organs collected for analyses.

High-resolution respirometry
Ex vivo mitochondrial function was measured in fresh liver and gastrocnemius muscle using the Oxygraph-2k (Oroboros Instruments, Austria) as described (17). Defined respiratory states were obtained by the following protocol: 2 mM malate, 10 mM pyruvate, 10 mM glutamate and 2.5 mM ADP (state 3, complex I), 10 mM succinate (state 3, complex I+II), 10 µM cytochrome c (mitochondrial membrane integrity check), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (stepwise increments of 0.25 µM up to the final concentration of max. 1.25 µM, state u) and 2.5 µM antimycin A. Addition of cytochrome c did not increase oxygen consumption indicating integrity of the outer mitochondrial membrane after saponin permeabilization.

Mitochondrial density

Tissue DNA was extracted with Qiagen Tissue and Blood Kit (Qiagen, Hilden; Germany) and concentration measured spectrophotometrically (GeneQuant, GE Healthcare, Munich, Germany). Mitochondrial DNA (mtDNA) copy number was quantified with RT-PCR (ABI Prism 7000, Applied Biosystems, Darmstadt, Germany) using specific primers and 5′-Fam–3′Tamra labeled probes (Eurogentec, Liège, Belgium) for ND1 (forward primer: 5′-CTA-CA-DGCC-TTT-GCA-GAC-GC-3′, reverse primer 5′- GGA-ACT-CAT-AGA-CTT-AAT-GC -3′, probe 5′- CCA-ATA-CGC-CCT-TTA-ACA-ACC-TC -3′), and for LPL (forward primer : 5′-GGT-TTG-GAT-CCA-GCT-GG-GG-CC. -3′, reverse primer 5′- GAT-TCC-AAT-ACT-TCG-ACC-AGG -3′, probe 5′- CTT-TGA-GTA-TGC-AGA-AGC-CC -3′). NDI and LPL DNA copy numbers were determined by comparison with log–linear standard curves. The ratio of mtDNA to nuclear DNA is a measure of tissue concentration of mtDNA per cell.

Laboratory analyses

Serum insulin (Mouse Insulin Elisa kit Mercodia, Uppsala, Sweden) and fetuin A (Mouse Fetuin A/AHSG DuoSet, R&D Systems, Abingdon, UK) were measured by ELISA. D-[6,6-


\[ \text{H}_2\text{glucose enrichment in deproteinized plasma was quantified with GC-MS (Agilent Technologies, Waldbronn, Germany) after derivatization of glucose to pentaacetate. Serum TG, cholesterol (Roche/Hitachi, Roche Diagnostics, Mannheim, Germany), FA and } \beta\text{-hydroxybutyrate (Wako Chemicals GmbH, Neuss, Germany) were assessed photometrically.}

\textbf{Assessment of lipid peroxidation}

Tissue concentrations of thiobarbituric acid-reactive substances (TBARS) were measured fluorometrically (BioTek, Bad Friedrichshall, Germany) in 10 mg of frozen gastrocnemius muscle and liver (18). Protein content in homogenates was assessed with the bicinchoninic acid assay (BCA protein kit, Thermo Fisher Scientific, Bonn, Germany).

\textbf{Insulin signaling \textit{ex vivo}}

Mice were intraperitoneally injected with insulin (1 U/kg body weight) or saline (control), euthanized after 10 min, and, liver and muscle tissues were snap-frozen in liquid nitrogen.

\textbf{RNA and protein analyses}

Total RNA extraction, cDNA synthesis and real time qRT-PCR expression analyses were performed as described (19). Data of gene specific probes (Assay on Demand™, Applied Biosystems, Darmstadt, Germany) were normalized to 18S RNA content (19). Proteins were extracted by homogenization and centrifugation (21,000 g, 15 min; 4°C) and measured by the BCA assay (Thermo Fisher Scientific, Bonn, Germany). Membrane and cytosol fractions were prepared employing differential centrifugation (20).

