Influence of Diet on Visceral Adipose Remodeling in NONcNZO10 Mice With Polygenic Susceptibility for Type 2 Diabetes

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Visceral adipose tissue (VAT) is a source of inflammatory cytokines that in obese subjects may contribute to low-level systemic inflammation and development of metabolic syndrome. Expansion of VAT involves adipocyte hypertrophy and hypertension and requires breakdown of the extracellular matrix and increased vascular outgrowth. To investigate changes of gene expression associated with VAT expansion and the role of combined genetics and diet, we implemented gene microarray analyses of VAT in NONcNZO10 (NZ10) and control SWR/J mice subjected to control chow (CD) or a diet of high protein and fish oil (HPO). NZ10 mice on CD showed increased body weight, hyperglycemia, and hyperinsulinemia at 25 weeks whereas those on HPO diet retained normal insulin levels and were normoglycemic. Two-way ANOVA revealed a significant interaction between diet and strain on blood glucose, serum insulin, and percent fat but not for body weight. Microarray heat maps revealed a remarkable combined effect of genetics and diet on genes that regulate extracellular matrix as well as angiogenic genes. Real time-PCR (RT-PCR) confirmed markedly increased expression of matrix metalloproteinases (MMPs) 2, 3, 11, and 12, vascular endothelial growth factor-A and C (VEGF-A and C), Von Willebrand Factor, and peroxisome proliferator-activated receptor-γ (PPAR-γ) selectively in the NZ10/CD group. MMP7 was significantly decreased. Protein levels of MMP2, 3, and 9 were significantly increased in the VA of NZ10 mice fed CD while those of MMP7 were downregulated. Microarrays also revealed diet-dependent two to fourfold increased expression of all four tissue inhibitor of metalloproteinases (TIMP) isoforms in NZ10 mice. Two-way ANOVA confirmed strongly interactive roles of diet and genetics on fat deposition and progression of type 2 diabetes in this polygenic mouse model.

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NONcNZO10/LtJ (NZ10) is a new polygenic mouse model of obesity-induced type 2 diabetes (T2D) that develops maturity-onset insulin resistance and hyperglycemia (1). The obesity/T2D phenotype of NZ10 mice is relatively diet-independent, the phenotype is supported even on moderately low fat chow and the mice do not have overtly dysregulated leptin gene expression (2,3). The NZ10 mice differ from the parent NZO/HILt strain in acquiring adiposity primarily in visceral adipose tissue (VAT) and this may contribute to the T2D susceptibility phenotype (4,5).

During the development of obesity, adipose tissue is remodeled, a process that requires changes in the expression of genes that regulate adipogenesis, adipocyte hypertrophy, and angiogenesis. Proteolysis of the extracellular matrix plays a key role in adipose expansion and includes changes in the activities of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) (6–9). Overexpression of TIMPs inhibits adipogenesis but may promote lipid accumulation and hypertrophy of mature adipocytes and although controversial, some studies suggest an essential role for enhanced TIMP activity (8,9). There is discrepancy over the contributions of individual MMPs and TIMPs to adipose expansion. Increased mRNA levels of MMP3, 11, 12, 13, 14, and TIMP1 were reported in gonadal fat depots of nutritionally-induced obese wild-type mice whereas MMP7, 9, 16, and 24 were decreased and there was no change in the levels of MMP2, 10, 15, 17, 19, 23, TIMP2, and 3 (10). Increased mRNA levels of MMP2, MMP3, MMP12, MMP14, and MMP19 were reported in white adipose tissue of obese ob/ob and db/db mice; MMP7 mRNA again was decreased (11). In db/db mice, it was found that ω-3 fatty
acid supplements significantly prevented obesity and blocked the expression of MMP12 and 14 mRNAs (7). Several studies have described increased levels of MMP2 and 9 in adipose and serum of obese human subjects (12,13). Adipose expansion in animal models and humans is associated with active angiogenesis and elevated expression of associated genes (14,15).

