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Abstract

Identification of new adipokines that potentially link obesity to insulin resistance represents a major challenge. We have recently shown that NOV/CCN3, a multifunctional matricellular protein, is synthesized and secreted by adipose tissue, with plasma levels highly correlated with body mass index. NOV has been previously involved in tissue repair, fibrotic and inflammatory diseases, and cancer. However, its role in energy homeostasis remains unknown. We investigated the metabolic phenotype of NOV-/- mice fed a standard or high fat diet (HFD). Strikingly, the weight of NOV-/- mice was markedly lower than that of WT mice but only on a HFD. This was related to a significant decrease in fat mass associated with an increased proportion of smaller adipocytes and to a higher expression of genes involved in energy expenditure. NOV-/- mice fed a HFD displayed improved glucose tolerance and insulin sensitivity. Interestingly, the absence of NOV was associated with a change in macrophages profile (M1-like to M2-like) and in marked decrease in adipose tissue expression of several proinflammatory cytokines and chemokines and to an enhanced insulin signaling. Conversely, treatment of adipocytes by NOV increased chemokine expression. Altogether, these results show that NOV is a new adipocytokine that could be involved in obesity-associated insulin-resistance.

Key words: Adipocytokine/inflammation/insulin-resistance/NOV/CCN3/obesity.

Introduction

Worldwide, more than 300 million people are currently obese, and this non-infectious epidemic affects both Western and emerging countries (1-3). In the absence of adequate care, obesity induces many co-morbidities and reduces life expectancy (4-7). Thus, there is a crucial need to better understand the pathophysiology of obesity, insulin-resistance and type 2 diabetes (T2D). In this context, the discovery of new adipocytokines involved in the control of energy balance is not only a challenge for understanding the mechanisms responsible for the abnormal development of obesity or insulin resistance but also offers new opportunities to manage this disease and its complications.

The NOV/CCN3 gene (nephroblastoma overexpressed gene) (8) is a founder of the CCN (Cyr61/CCN1, CTGF/CCN2, NOV/CCN3) family. The CCN genes encode matricial multifunctional proteins that are involved in organogenesis (9). In addition, these proteins play key roles in inflammation (10), wound healing, fibrosis and cancers (9). Several studies have also shown that NOV is involved in the adhesion, migration, proliferation, differentiation and survival of different cell types (11-16). NOV also modulates the expression of inflammatory molecules and their effects (17-20). These different functions are mediated through the interaction of NOV with specific integrins and, in some cases, by the Notch pathway (9).

Measurements of plasma NOV concentrations in a large cohort of patients screened for cardio-metabolic diseases in which 60% of the patients had a BMI above 30 kg/m^2 revealed, for the first time, a strong relationship between plasma NOV concentrations and obesity (21). Plasma NOV levels were also correlated with weight loss in patients who had undergone bariatric surgery. Accordingly, we found that in adipose tissue from obese patients and in human primary cultures the NOV protein was synthesized and secreted by adipocytes and

macrophages (21). Moreover, we demonstrated that the induction of NOV during adipose tissue expansion was not restricted to humans. Indeed, in mice fed a high fat diet (HFD), plasma NOV levels and its expression in adipose tissue were also increased compared to mice fed a standard diet (SD) (21).

Even though a high BMI is often associated with impaired glucose tolerance and T2D, we did not find any correlation between NOV and fasting glycemia. However, there was a positive correlation between plasma NOV and HbA1c (21). Thus, it is conceivable that a relationship could exist between NOV and carbohydrate metabolism. In this context, a possible link between NOV and insulin was suggested by Shimoyama et al. (22), who reported that NOV expression was reduced in rat aortas after streptozotocin injection and was increased by insulin treatment. Moreover, the *nov* gene is localized on chromosome 8q24 (23), which is a susceptibility locus controlling β-cell function in linkage studies of patients with diabetes (24). Taken together, the above results and a recent report (25) showing that NOV is a direct target of transcription factor FOXO1 and that NOV impairs insulin secretion in pancreatic β-cells reinforce the possibility that this protein could be involved in energy homeostasis. However, there has been no data reported to date on the function of NOV in adipose tissue or in whole body metabolism. Thus, the main focus of this study was to identify whether targeted disruption of the *nov* gene modulates energy balance in mice fed a normal or obesogenic diet.

Research Design and Methods

Mice

NOV-/- mice were generated by Shimoyama et al (22) using a targeting vector to delete the genomic region encompassing exons 1, 2 and a part of 3 for complete elimination of the NOV transcription. Mice heterozygously transmitting the NOV-knockout genome were generated and their descendants were backcrossed to C57Bl/6J for at least 6 generations and maintained as heterozygotes. C57Bl/6J heterozygous males were then mated with 129sv/PAS females. Embryos derived from these crossings were reimplanted in pseudopregnant females in order to get a F1 on a mixed background (C57Bl/6J-SV129) with the EOPS status.