Ten micrograms of protein were loaded onto SDS-Page gels, and transferred to polyvinylidene difluoride membranes (Merck Millipore, Schwalbach, Germany). Membranes were blocked with 5% milk in phosphate buffered saline and incubated with antibodies recognizing total Akt isoforms 1 and 2, extracellular-signal regulated kinase phosphorylated
at Thr202 and Tyr204 (pERK-Thr202/Tyr204), e-Jun N-terminal kinase phosphorylated at Thr183 and Tyr186 (pJNK-Thr183/Tyr185), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells phosphorylated at Ser536 (pNFkB-Ser536), p38 mitogen-activated protein kinase phosphorylated at Thr180 and Tyr182 (p-p38-Thr180/Tyr182), pIRS1-Ser1101, pIRS1-Ser307 (Cell Signalling Technology, Danvers, MA, USA), GLUT4 (Abcam, Cambridge, UK), PKCε, PKCθ (BD Biosciences, Franklin Lakes, NJ, USA) and the insulin receptor β-subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horse radish peroxidase-conjugated secondary antibodies (Promega GmbH, Mannheim, Germany) and enhanced chemiluminescence (Merck Millipore, Schwalbach, Germany) were used for detection. Immunoblots were quantified using Versadoc 4000 MP (Biorad) and Quantity One software (Biorad, version 4.6.9). Stripped membranes were reprobed with α-tubulin (Calbiochem, Darmstadt, Germany) and GAPDH (ABCAM, Cambridge, UK) antibodies to allow for loading corrections.

Quantification of lipids

Oil red O (ORO) staining was performed in fresh right liver lobe fixed in 4% paraformaldehyde (21). Stained sections were examined using light microscopy at 40fold magnification (Leica DMRBE, Wetzlar, Germany). Liver and muscle samples were solubilized in ethanolic KOH and glycerolipids measured as described (22) using a TG standard (Lyonorm Calibrator, Pliva-Lachema Diagnostika, Brno, Czech Republic).

Myocellular lipid metabolites

Membrane and cytosol fractions were extracted by homogenization and centrifugation (52) and DAGs and ceramides measured by LC-MS (23).

Immunohistochemistry
Pancreas tissue was embedded in paraffin, stained by hematoxylin/eosin and examined with light microscopy (24). For β-cell and α-cell detection, double immunofluorescence staining was performed using anti-insulin and anti-glucagon antibodies (DAKO, Hamburg, Germany), respectively. Images were acquired using inverted microscope (Leica, Wetzlar, Germany) and digital camera (Olympus Europa, Hamburg, Germany).

**Calculations and statistical analyses**

Glucose disposal (Rd) was calculated by Steele’s single-pool non–steady-state equations (25). Endogenous glucose production (EGP) is given as the difference between Rd and glucose infusion rate.

Surrogate indexes of insulin sensitivity and secretion were calculated from fasting blood glucose (G) and serum insulin (I) levels: quantitative insulin check index of insulin sensitivity (QUICKI = 1/[log(I in µU/ml) + log(G in mg/dl)]) (26) and homeostasis model index of insulin β-cell function (HOMA-B = 20*(I in µU/ml)/(G in mmol/l)-3.5) (27).

Data are presented as means±standard deviations (SD) in the text and tables and as means±standard error of the mean (SEM) in figures. Groups were compared using Bonferroni test or non-parametric unpaired t-test (Mann-Whitney) with Hochberg post hoc analysis. P values <0.05 were considered to indicate statistically significant differences.
Results

Both diabetic and non-diabetic NOD mice are insulin resistant

Body weight was similar among A-DM, N-DM and C-DM and slightly higher than in WT mice (Table 1). In the fed state, blood glucose was 4-fold higher in A-DM and C-DM, versus N-DM and WT (Table S1). Insulin levels were lower in A-DM compared to N-DM (Table 1). During fasting, circulating glucose, TG and FA were increased in A-DM compared to N-DM and WT (Fig. 1A,B,C). QUICKI was 13% and 9% lower in A-DM and N-DM, than in WT, suggesting glucose-independent insulin resistance in the NOD model (Table 1). HOMA-B was 3.5- and 5.5-fold higher in N-DM compared to WT and A-DM, fitting with the previous observation that prediabetic NOD mice have higher in vivo and ex vivo glucose-stimulated insulin secretion as compared to C57BL6 mice (28). A-DM had lower HOMA-B and insulin levels in fed state compared to N-DM, illustrating the impaired β-cell function, which is due to severe insulitis (data not shown) typical for the NOD model (24).