In this study, we used microarray, real time-PCR (RT-PCR), and western blotting to quantify MMPs, TIMPs, and angiogenic genes in VAT of NZ10 mice. The results indicate strong interaction between diet and genetics on VA deposition and T2D phenotypes with selective diet/genetics-dependent induction of MMPs 2, 3, 11, 12, multiple angiogenic genes and all four TIMPs. Consistent with two other murine models, MMP7 was downregulated in the VAT of NZ10 mice fed CD.

MATERIALS AND METHODS

Animals and diets

Four-week-old male NZ10 mice and their nondiabetic SWR/J counterparts were purchased from The Jackson Laboratory (Bar Harbor, ME). After 2 weeks of acclimatization, mice were randomized into two groups (at least 10 mice per group) to receive control chow (CD) or high-protein and fish oil (HPO) diets for a period of 19 weeks. CD is a semi-defined open formula custom diet with macronutrients based on that of Purina 5K20 that provides 27% calories from fat, 19% from protein, and 54% from carbohydrate. This diet contains lower fat than the standard western style diet for rodents such as Purina 5TJN (40% fat). The HPO diet is Purina LabDiet 5TWH, based on the South Beach diet and optimized for high protein and "good" fat (ω-3) with casein, canola, and menhaden fish oils. Food was provided ad-lib and weekly food intake was measured by providing preweighed chow; there was no significant difference in food consumption (data not shown). For tissue harvest, mice were fasted for 12-h, sacrificed by cervical dislocation and the VA depots including epididymal, perirenal, and retroperitoneal rapidly dissected and processed for RNA analysis. Percent body fat was estimated using Lunar PIXimus II densitometer (Lunar, Madison, WI) before sacrifice. Blood was collected from 12-h-fasted mice at age 25 weeks for measurement of glucose and plasma insulin.

RNA preparation

Total RNA was isolated from VAT using TRIzol Reagent (Invitrogen, Carlsbad, CA). The samples were processed using an RNeasy Mini Kit (Qiagen, Valencia, CA) and digested with RNase free DNase (Qiagen). The integrity and size distribution of total RNA was monitored using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). The concentration of RNA was determined by spectrophotometry, using Nanodrop-1000 (Nanodrop Technologies, Wilmington, DE). Reverse transcription was performed on 200ng/μl of RNA with High-Capacity cDNA Reverse Transcription kit using random primers (Applied Biosystems, Foster City, CA).

Microarray

Details of our cRNA and microarray procedures are described elsewhere (16). Equal amounts of RNA were pooled from four animals per group for each experimental group. Hybridizations were implemented by Ocean Ridge Biosciences (Palm Beach, Florida) using a CodeLink Bioarray (Applied Microarrays, Tempe, Arizona) using standard procedures. For the microarray data statistical analysis, samples were binned into two treatment groups (NZ1 and SWR). The log2-transformed and normalized spot intensities for the 25,173 detectable probes were examined for differences between the treatment groups by two-way ANOVA using National Institute of Ageing Array Analysis software. The ANOVA was conducted using the Bayesian Error Model and 10 degrees of freedom. Statistical significance was determined using the False Discovery Rate method. A total of 181 probes showed significant differences with P < 0.05 (Supplementary Table S1 online). Principal Component Analysis was performed on the 25,173 detectable probes using National Institute of Ageing software.

Figure 1 Physiological and biochemical parameters of NZ10 and SWR mice after 19 weeks on diet. (a) Body weight; (b) Percent lean tissue by PIXimus densitometry; (c) Percent fat by Lunar PIXimus densitometry (Lunar, Madison, WI); (d) Fasting blood glucose; (e) Serum insulin. Data represent mean ± s.e.m. and were analyzed by two-way ANOVA as described in Methods (*P < 0.01; n = 10 or larger). CD, control chow; HPO, high protein and fish oil.
RT-PCR
Gene expression levels were assayed by RT-PCR using the ABI 7900HT thermal cycler with custom assays (eight pooled samples per group). The following Assay IDs Mm00439506_m1, Mm01168406_g1, Mm00485048_m1, Mm00500554_m1, Mm01168420_m1, Mm00437306_m1, Mm01202432_m1, Mm00550376_m1, Mm01184322_m1 were used for MMP2, 3, 11, 12, VegfA, C, Von Willebrand Factor, and peroxisome proliferator-activated receptors-γ (PPAR-γ) genes, respectively. Eukaryotic 18S rRNA (Assay ID Hs99999901_s1) was used for normalization.