C57Bl/6-SV129 NOV-/- mice and the WT controls were derived from the same heterozygous crossings. Mice were kept on a 12 h-12 h light–dark cycle with *ad libitum* access to food and tap water. Six-wk-old male mice were fed either a SD (4% fat, Genestil 1326 Royaucourt, France) or a HFD (42% of fat, TD.88137, Harlan Teklad, Gannat, France) for 16 wks. Mice were bred according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication N° 85-23, revised 1996). The animal facility was granted approval (B-75-12-01) given by the French Administration. All procedures were approved by a local ethic committee (N° Ce5/2012/091). Mice genotypes were performed as previously described (22). The genotypes were further checked by NOV mRNA levels in epididymal white adipose tissue (eWAT) and subcutaneous white adipose tissue (scWAT), the plasma level of the protein and its expression in heart where NOV is normally highly expressed (Fig S1).

Body composition

Body weight (BW) was measured using a laboratory scale (Ohaus CS 200 Series, Sigma-Aldrich, l'Isle d'Abeau, France). Body mass composition was analyzed using an Echo

Medical systems EchoMRI 100 (Whole Body Composition Analyzers, EchoMRI, Houston, USA) according to the manufacturer's instructions (26).

Indirect calorimetry measurements

For measurement of energy expenditure (EE), respiratory exchange ratio (RER) and spontaneous locomotor activity, mice were placed in an indirect calorimetry system (Labmaster, TSE Systems Gmbh, Bad Homburg, Germany).

Metabolic parameter exploration

Oral glucose tolerance tests (OGTTs) and insulin tolerance tests (ITTs) were performed after 14 and 15 wks of diet, respectively, on food-deprived (6 h and 4 h, respectively) non-anesthetized mice. For the OGTT, animals were weighed and orally fed using gavage needles (Ref 18060-020, Phymep, Paris, France) with a 1 g/kg BW of a 20% glucose solution. Whole-tail vein blood (30 µL) was sampled at baseline and 15 min to measure plasma insulin. For the ITT, animals were weighed and i.p. injected with human insulin (0.75 mU/kg). For both tests, glucose levels were obtained from whole-tail vein blood using an automatic glucometer (Accu-Chek Performa, Roche, Meylan, France) at indicated times. For insulin signaling experiments , mice were i.p. injected with 0.5 units of regular human insulin (Actrapid Penfill, NovoNordisk, Paris, France). Ten minutes later, tissues were snap frozen in liquid nitrogen.

Plasma measurements

Total cholesterol, HDL and LDL cholesterol, triglycerides and non-esterified fatty acids levels were determined by enzymatic colorimetric assays (Sobioda, Montbonnot–Saint-Martin, France). Leptin, adiponectin and NOV levels were measured using ELISA specific kits (R and D Systems, Lille, France).

Pancreatic measurements

The pancreatic and islet insulin content was extracted at −20°C in acidic ethanol (1.5% [vol/vol] HCl in 75% [vol/vol] ethanol). Pancreatic islets from NOV-/- and WT mice were isolated after collagenase digestion and cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37°C for 24 h. Batches of 30 islets were incubated in Krebs buffer containing 2.8, 16.7 mmol/L glucose or 50 mmol/L KCl for one hour at 37°C. Plasma insulin, total pancreatic and islet insulin content, and insulin secreted in response to glucose were measured using the Ultra Sensitive Insulin ELISA kit (Crystal Chem, Inc, Downers Grove, USA).

Histomorphological procedures

Paraffin-embedded adipose sections were analyzed after hematoxylin and eosin staining. Five fields were randomly observed at 10x magnification by an optical microscope (BX61 Olympus). Adipocyte quantification was performed using the ImageJ software (http://rsbweb.nih.gov/ij/) on approximately 500-600 cells per mouse (4 animals per group and per genotype were analyzed).

Considering that 90% of the adipose tissue volume was constituted of adipocytes, the number of cells was estimated by dividing the tissue mass per the mean volume of cells. The results are expressed in cells/g of tissue.

Sections (3 µm thick) were stained with Sirius red and interstitial fibrosis was assessed semi-quantitatively by selecting randomly 10 fields per section that covered the entire surface at x200 magnification. Fibrosis was then quantified using computer-based morphometric analysis software (Analysis, Olympus) that allowed the formation of a binary image, in which the stained area could be automatically calculated as a percentage of the image area (27).

Isolation of stroma-vascular fraction and flow cytometry analysis

eWAT from WT and NOV-/- mice were removed, minced and digested with collagenase A (1 mg/mL in PBS, Sigma-Aldrich) at 37°C for 45 min. Homogenates were passed through a 70-µm mesh and the effluent was centrifuged at 500 g for 3 min. Cells from pellets defined as the stroma-vascular fraction (SVF) were resuspended in FACS buffer (PBS with 5% FBS). Extracellular staining was preceded by incubation with FcR-blocking antibody (2.4G2, BD Biosciences, Franklin Lakes, NJ) to avoid non-specific staining. For lymphocytes cytometry analysis, cells were first stained with mAbs to cell-surface markers purchased from BD Biosciences; FITC-conjugated anti-CD3 (145-2C11), APC-conjugated anti-CD4 (RM4-5), and Per-CP-conjugated anti-CD8 (53-6.7). Cells were then fixed, permeabilized and incubated with PE-conjugated anti-Foxp3 (FJK-16s, eBioscience) for intracellular staining according to the manufacturer's specifications. For macrophages staining analysis, cells were surface-stained with BV421-conjugated anti-F4/80 (T45-2342), Alexa Fluor647-conjugated anti-CD206 (19.2) and Biotin-conjugated anti-CD11b (M1/70) revealed with PE conjugated Streptavidin all from BD Bioscience. FITC-conjugated anti-CD11c (N418 was from eBioscience. Stained cells were analyzed using a Gallios flow cytometer (Beckman Coulter, Inc., Villepinte, France), and data were processed using Kaluza sofware (Beckman Coulter).