Diabetic NOD mice lose fat mass and rely on fat oxidation

A-DM had substantially (60-75%) lower visceral and subcutaneous fat masses (Fig. S1A,B). Muscle TG was unchanged except for a small increase in ad libitum-fed N-DM (Fig. S1C). Liver weight was comparable between groups, while hepatic TG was lower in fasted A-DM (Fig. S1A,B,C,D and Fig. S2).

Only WT and N-DM displayed normal diurnal energy expenditure patterns (Fig. 2A,B). A-DM showed a shift in RQ to 0.81 with preferential lipid oxidation (Fig. 2C,D), which was supported by a trend towards higher serum β-hydroxybutyrate levels (0.62±0.22 versus N-DM: 0.25±0.07 mmol/l, p=0.083). Finally, A-DM exhibited lower physical activity during the dark cycle (Fig. 2D) and increased overall food and water intake (Fig. 2F,G). Energy expenditure was similar among groups (Fig. 2H).
Diabetic and non-diabetic NOD mice show tissue-dependent differences in insulin sensitivity

During the hyperinsulinemic-euglycemic clamps, steady-state blood glucose (Fig. 3A) and serum insulin (not shown) were comparable. A-DM had higher fasting EGP versus WT and N-DM (Fig. 3B). In A-DM, EGP was positively related to fasting glycemia (r=0.95, p<0.05, n=9). Both A-DM and N-DM displayed lower insulin-mediated suppression of EGP versus WT (Fig. 3C) indicating hepatic insulin resistance. A-DM showed impaired insulin-stimulated Rd, which was 62% and 66% reduced compared to WT and N-DM (Fig. 3D). Uptake of 2-deoxy-glucose by gastrocnemius and soleus muscles was also lower in A-DM (Fig. 3E,F). In A-DM, plasma FA decreased similarly as in WT and N-DM during the first 15 min, but remained higher from the 30 min to the end of the clamp (Fig. 2G). Overall percent suppression of FA in A-DM was comparable to N-DM (Fig. 2H) suggesting intact insulin sensitivity of adipose tissue.

Diabetic and non-diabetic NOD mice show lower fasting muscle mitochondrial oxidation, but increased lipid peroxidation in the fed state

In the fed state, complex I (CI) respiration was decreased by 38% in C-DM (Fig. 4A). During fasting, CI+CII and maximal ETS capacities were decreased by ~20% in A-DM and N-DM (Fig. 4B). Results were normalized to mtDNA copy number, which did not differ between groups (not shown). Differences in mitochondrial respiration did not associate with changes in gene expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), mitochondrial transcription factor A (TFAM) nor nuclear respiratory factor-1 (NRF-1) (Fig. 4C,D).

Measurement of TBARS in the muscle revealed that WT, unlike N-DM or A-DM, have lower lipid peroxides in the fed compared to fasting state, (p<0.01, Fig. S1E,F). Moreover, fed N-DM, A-DM and C-DM mice had higher TBARS than WT (Fig. S1E).
Diabetic NOD mice show increased PKCθ-mediated serine phosphorylation of IRS1 in gastrocnemius muscle

Upon intraperitoneal insulin injection, A-DM displayed reduced basal levels of insulin receptor (IR, -32%) and insulin receptor substrate-1 (IRS-1, -30%) in muscle compared to WT (Fig. 5A,B). Serine phosphorylation of IRS1 was increased by 149% at Ser1101 and by 246% at Ser307 (Fig. 5C,D). A-DM showed profoundly decreased insulin-stimulated membrane-to-cytosol ratio of Akt (-61%, Fig. 5E) and glucose transporter 4 (GLUT4, -76%, Fig. 5F). N-DM had reduced IR and intermediate decreases in Akt and GLUT4 membrane-to-cytosol ratio (Fig. 5A,E,F).