Western blotting
Samples (three mice per group) containing equal amounts of protein were subjected to SDS-PAGE and western blotting as described earlier (17). Antibodies were MMP2 (Santa Cruz Biotechnology, CA), MMP3 (Abcam, Cambridge, MA), MMP7 (Cell Signaling Technology, Danvers, MA), MMP9 (Abcam) Beta-Actin (Millipore, Billerica, MA) antibodies.

Statistical analysis
Data are expressed as means ± s.e.m. Differences between two groups were analyzed by Student’s t-test and Mann-Whitney U test. A two-way between groups ANOVA was used to evaluate diet and genetic strain interaction effects for dependent variables; a significant interaction was interpreted by a subsequent simple-effects analysis with Bonferroni correction.

RESULTS AND DISCUSSION
NZ10 mice fed CD for 19 weeks displayed hyperglycemia and hyperinsulinemia with significantly increased percent fat compared with their counterparts fed HPO (Figure 1). Two-way ANOVA revealed very significant interaction between diet and genetic strain for blood glucose, serum insulin, and percent fat (all \( P < 0.01 \)). The interaction effect between strain and diet did not reach significance for body weight (\( P = 0.10 \)) or lean tissue (\( P = 0.32 \)), but there was a significant main effect of strain on both of the latter parameters (\( P < 0.001 \)). For SWR mice, there was no significant diet-related differences in blood glucose, insulin level, fat deposition, or body weight. Elevated blood glucose and plasma insulin in the NZ10-CD group

**Figure 2** Gene expression analysis of visceral adipose tissue (VAT) using microarray and real time PCR. (a) Microarray analysis. Heat maps show differences in transcript levels of MMPs, TIMPs, and angiogenic factors in the VAT of NZ10 and SWR mice fed either diet for 19 weeks. Pooled visceral adipose (\( n = 4 \)) was used for performing the microarrays. Color scale represents fold increase (red) or decrease (green) from baseline. (b) Real-time PCR (RT-PCR) analysis of MMPs and angiogenic transcripts comparing effect of diet on transcripts levels in VAT after 19 weeks. Top panel, ratios of MMP 2, 3, and 9 transcript levels; middle panel, ratios of MMP7, 11, and 12 transcript levels; bottom panel, ratios of vascular endothelial growth factor, VWF, and PPAR-γ transcript levels. Dark bars: NZ10-CD/NZ10-HPO; Light bars: SWR-CD/SWR-HPO. Data represent mean ± s.e.m. (* \( P < 0.05 \); ** \( P < 0.01 \); \( n = 8 \)). CD, control chow; CtsD, cathepsin D; CtsK, cathepsin K; FABP4, fatty acid binding protein 4; HPO, high protein and fish oil; MMP, matrix metalloproteinase; NS, non significant; PPAR-G, peroxisome proliferator-activated receptors-γ; TIMP, tissue inhibitor of metalloproteinase; VEGF-A, vascular endothelial growth factor-A; VWF, Von Willebrand Factor.
were almost normalized by HPO diet. There were no apparent adverse effects (behavior, grooming, feeding) of long-term HPO feeding in either group.