Adipose tissue and liver biochemical measurements

eWAT from WT and NOV-/- mice were removed and digested as described above. Homogenates were centrifuges and isolated adipocytes were obtained after centrifugation. Glycerol secreted from isolated adipocytes was used as an index of lipolysis, and measured with the Free-Glycerol reagent (Sigma-Aldrich). Triacylglycerols were extracted from adipocytes by the Dole's method (28) and from liver by acetone, then tested with the Triglycerides reagent (Sigma-Aldrich).

Primary preadipocytes and 3T3-L1 culture

Primary preadipocytes derived from SVF of either scWAT or eWAT were plated on 6-well culture dishes and cultured in DMEM (4.5 g/L D-glucose) with 10% fetal calf serum (FCS) (Eurobio, Les Ulis, France). Cell proliferation was assessed by BrdU incorporation according to the manufacturer (Cell Signaling, Danvers, USA). Adipocyte differentiation was initiated in confluent cultures of primary and 3T3-L1 preadipocytes with DMEM supplemented with 10% FCS and 0.1 mmol/L 3-isobutyl-1-methylxanthine, 200 nmol/L dexamethasone and 100 nmol/L insulin (Sigma-Aldrich) for 3 days. Adipocytes were further maintained for 6 days in DMEM supplemented with 10% FCS and 100 nmol/L insulin.

Primary macrophages culture

Bone marrow mononuclear phagocytic precursor cells were isolated from femurs and tibiae, from HFD WT and NOV-/- mice as described (29). These precursors were differentiated into adherent mature macrophages (BMM) for 6 days in non-coated petri dishes in DMEM (4.5 g/L D-glucose) with 20% decomplemented fetal calf serum (FCS) (Eurobio) containing 20 ng/mL M-CSF (Peprotech, Neuilly-sur-Seine, France). BMM were seeded at 2 X 10^6 cells/well in 6-well plates and polarized towards a M1 phenotype with 10ng/mL LPS (Sigma-Aldrich) or a M2 phenotype with 10ng/mL IL4 (Peprotech) for 24h. Cytokine secretion into the cell culture medium of BMM was subsequently analyzed using TNF-α for M1 or IL-10 factor for M2 phenotype Elisa kit (Peprotech).

Peritoneal macrophages were derived from HFD WT and NOV-/- mice, following an intraperitoneal administration of PBS 1X (5 mL). The peritoneal wash was recovered from injected mice and peritoneal macrophages (PEM) were collected by centrifugation at 1500

rpm at 4°C for 5 min. PEM at a density of 10^6 cells/well in 6-well plate and polarized towards a M1 or a M2 phenotype as described for BMM.

Gene expression analysis

Total RNA was extracted from mouse tissues, primary preadipocytes, adipocyte cultures and from 3T3-L1 cells using RNeasy Lipid Mini or RNeasy Mini Kits (Qiagen, Les Ulis, France). Reverse transcription and semi-quantitative real-time PCR amplification (RT-qPCR) on an ABI 7300 apparatus (Applied Biosystems) were performed as previously described (13). Specific primers (Proligo, Sigma-Aldrich) used for the amplification of target genes are presented in Table S1. The comparative Ct method (30) was used to calculate gene expression values. The ribosomal S26 was used for normalization as a housekeeping gene.

siRNA and transfection

Small interfering RNAs (siRNA) sequences predesigned by Qiagen are listed in Table S2. 3T3-L1 cells plated at $9x10^3$ cells/cm^2 in DMEM containing 10% FCS were transfected, one day later, with siRNA (120 pmoles in 5 µL) using the RNAi Max reagent (Qiagen). Seventy-two hours after transfection (day 0), differentiation was initiated. On day 3 after the induction of differentiation, total RNA extracted from the cells was subjected to RT-qPCR. For the study of insulin signaling, 3T3-L1 cells were transfected on day 3 with siRNA (240 pmoles in 10 µL); then, on day 5, the cultures were deprived of serum and insulin and were further treated on day 6 with insulin (10^{-8} mol/L) for 8 min. RNA was obtained from parallel cultures and subjected to RT-qPCR.

Protein analysis

3T3-L1 adipocytes or mouse tissues were lysed, and equal protein amounts (20 µg) were separated by SDS-PAGE. The conditioned medium corresponding to 10^6 3T3-L1 adipocytes was used for ELISA tests or for NOV expression.