Levels of pERK-Thr202/Tyr204, p-p38-Thr180/Tyr182, pIkB-Ser32 (Fig. S3) and pNFκB-Ser536 (Fig. 5I) were unchanged. Phosphorylation of JNK at Thr183 and Tyr186 was increased in A-DM and N-DM (Fig. 5H). Only the higher membrane-to-cytosol ratio of PKCθ clearly associated with decreases in peripheral insulin sensitivity in A-DM (+148% versus WT, +53% versus N-DM, Fig 5G).

A-DM had higher membrane-to-cytosol DAGs (Fig. 6A) due to changes in several specific DAG species (Fig. 6B). Neither total nor individual species of ceramides were different between the groups (Fig. 6C,D).

NOD mice have increased hepatic lipid peroxidation, but only diabetic NOD mice show augmented hepatic mitochondrial oxidation

In the fed state, hepatic O₂ flux rates through CI, CI+CII and maximal ETS capacity were increased in A-DM compared to N-DM and WT (Fig. 4E). In C-DM, CI and CI+CII respiration was increased versus WT, but lower versus A-DM (Fig. 4E). No differences were observed in the fasted state (Fig. 4F). Hepatic mtDNA copy number was similar among all groups (not shown).
In contrast to muscle, increased respiration in the liver of A-DM was accompanied by higher transcription of PGC-1α TFAM and NRF1 compared to N-DM and WT (Fig. 4G).

Hepatic TBARS were unchanged in the fasted state, but higher in fed N-DM, A-DM (Fig. S1E,F) and even more pronounced in C-DM. Similar to muscle, hepatic TBARS decreased during feeding in WT only (p<0.001).

**JNK signaling is increased in the liver of NOD mice**

Protein abundance of hepatic IR, IRS-2 (Fig. 5J,K), and IRS-1 (not shown) were similar in all groups. However, A-DM had 125% and 74% higher levels of phosphorylated IRS1 at Ser1101 and at Ser307 than WT at baseline (Fig. 5L,M). N-DM mice also showed 137% higher IRS1 phosphorylation at Ser1101 (Fig. 5L). Insulin-stimulated translocation of Akt to the membrane was suppressed by 76% in A-DM and to a lesser extent (by 40%) in N-DM (Fig. 5N).

Levels of pERK-Thr202/Tyr204, p-p38-Thr180/Tyr182, IκB, pIκB-Ser32 (Fig. S4) and pNFκB-Ser536 (Fig. 5R) were unchanged. Intriguingly, the membrane-to-cytosol ratio of PKCε was 36% and 27% lower in A-DM and N-DM, respectively (Fig. 5O). This was exclusively due to an increase in PKCε in the cytosolic fraction (Fig. 5P). Importantly, phosphorylation of JNK at Thr183 and Tyr186 was increased by 64% in A-DM and by 67% in N-DM (Fig. 5Q) rendering pJNK as the only factor associated with changes in hepatic insulin sensitivity.

**Circulating fetuin A levels are increased in NOD mice**

In A-DM and N-DM, fasting serum concentrations of fetuin A were 2.0- and 2.9-fold higher than in WT (Fig. 1D).
Discussion

This study describes the early metabolic events occurring before and at the onset of insulin-dependent diabetes in NOD mice. The NOD model spontaneously develops autoimmune insulitis with insulin deficiency resembling human T1DM. As chemical (29; 30) or surgical (31) induction of insulin deficiency may per se induce insulin resistance, NOD mice currently represent the most suitable model for studying metabolic changes associated with autoimmune diabetes. The study shows that the initiation of diabetes is associated with tissue-specific differences in metabolic flexibility and insulin sensitivity. Normoglycemic, non-diabetic NOD mice already exhibit hepatic insulin resistance associated with increased JNK phosphorylation and lipid peroxidation as well as lower muscle glucose transport. Acutely diabetic NOD mice additionally display muscle insulin resistance associated with increased intramyocellular DAG and PKCθ activation. Moreover, the liver transiently enhances its mitochondrial oxidative capacity at diabetes onset, possibly as an adaptation to increased lipolysis, while muscle oxidative capacity declines during later stages of diabetes.