Microarray heat maps (Figure 2a) suggested diet and strain-dependent increase in the levels of MMPs and angiogenic genes as well as TIMPs and cathepsins D and K. Responsive MMP and angiogenic genes were confirmed and quantified using RT-PCR (Figure 2b). The following transcripts were increased by three to sevenfold in VAT of NZ10 mice fed CD relative to their counterparts fed HPO: MMP2, 3, 11, 12, VEGF-A, VEGF-C, and Von Willebrand Factor. Protein expression of pro-MMP2, 3, and 9 was also significantly increased in the VAT of NZ10 mice (Figure 3). These results are consistent with previous reports of increased levels of MMPs 3, 11, and 12 in the VA of obese wild-type mice (10) and elevated MMP2 in obese db/db mice and humans (11). It was recently reported that MMP3 and 12 are part of a panel of MMPs that are induced by inflammatory cytokines (18). None of these transcripts were diet-regulated in the SWR group. MMP7 expression was not regulated but MMP7 transcripts were markedly decreased in NZ10-CD relative to NZ10-HPO or SWR groups. Two other studies have reported similar findings for MMP7 in different models suggesting that decreased MMP7 may be an obesity marker (10,11). Microarrays indicated increased levels of TIMPs 1–4 transcripts in the NZ10-CD group relative to HPO or SWR groups (Table 1) suggesting components of diet and genetics in the regulation of these transcripts. A complete list of differentially expressed genes is presented in the (Supplementary Table S1 online).

Earlier work has shown positive correlations between PPAR-γ gene expression and the obese phenotype of mice fed high fat (19). Our studies also showed a marked increase in VAT expression of PPAR-γ only in the NZ10-CD group confirming PPAR-γ as a genetics- and diet-responsive transcript also in this model.

CONCLUSION

Our results confirm that a diet of high protein and fish/vegetable oil decreases the risk of obesity and T2D in NONcNZ10 mice. The studies highlight roles for MMPs 2, 3, 11, and 12, and provide further evidence that depressed expression of MMP7 is a marker of obesity and implicate all four TIMPs and cathepsins in VA expansion in this model. Because some humans are genetically predisposed to obesity and T2D, NZ10 mice may provide an appropriate model for dissecting the underlying genes that confer the susceptibility phenotype. This is the first study to describe

Table 1 Comparison of selected diet-regulated genes in NZ10 and SWR mouse strains by Microarray

| Diet-regulated genes from microarray | NZ10 CD/NZ10 HPO | SWR CD/SWR HPO |
|-------------------------------------|------------------|----------------|
| MMP2                               | 4.41             | 1.35           |
| MMP3                               | 9.13             | 1.09           |
| MMP7                               | −44.32           | −1.35          |
| MMP8                               | 3.43             | 1.60           |
| MMP9                               | 1.37             | 1.00           |
| MMP11                              | 3.53             | 1.20           |
| MMP12                              | 5.86             | 0.74           |
| MMP19                              | 2.91             | 0.47           |
| MMP25                              | 2.04             | 1.44           |
| TIMP1                              | 4.63             | 0.87           |
| TIMP2                              | 4.38             | 0.98           |
| TIMP3                              | 2.23             | 1.27           |
| TIMP4                              | 2.83             | 0.82           |
| CTSD                               | 3.41             | 1.39           |
| CTSK                               | 8.17             | 1.14           |
| CTSZ                               | 3.36             | 0.77           |
| CTSB                               | 2.71             | 0.73           |
| FABP4                              | 3.01             | 0.88           |
| PPAR-γ                             | 4.32             | 0.96           |

The table shows the ratio of transcript levels for the CD vs. high protein and fish oil diet after 19 weeks on diet for each mouse strain. CD, control chow; CTSD, cathepsin D; CTSB, cathepsin B; CTSK, cathepsin K; CTSZ, cathepsin Z; FABP4, fatty acid binding protein 4; MMPs, matrix metalloproteinases; PPAR-γ, peroxisome proliferator-activated receptors-γ; TIMP, tissue inhibitor of metalloproteinases.
diet-regulation of obesity-related genes in any murine model of polygenic susceptibility for obesity and T2D. Further studies with larger sample sizes are warranted to accurately define the molecular basis of diet-regulated obesity in these mice.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

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DISCLOSURE
The authors declared no conflict of interest. See the online ICMJE Conflict of Interest Forms for this article.

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