Antibodies and chemicals

Recombinant human NOV protein was obtained as previously described (15; 17). For NOV protein detection, we used a homemade affinity-purified rabbit antibody (referred as CT-Mu, anti-mouse NOV antibody) (31).

Rabbit polyclonal antibodies against total Akt, phosphorylated Akt (P-Ser 473), total Erk, and phosphorylated Erk, were from Cell Signaling, UCP-1, PGC1-α, parp-1 were from Abcam (Paris, France) and mouse monoclonal antibodies against β-actin, β-tubulin, GAPDH, were from Sigma Aldrich.

Statistics

The results are expressed as the mean ± SEM. Variance equality was analyzed by an F-test (Microsoft Excel, Issy-Les-Moulineaux, France). Comparisons between groups in animal studies, were carried out using a parametric Student's t test or by a nonparametric Mann–Whitney–Wilcoxon's test (Minitab, Paris, France). A P value of <0.05 was considered statistically significant.

Results

NOV-/- mice are protected against HFD-induced obesity, glucose intolerance and insulin resistance

We addressed the impact of NOV deficiency on weight gain in mice kept on either a SD or HFD for 16 wks. No difference was observed on a SD between the 2 groups of mice (Fig 1A,B). However, on a HFD, NOV-/- mice gained less weight than WT controls (Fig 1C). The body weight gain (BW) of NOV-/- mice was markedly lower compared with WT mice (Fig 1D). NOV-/- mice fed a HFD also displayed a decrease in fat and relative fat mass (Fig 1E,F) and a slight increase in relative lean body mass compared to their respective controls, but the absolute lean mass was unchanged (Fig 1G,H) and there was no difference in mouse-tail length (96±0.83 and 96.5±1.32 mm for WT and NOV-/-, respectively). Mice were subjected to an intensive analysis of energy intake and energy expenditure using indirect calorimetry. No significant change in food intake (Fig 1I), or spontaneous locomotor activity (Fig 1K and S2C) was observed. However a slight increase in energy expenditure was detected in NOV -/- mice, but limited to the day-light period (Fig 1J and S2B). The respiratory exchange ratio (RER; VCO_2/VO_2) did not change between NOV -/- and WT mice (Fig 1L and S2D).

To examine whether the marked differences in BW gain and adiposity between the 2 genotypes were associated with changes in carbohydrate metabolism, we evaluated glycemia and insulinemia under basal and dynamic conditions (Fig 2). During OGTT, HFD-fed (Fig 2B) but not SD-fed (Fig 2A) NOV-/- animals showed improved glucose tolerance compared with WT mice. This correlated with a significant decrease in the AUC for NOV-/- mice compared to controls (Fig 2B, upper panels). Of note, fasting glucose levels were slightly lower in HFD-fed NOV-/- mice compared to their WT controls. We then conducted an ipITT (Fig 2 C,D), which demonstrated in HFD-fed mice, an improved insulin sensitivity in NOV-/- mice compared with their WT littermates (Fig 2D). The improved glucose tolerance observed

in HFD-fed NOV-/- mice was not accompanied by an increased insulinogenic index after the oral glucose challenge compared with the WT controls (Fig 2E). Because a previous work reported decreased insulin release subsequent to NOV overexpression in a rat β-cell line (25), insulin secretion was tested in islets isolated from WT and NOV-/- mice fed a HFD. Insulin secretion similarly increased in both control, and NOV-/- islets when exposed to 16.7 mM glucose or 50 mM KCl compared to 5.5 mM glucose (Fig 2F). The pancreatic and islet insulin contents were also similar between the two genotypes. Further pancreatic morphometry analysis showed that beta-cell fraction, beta-cell mass, mean islet density and mean islet size were similar in NOV -/- and WT mice fed on a HFD (Fig S3).

Collectively, these observations show that on a HFD, NOV deficiency decreased weight gain and improved glucose tolerance and insulin sensitivity, while insulin production remained unchanged.

NOV-/- mice on a HFD display less adipose tissue, a higher proportion of small adipocytes and improved lipid and hepatic parameters

On a SD, the organ weights to BW ratios were not significantly different between the two genotypes (Table 1). However, on a HFD, a striking and similar decrease of these ratios was observed in NOV-/- mice compared with the WT controls for all the white adipose tissue depots. We also assessed the adipocyte size in H&E stained epididymal and subcutaneous fat tissue from WT and NOV-/- mice fed a HFD. Of interest, we observed that NOV deficiency influenced the adipocyte size distribution in both fat tissues (Fig 3). The mean adipocyte surface area in both eWAT and scWAT was markedly reduced in NOV-/- compared to WT mice (Fig 3A,B). Moreover, Chi-squared analysis on more than 1500 independent determinations showed that in both eWAT and scWAT, NOV-/- displayed a higher proportion of small adipocytes compared to those of the WT mice ($p<0.0001$) (Fig 3C,D). Adipocyte

cellularity was also evaluated in eWAT and scWAT with the assumption that adipocytes represent approximately 90% of adipose tissue volume (32). The results revealed no difference in the number of adipocytes in scWAT between WT and NOV-/- mice (3.10±0.41 and 3.1±0.46 $x10^6$ cells/g of tissue, respectively). In contrast, the adipocyte number in eWAT was significantly increased in NOV-/- mice (3.3±0.47 *vs* 2.1±0.32 $x10^6$ cells/g of tissue, respectively, $p<0.05$).