A-DM mice show a reduction of visceral and subcutaneous fat, which reflects excessive fasting lipolysis and/or impaired stimulation of lipid synthesis by insulin in adipocytes. Blunted inhibition of lipolysis has been linked to the development of peripheral insulin resistance in adolescents with poorly controlled T1DM (32). In the present study, the intact reduction of FA during hyperinsulinemic clamps - unlike in humans - indicates that the absence of insulin-dependent control of lipid metabolism results rather from reduced insulin secretion than from insulin resistance of adipose tissue in NOD mice. The resulting rise in circulating FA and TG would favor redistribution of lipids towards ectopic fat storage in liver or muscle, which associates with insulin resistance in these tissues (7; 13). Importantly, A-DM mice have unchanged muscle and even reduced liver TG content indicating permanently ongoing lipid oxidation in the fed state. This was supported by constant reduction and lack of
the diurnal oscillation of RQ, reflecting restriction to fat oxidation, impaired switching to glucose utilization and a trend towards ketonemia within three days after onset of hyperglycemia in A-DM. Likewise, T1DM patients with poor metabolic control have lower intrahepatic fat content along with moderate peripheral insulin resistance and increased whole-body lipid oxidation (2). On the other hand, N-DM mice had increased muscle fat content likely reflecting higher lipid storage leading to unchanged circulating FA and TG. Both A-DM and N-DM mice also accumulated lipid peroxidation products in muscle and liver indicating greater ROS production, which can further aggravate insulin resistance (33). Taken together, NOD mice are characterized by hyperlipidemia and oxidative stress in muscle and liver.

A-DM mice display markedly reduced muscle insulin sensitivity as assessed both in vivo and ex vivo. Impaired insulin-mediated glucose disposal and muscle glucose uptake, along with decreased membrane translocation of GLUT4 and Akt, could result from lipid-mediated intracellular alterations. In human T1DM, muscle insulin resistance is accompanied by a decreased glucose transport into myocytes (34; 35) and insulin-stimulated upregulation of GLUT4 mRNA (36) but increased muscle lipid contents (37). FA-induced inhibition of insulin-stimulated glucose transport/phosphorylation (38) has been linked to intracellular accumulation of DAG, PKCθ activation and serine phosphorylation of IRS1 (6). Supporting this contention, A-DM mice showed increased membrane DAG, PKCθ translocation and Ser1101/Ser307 phosphorylation of IRS1 in skeletal muscle (Fig. 7). Moreover, the hyperglycemia-induced basal glucose uptake in A-DM mice could also contribute to nPKC activation (39). On the other hand, plasma levels of fetuin A, a FA-induced hepatokine, were increased in A-DM and N-DM mice. Fetuin A positively correlates with hyperglycemia and insulin resistance during hyperlipidemia (40) and promotes lipid-induced insulin resistance by mediating the binding of FA to TLR4 (41). We found no changes in signaling downstream of
TLR4, such as NFκB or ceramides, questioning the contribution of this pathway to muscle insulin resistance. Recent data also indicate that both high saturated and unsaturated fat diets induce insulin resistance independently of TLR4 signaling (42).

In vivo hepatic insulin sensitivity is impaired in both N-DM and A-DM mice, implying that hepatic insulin resistance can develop before the onset of hyperglycemia. In the absence of hyperglycemia and hyperlipidemia, the mechanisms of hepatic insulin resistance in N-DM mice likely differ from that observed in muscle of A-DM. Indeed, inhibition of IRS1 in livers of N-DM and A-DM did not associate with changes in PKCε, but rather with an increase in pJNK. Although previous studies yielded conflicting data on protective effects of liver-specific JNK-deficiency against lipid-induced insulin resistance, it should be emphasized that the enhanced phosphorylation of JNK in the NOD model occurs independently of lipids. Alternatively, increased fetuin A (43) and hepatic oxidative stress (44), which have been linked to JNK activation, could account for increased pJNK, inhibition of IRS-1 and hepatic insulin resistance.

We found no association of mitochondrial respiration with insulin sensitivity, excluding mitochondrial function as a potential mediator of insulin resistance in NOD mice. In muscle, mitochondrial oxidative capacity and transcript levels of mitochondrial biogenesis-related genes were comparable between A-DM and N-DM. However, chronically diabetic mice (C-DM) showed lower oxidative capacity, in line with the observation that muscle ATP synthesis is decreased in vivo in human T1DM (1). This secondary decrease of mitochondrial function could result from mitochondrial damage caused by lipid peroxide accumulation (45), inhibitory effects of prolonged insulin treatment on mitochondrial biogenesis and function (46) or direct effects of the chronic diabetic state (1). In contrast, hepatic mitochondrial oxidative capacity as well biogenesis were clearly higher in A-DM mice. Increased oxidative
capacity may represent an early adaptation to increased lipid and glucose flux after diabetes onset. Expression of genes related to oxidative phosphorylation was upregulated in the livers of NOD mice two weeks after diabetes onset (47) as well as in the livers of obese with T2DM (48). Decreased Akt could mediate the increase in hepatic PGC-1α, fatty acid oxidation (49) and overall mitochondrial respiration (50). Importantly, upregulated hepatic mitochondrial oxidative capacity seems to be transient, as it declines in C-DM. Similar to muscle, this could be a consequence of mitochondrial damage due to lipid peroxide accumulation.

In conclusion, insulin resistance in a murine non-obese model of T1DM develops in the liver before the onset of diabetes and associates with increased oxidative stress and pJNK, as possible cellular mediators. Muscle insulin sensitivity is impaired already three days after the onset of diabetes and accompanied by increased levels of DAG, PKCθ and serum fetuin A. Furthermore, mitochondrial oxidative capacity is transiently enhanced in the liver and declines in muscle with longer disease duration. Knowledge of these mechanisms could help to develop new strategies to prevent and treat insulin resistance and subsequent complications in patients with type 1 diabetes.
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T.J and G.S. conceived the experiments, researched data, contributed to discussion, and wrote the manuscript. K.K, D.M.O, E.P., J.K. and B.K. researched data and edited/reviewed the manuscript. J.W. A.L.R, L.J., P.N., H.J.P. and D.Z. researched data. G.I.S. contributed to discussion and edited/reviewed the manuscript. J.S. conceived the experiments, contributed to discussion and edited/reviewed the manuscript. M.R. conceived the experiments, contributed to discussion and wrote the manuscript. M.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1: Characteristics of WT, N-DM, A-DM and C-DM mice in the fasted and fed state

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<td>25</td>
<td>26</td>
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<td>Age [days]</td>
<td>152±33</td>
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<td>156±29</td>
<td>154±36</td>
<td>165±28</td>
<td>145±40</td>
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<td>Body weight [g]</td>
<td>22±2</td>
<td>24±2*</td>
<td>24±2*</td>
<td>23±2</td>
<td>26±2*</td>
<td>25±2*</td>
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<tr>
<td>Insulin [pmol/l]</td>
<td>86±45</td>
<td>267±143</td>
<td>224±404</td>
<td>99±73</td>
<td>583±623*</td>
<td>227±390†</td>
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<tr>
<td>QUICKI</td>
<td>0.32±0.02</td>
<td>0.29±0.03#</td>
<td>0.28±0.03#</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>HOMA-B</td>
<td>74±39</td>
<td>260±184#</td>
<td>47±62§</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</table>

All data are given as means±SD. *p<0.01 vs. WT, #p<0.001 vs. WT, †p<0.01 vs. N-DM, §p<0.001 vs. N-DM in respective metabolic state. ANOVA with Bonferroni post hoc analysis or non-parametrical test (Mann-Whitney) with Hochberg post hoc analysis. NA: not assessed.
Figure Legends

Figure 1

Serum concentrations of glucose (A), triglycerides (TG, B), fatty acids (FA, C) and fetuin A (D) of WT, N-DM and A-DM mice in the fasted state. Data are given as means±SEM (n=8-26 per group). **p<0.01, ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure 2

Energy balance of WT, N-DM and A-DM mice. Time course of respiratory quotients (RQ) of (A) WT, (B) N-DM and (C) A-DM during light and dark cycles. (D) Average RQ values, (E) activity counts, (F) food intake, (G) water intake and (H) energy expenditure during light (L) and dark (D) cycles. Data are represented as means±SEM (n=5 per group). *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure 3