We also analyzed several plasma metabolic parameters, which are summarized in Table 1. On a SD, no differences could be detected between the 2 genotypes. In WT mice, HFD induced a significant increase in the concentrations of cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), nonesterified fatty acids (NEFA) and leptin. In NOV-/- mice, HFD also provoked an increase in cholesterol, triglycerides, HDL-C and leptin, but not NEFA. However, compared to WT, the increases in cholesterol, HDL-C and leptin were significantly reduced in NOV-/- mice. In these mice, a trend ($p<0.1$) towards a lower concentration of low-density lipoprotein cholesterol (LDL-C) was also observed.

Basal or induced lipolysis was also investigated in adipocytes isolated from eWAT fat depot from HFD mice. As shown in Fig S4, both basal and isoproterenol-stimulated lipolysis was stimulated in adipocytes from NOV-/- as compared to those of WT animals.

The liver phenotype of WT and NOV-/- mice fed a HFD was also characterized. Analysis of hepatic histology illustrated in Fig S5A revealed less steatosis in NOV-/- mice. Indeed, the hepatic triacylglycerol (TG) content, was reduced by ~ 3-fold (Fig S5B). This observation was consistent with decreased mRNA levels of the fatty acid transporter CD36 and its main transactivator PPARγ (Fig S5C). By contrast, no significant difference was detected for genes involved in fatty acid oxidation (PPARα, ACOX1), lipogenesis (SREBP-1c, ACC) or neoglucogenesis (PEPCK1, G6Pase). Interestingly, we did not detect any change in several liver inflammation markers (CCL2, TNFα, CD68, F4/80) (Fig S5D).

In vitro preadipocyte differentiation is increased by NOV deficiency

As a difference in adipogenesis could at least, in part, explain the reduced adipocyte size and adipose mass found in HFD-fed NOV-/- mice, we first investigated NOV expression in differentiating 3T3-L1 cells. As shown in Fig S6A, NOV mRNA and protein expression was strongly induced during differentiation. In agreement with this observation, there was an approximately 10-fold increase in the NOV transcript level after adipocyte differentiation in primary cultures (Fig S6B). These findings suggested that NOV could be involved in adipogenesis. Thus, we then analyzed the expression of the three major transcription factors induced during adipogenesis in eWAT, perirenal WAT (prWAT) and scWAT. As shown in Fig 4 (A,B,C), we did not find any significant differences in the expression of SREBP-1c, PPARγ and C/EBPα between WT and NOV-/- mice in the three fat depots, but the pattern of these mRNAs did not exclude the possibility that NOV could modulate adipogenesis. To examine whether an alteration in adipocyte differentiation was an intrinsic property of NOV-deficient mice, we isolated stroma vascular fractions (SVF) from eWAT and scWAT and tested their *in vitro* adipogenic capacity. Interestingly, we showed (Fig 4D,E) that preadipocytes originating from NOV-/- mice had a greater adipocyte differentiation ability than those from WT mice, as assessed by the increase in SREBP-1c, PPARγ and C/EBPα expression. In line with this result, when NOV expression was suppressed in 3T3-L1 preadipocytes by transfection with an siRNA specific for NOV, the expression of SREBP-1c, PPARγ and C/EBPα was also increased compared to the same cells transfected with a non-silencing siRNA (Fig 4F). We did not observe any difference in the proliferation capacity of preadipocytes derived from WT or NOV-/- mice (Fig S6C).

These results show that under HFD, the relative resistance to obesity and insulin resistance of NOV-/- mice might be related to a higher proportion of small adipocytes that likely exhibit better intrinsic adipogenic ability in the absence of NOV.

Inflammation is reduced in adipose tissue from NOV-/- mice

NOV has been involved in the regulation of various inflammatory molecules (10; 20) that are known to impair insulin signaling and promote insulin resistance in metabolic tissues. Consistent with enhanced insulin action and sensitivity (Fig 2), we observed a specific pattern of altered inflammatory gene expression in different adipose tissues from HFD-fed NOV-/- mice (Fig 5). In eWAT and prWAT, transcript levels of TNFα, CCL2 and F4/80 were decreased (Fig 5A,B), whereas the expression of these genes did not differ from WT in scWAT (Fig 5C). We also detected a decrease in leptin levels in eWAT and scWAT in NOV-/- animals, consistent with their reduced leptin plasma levels (Table 1). Thus, in NOV-/- mice on a HFD, the reduced proinflammatory pattern of gene expression essentially affects the visceral fat depots, which are generally considered to be crucial in the control of metabolic status. Consistent with these data, we found that treatment of 3T3-L1 cells with NOV recombinant protein led to a striking increase in CCL2 expression at both the mRNA and protein level (Fig 5 D,E).

It is now largely recognized that rodent or human obesity is associated with a low grade inflammation of adipose tissue and its infiltration by immune cells, including lymphocytes and macrophages that shift from a M2-like anti-inflammatory to a M1-like pro-inflammatory phenotype (33). In addition, these changes in macrophage phenotypes are likely involved in obesity-linked insulin resistance (34). Given the metabolic phenotype and the profile of gene expression in deep fat depots of NOV-/- animals, we further characterized the immune cell populations present in eWAT of NOV-/- and WT mice. The absence of NOV was associated

with an increase in a population of macrophages expressing high levels of the M2-like marker CD206 (Fig 5F) and with a trend to less macrophages displaying an apparent M1-like profile (CD11c+). No difference in the overall frequency of CD3+ lymphocytes population was detected between the two genotypes (data not shown). Whereas, no difference was observed in the frequency of CD8+T cells, percentages of CD4+ lymphocytes among infiltrating T cells were strongly decreased in NOV-/- compared to WT (Fig 5G left panel). This was related to significantly reduced frequency of conventional CD4+Foxp3- T cells with no difference in percentages of CD4+Foxp3+ regulatory T cells (Fig 5G right panel). Interestingly, a large proportion of infiltrating CD3+ lymphocytes were identified CD4-CD8- double negative (DN) T cells, the frequency of which was significantly increased in NOV-/- as compared to WT mice (Fig 5G left panel).

We further analyzed whether NOV deletion can affect M1 *vs* M2 polarization in myeloid/macrophages cells. Macrophages were derived from bone marrow (or peritoneal macrophages) of WT and NOV-/- HFD mice. NOV expression was reported in human macrophages (21), however its expression in macrophages from HFD WT mice has not been investigated so far. In our experimental conditions the levels of NOV mRNA in BMM or PEM derived from HFD WT mice were below the threshold of detection (data not shown). Nevertheless, as a transient expression of NOV during macrophage maturation or differentiation could affect their susceptibility to pro-inflammatory molecules we subjected WT and NOV-/- BMM or PEM cultures to polarization to a M1-like or M2-like phenotype with LPS and IL4, respectively (32). As compared to untreated control cultures, the mRNA levels of M1-like pro-inflammatory markers such as TNFα and CCL2 were significantly induced in BMM cultures treated with LPS, whereas IL4 markedly enhanced the M2-like marker CD206 (Fig S7A). In our experimental conditions, we also observed as previously described (35) that the mRNA levels of the M2-like marker IL10 was considerably induced

by LPS. Both TNFα and IL10 proteins analysis confirmed the mRNA results (Fig S7B). However, any significant difference could be detected between WT and NOV-/- BMM under these different treatments (Fig S7). Similar results were also obtained with PEM (data not shown). Thus, whereas NOV-/- were protected against inflammation in adipose tissue it does not seem to be due to its absence in macrophages.

~~Thus, NOV-/- mice were protected against inflammation, and~~ As limited inflammation and reduced fibrosis were previously described in NOV-/- mice in the unilateral ureteral obstruction (UUO) experimental model of nephropathy (36) and as ~~As~~ fibrosis in adipose tissue is also linked to insulin-resistance (37), we also studied the impact of NOV deletion on fibrosis of the adipose tissue. Analysis of the interstitial collagen accumulation, assessed by Sirius Red coloration, revealed a trend for decreased fibrosis in eWAT of NOV-/- animals and no significant difference between WT and NOV-/- mice in the scWAT (Fig S8).

Expression of genes involved in energy expenditure is increased by NOV deficiency in adipose tissue

A slight increase in energy expenditure was observed in NOV -/- mice (Fig 1J). As an increased turnover of adipose cells in these mice could be energetically more expensive, we first analyzed markers of apoptosis in adipose tissue. Western blots did not reveal any significant differences in the Parp-1 cleavage in scWAT of both genotypes on a HFD, whereas in eWAT Parp-1 cleavage was even decreased in NOV-/- animals (Fig S9). Thus, these results could not account for the lower gain weight of NOV-/- mice.

We next analyzed in the brown adipose tissue (BAT) and WAT the expression of genes known to be involved in energy expenditure. In HFD-fed NOV-/- mice, the expression of the uncoupling protein 1 (UCP-1) was increased in both BAT and eWAT at both mRNA and protein levels (Fig 6). The expression of PGC1α, a major co-regulator that cooperates with

PPARγ to transactivate the UCP-1 gene promoter (38) was also significantly increased in BAT of these mice (Fig 6).

In vitro and in vivo NOV deficiency increases insulin sensitivity

To further delineate the molecular mechanisms involved in the improved insulin sensitivity in NOV-/- mice, we analyzed the effect of NOV deficiency on insulin signaling. We found that in 3T3-L1 adipocytes transfected with two different siRNAs specific for NOV, the decrease in NOV expression was accompanied by a marked increase in insulin-stimulated phosphorylation of Akt (p-Akt) and ERK (p-Erk) compared to the same cells transfected with a non-silencing siRNA as a control (sc) (Fig 7A,B,C). *In vivo*, we also observed that an acute exposure to insulin resulted in a significant increase in phosphorylation of AKT(p-Akt) in eWAT of NOV-/- compared to WT mice (Fig 7D,E) but not in scWAT (data not shown).