Insulin sensitivity in WT, N-DM and A-DM mice. (A) Blood glucose levels before and during the hyperinsulinemic-euglycemic clamp. (B) Endogenous glucose production (EGP) under basal conditions. (C) Insulin-mediated suppression of EGP. (D) Insulin-mediated stimulation of peripheral glucose disposal (Rd). Insulin-mediated 2-deoxy-glucose uptake by (E) gastrocnemius and (F) by soleus muscles. Plasma fatty acids (G) concentrations and (H) percent suppression during the clamp. Data are represented as means±SEM (n=6-10 per group (A-D); n=3-4 per group (E-H)). *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure 4
Mitochondrial function and transcript levels of genes related to mitochondrial biogenesis in gastrocnemius muscle (A-D) and liver (E-H) of WT, N-DM and A-DM mice. Mitochondrial respiration capacity through complex I (CI), CI+CII as well as maximal respiratory capacities of electron transport system (ETS) in gastrocnemius muscle (A and B) and liver (E and F) of fed and fasted mice (n=5-14 per group). Respiratory data were individually corrected for mitochondrial content. Expression levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM) in gastrocnemius muscle (C and D) and liver (G and H) of fed and fasted mice (n=5 per group). Data are represented as means±SEM. *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure 5
Protein levels of cellular signaling pathway components in gastrocnemius muscle (A-I) and liver (J-R) of WT, N-DM and A-DM mice. (A-F, J-N) Basal and insulin-stimulated expression levels (n=6 per group) of insulin receptor (IR) (A,J), IR substrate-1 (IRS1) (B), IR substrate-2 (IRS2) (K), serine-1101 phosphorylation of IRS1 (pIRS1-Ser1101) (C,L), serine-307 phosphorylation of IRS1 (pIRS1-Ser307) (D,M), membrane-to-cytosol ratio of Akt (E, N) and glucose transporter 4 (GLUT4) (F).

(G-I, O-R) Fasted conditions: membrane-to-cytosol ratio of protein kinase Cθ (PKCθ) (G) and PKCe (O) (n=7-8 per group), cytosolic PKCe (P), phosphorylation of c-Jun N-terminal kinase pJNK (H,Q) (n=6 per group) and phosphorylation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells NFκB (I,R) (n=6 per group). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control for all proteins. Data are represented as means±SEM. *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.
Figure 6

Myocellular content of diacylglycerols (DAG) and ceramides in WT, N-DM and A-DM mice. Membrane-to-cytosol total (A) or individual species (B) of DAG and whole-tissue content of total (C) or individual species (D) of ceramides in gastrocnemius muscle (n=8 per group). All data are represented as means±SEM. *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure 7

Mechanisms of tissue-specific changes in glucose homeostasis occurring in diabetic and non-diabetic NOD mice. Effects occurring at the onset of T1DM (A-DM mice) are shown in red arrows and marks. Effects of the NOD genotype occurring independently of diabetes (both N-DM and A-DM mice) are shown in blue arrows and marks. The progression of T1DM is associated with increased circulating glucose and free fatty acids (FA) levels, resulting from impaired control of glucose and lipid metabolism by insulin. Intramyocellular lipotoxic intermediates diacylglycerols (DAG) activate protein kinase Cθ (PKCθ), which results in enhanced serine-1101 and -307 phosphorylation of insulin receptor substrate 1 (pSer1101- and pSer307-IRS1). This inhibits IRS1 activity, and the membrane translocation of Akt and GLUT4 and thereby decreases muscle glucose disposal. In the liver, increased production of reactive oxygen species (ROS) and lipid peroxidation is associated with increased phosphorylation of c-Jun N-terminal kinase (pJNK) and IRS1 (pSer307- and pSer1101-IRS1). This leads to lower translocation of Akt and impaired insulin-mediated suppression of endogenous glucose production (EGP). Furthermore, fetuin A (fetA) levels in serum are increased, which could contribute to the downregulation of the insulin signaling pathway by binding to insulin receptor β-subunit and shutting down the phosphorylation at tyrosine (pTyr) (51) or by increasing pJNK phosphorylation via toll-like receptor 4 (TLR4) (43).
Figure 1
161x115mm (300 x 300 DPI)
Figure 3
175x214mm (300 x 300 DPI)
Figure 4
160x75mm (300 x 300 DPI)
Figure 5
267x368mm (300 x 300 DPI)
Figure 6
191x141mm (300 x 300 DPI)
Supplementary Material