Discussion

In this study, we report for the first time that on a HFD, NOV deficiency protects mice against obesity, likely through an increase in energy expenditure. We also report that in NOV-/- mice on a HFD, the inflammation of the eWAT and prWAT was reduced compared to WT mice. Taken together, these differences between WT and NOV-/- mice likely account for the improved glucose tolerance, insulin sensitivity and metabolic profile of NOV-/- mice.

The increased population of small adipocytes in eWAT could be due to the better proliferation or the better differentiation ability of the resident preadipocytes. Our data are in favor of the second hypothesis because we did not observe any difference in the proliferation rate of primary preadipocytes derived from WT or NOV-/- mice. In contrast, the absence of NOV had a positive impact on the differentiation ability of these cells. Moreover, different data have reported that NOV could play an effective role in the control of cell differentiation, as it can inhibit myoblast and osteoblast differentiation (13; 39; 40). It is also crucial for primitive hematopoietic progenitor cell activity (14). However, while the improved ability to differentiate in NOV-/- preadipocytes was observed in both eWAT and scWAT, the cellularity was only increased in eWAT. This observation is in accordance with recent data (41) showing that in mice fed a HFD for over one month, adipogenesis is preferentially initiated in eWAT compared to scWAT. This potentially improved ability for preadipocyte differentiation, in which NOV is disrupted or inhibited, is apparently contradictory with the decreased fat mass in NOV-/- mice and with the increase in NOV expression *in vitro* during the differentiation process. Our results suggest that the absence of NOV favors the recruitment of smaller adipocytes that are more sensitive to insulin. However, a longer follow-up of WT and NOV-/- mice would be required to examine whether the recruitment of small adipocytes maintains the limited expansion of fat mass in NOV-/- mice or whether this is overcome over time by increased cellularity.

Taking into account the whole body, this fat mass loss occurs while there is no decrease in food intake and preliminary analysis of lipids in feces did not reveal any significant difference in gut absorption of lipids between NOV-/- and control mice (Fig S10). However, there is a slight, but significant increased energy expenditure during the day-light period. Remarkably, this enhanced energy expenditure is associated with an induction in BAT and eWAT in the expression of UCP1 and PGC1-α, two key effectors of the thermogenic pathway. This could represent a major phenomenon to protect NOV-/- mice from HFD-induced obesity and insulin resistance. However, the molecular mechanisms by which the absence of NOV leads to UCP1 induction remain unknown. For instance, the increased lipolytic activity of NOV-/- adipocytes could also promote substrate availability for thermogenesis.

In this study, we report that the impact of the absence of NOV is only detectable when mice are challenged with a HFD. Indeed, two reports describing the phenotype of NOV-/- mice revealed that they are viable and apparently normal and fertile (22; 40). However, following a lesion of the femoral artery, the null mice presented an enhancement of neointimal thickening compared with wild-type mice during vascular repair (22). Another example showed that following an injury to the femur, bone regeneration in NOV-/- mice was accelerated compared with WT mice (40). More recently, we also found that during the progression of obstructive nephropathy, NOV-/- mice displayed limited inflammation and renal interstitial fibrosis compared to WT mice (36).

A HFD is known to induce low grade inflammation in adipose tissue (42) and is associated with the increased expression of NOV mRNA in adipose tissue and NOV plasma levels (21). Under these specific nutritional conditions, the absence of NOV not only limits adipose tissue expansion but is also associated with improved glucose tolerance. This is primarily related to better insulin sensitivity, as supported by insulin tolerance tests and by the normalization of plasma NEFA levels in NOV-/- mice fed a HFD, suggesting the restoration of the antilipolytic

effect of insulin. These results are strengthened by *in vitro* and *in vivo* experiments showing that NOV deficiency in adipocytes promotes insulin signaling.

Our results also strongly suggest that NOV could be a key player in adipose tissue inflammation and that this in turn could promote insulin resistance. Recent studies have shown that CD4+ and CD8+ T cells play a major role in promoting adipose tissue inflammation by secreting cytokines and recruiting macrophages during HFD (43). NOV deficiency led to a strong decrease in the frequency of infiltrating conventional CD4+ T cells, whereas no difference was observed neither for CD8+ nor CD4+ regulatory T cells.