Table S1: Glucose and lipid levels in the serum of WT, N-DM, A-DM and C-DM mice in the fed state

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<th>WT</th>
<th>N-DM</th>
<th>A-DM</th>
<th>C-DM</th>
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<tbody>
<tr>
<td>Glucose [mg/dl]</td>
<td>124±26</td>
<td>131±34</td>
<td>465±59</td>
<td>490±22</td>
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<tr>
<td>Triglyceride [mg/dl]</td>
<td>128±53</td>
<td>139±59</td>
<td>462±278</td>
<td>178±39</td>
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<tr>
<td>Free fatty acids [mmol/l]</td>
<td>0.87±0.30</td>
<td>0.77±0.40</td>
<td>1.32±0.84</td>
<td>1.07±0.24</td>
</tr>
</tbody>
</table>

All data are represented as means±SD. *p<0.01 vs. WT, #p<0.001 vs. WT, ‡p<0.01 vs. N-DM, §p<0.001 vs. N-DM, †p<0.05 vs. A-DM by ANOVA with Bonferroni post hoc analysis or non-parametrical test (Mann-Whitney) with Hochberg post hoc analysis.
Supplementary Figure Legends

Figure S1

Determination of body fat storage and degradation in WT, N-DM, ADM and C-DM mice. (A and B) Total weights of liver, visceral fat and subcutaneous fat under fed (n=10 per group) and fasted (n=5-10 per group) conditions. (C and D) Triglyceride content in gastrocnemius muscle and liver of fed (n=5-12 per group) and fasted (n=5-12 per group) mice. (E and F) TBARS levels in gastrocnemius muscle and liver of fed (n=5-12 per group) and fasted (n=5 per group) mice. Data are represented as means±SEM. *p<0.05, **p<0.01 and ***p<0.001 by ANOVA with Bonferroni post hoc analysis.

Figure S2

Detection of lipid droplets with oil red O (ORO) staining in frozen liver sections. Liver sections of (A) WT, (B) N-DM and (C) A-DM mice at fasted state were stained by ORO for specific detection and quantification of lipids. Thereby, WT mice displayed the highest lipid content with ~30% ORO staining of total tissue area. Compared to this, N-DM showed decreased content with ~15% ORO-stained area. A-DM had the lowest lipid content with ~10% ORO-stained area of total section.

Figure S3

Protein levels of cellular inflammatory pathway components in gastrocnemius muscle of WT, N-DM and A-DM mice. Basal (n=6 per group) levels of extracellular-signal regulated kinase phosphorylated at Thr202 and Tyr204 (ERK) (A), p38 mitogen-activated protein kinase phosphorylated at Thr180 and Tyr182 (p38MAPK) (B), nuclear factor 'kappa-lightchain-enhancer' of activated B-cells (NFκB) phosphorylated at Ser536 (C) and inhibitor of kappa B phosphorylated at Ser32 (pIκB) (D). Glyceraldehyde 3-phosphate dehydrogenase
(GAPDH) was used as loading control for all proteins. Data are represented as means±SEM and tested by ANOVA with Bonferroni post hoc analysis.

Figure S4

Protein levels of cellular inflammatory pathway components in the liver of WT, N-DM and A-DM mice. Basal (n=6 per group) levels of extracellular-signal regulated kinase phosphorylated at Thr202 and Tyr204 (ERK) (A), p38 mitogen-activated protein kinase phosphorylated at Thr180 and Tyr182 (p38MAPK) (B), as well inhibitor of kappa B (IκB) (C) and its phosphorylated form at Ser32 (pIκB) (D). Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) was used as loading control for all proteins. Data are represented as means±SEM and tested by ANOVA with Bonferroni post hoc analysis.
Figure S1
180x219mm (300 x 300 DPI)
Figure S2
160x47mm (300 x 300 DPI)
Figure S3
180x124mm (300 x 300 DPI)
Figure S4

180x131mm (300 x 300 DPI)