Of note, NOV-/- mice displayed a significantly increased sub-population of CD3+ T lymphocytes that were double negative CD4-CD8- double negative. These particular T cells that lack the expression of both CD4 and CD8 T cell co-receptors have been studied in both mice and humans for their contribution to peripheral tolerance and disease prevention (44). Our current findings are consistent with these observations. The reduced TNFα and F4/80 expression in two visceral WAT (eWAT and prWAT) from HFD-fed NOV-/- mice, reflects the decreased inflammation in these fat depots and is in agreement with the involvement of these tissues in insulin sensitivity (45-47). Interestingly, a decrease in TNFα and F4/80 expression was not detected in scWAT, which is generally considered to have an accessory role in the insulin resistance phenotype. FACS analysis on eWAT suggests that under HFD, the absence of NOV was associated with a switched overbalance from M1-like to M2-like macrophage population. However, our *in vitro* analysis revealed that NOV is not expressed by two different types of macrophages thus, this strongly suggests that the switch from M1-like to M2-like macrophage population does not result from an autocrine action of NOV on macrophages but rather from an indirect role of NOV on the expression of proinflammatory molecules by adipocytes and possibly also by other cells present in the adipose tissue. Given the documented protective role of such M2-like populations against obesity-linked insulin-

resistance, this suggests that these changes may also contribute to the improved insulin sensitivity and glucose tolerance of NOV-/- mice.

Our *in vitro* and *in vivo* experiments revealed that CCL2 expression is modulated by NOV. This regulation may be considered to be pivotal in the development of insulin resistance because targeted deletions in mice for the CCL2 and/or CCR2 genes result in reduced macrophage content, decreased inflammation in fat and protection from HFD–induced insulin resistance; conversely, mice overexpressing CCL2 in adipose tissue have increased local macrophage infiltration along with insulin resistance (47-49). The specific macrophage marker F4/80 expression is reduced in NOV-/- mice WAT. This suggests that the absence of NOV could decrease CCL2 with a subsequent reduction in macrophage number within the adipose tissue. However, the regulation of CCL2 by NOV could also have an impact on insulin sensitivity through additional pathways because it has also been shown that an increase in the plasma levels of CCL2 is sufficient to induce insulin resistance independent of macrophage infiltration into adipose tissue (50). Accordingly, CCL2, through its angiogenic effect on endothelial cells, could also contribute to the expansion and remodeling of adipose tissue (51).

During OGTT, we did not observe higher plasma insulin concentration in NOV-/- mice compared to WT controls. In isolated islets, insulin release was also comparable between the two genotypes, and the total content of insulin either in the whole pancreas or in isolated islets was similar in NOV-/- and WT mice. These results are apparently discordant with those reported by Paradis et al. (25). Nevertheless, the experimental conditions used by that study and ours substantially differ because they used a rat cell line overexpressing NOV. Taken together, our results suggest that the increase in insulin sensitivity is the major mechanism by which glucose tolerance is improved in NOV-/- mice.

In summary, the present study demonstrates that NOV participates in the development of obesity-induced insulin-resistance. These results indicate that the use of counteracting agents against NOV represents a potential strategy for new therapies against obesity and its metabolic comorbidities.

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CM, BF designed the research; MG, TTHD, BA, MM, GD, CK, MA, MB, TL, POM, MF, AB, BB, RD performed the research; HK, SH and CEC contributed new reagents /analytic tools; CM, BA, MM, GD, BB, RD, SL, BF analyzed data; and CM, BF wrote the paper.

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Pr B. Feve 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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Figure Legends

Figure 1: Body weight of WT (open symbols and bars) and NOV-/- (black symbols and bars) mice (n=5-9 for each group) during a 16 wk time course with a SD (A) or HFD (C). Body weight gain in WT and NOV-/- mice under a SD (B) or HFD (D). Mean fat content (E), lean body mass (G) content and relative fat content (g/body weight) (F) and relative lean body mass (g/body weight) (H) of WT and NOV -/- mice fed a HFD for 14 wks (n=5 for each group). Food intake (I). Whole energy expenditure was expressed as kcal/kg/hr (J), spontaneous locomotor activity (Beam Break per hour) (K), Respiratory Exchange Ratio (VCO_2/ VO_2) (L) of WT and NOV-/- mice fed a HFD for 14 wks (n=5 for each group) was expressed by the mean of 4 days and 4 nights.

Values are expressed as the means ± SEM. * $p<0.05$; **$p<0.01$, NOV-/- *vs* WT mice.

Figure 2: Glucose tolerance, insulin sensitivity and insulin secretion in WT (open symbols and bars) and NOV-/- (black symbols and bars) mice.

OGTT (A,B) and ITT (C,D) performed after 14 and 15 wks, respectively, for SD (A,C) or HFD-fed animals (B,D). OGTT mice (n=10-14 per group) were subjected to a 6-h fast. The area under the curve (AUC) is presented in the upper panels (A,B). The results are expressed as the means ± SEM. ITT mice (n=8-11 per group). (C,D) were subjected to a 4 h fast. The results are expressed as the percentage of basal glycemia The AUC is presented in the upper panels (C,D). Insulinogenic index (E) was determined at 0 and 15 min following oral feeding with glucose and expressed as the ratio Ins15min-Ins0 (mU/L)/G15min–G0(mmol/L). Insulin secretion (F) assayed after *in vitro* incubation of islets in 5.5 mM, 16.7 mM glucose or 50 mM KCl (n=4-5 per group). *$p<0.05$; *** $p<0.001$, NOV-/- *vs* WT mice.

Figure 3: Adipocyte size and cellularity in WT and NOV-/- mice fed a HFD for 16 wks.

(A-B) Representative H&E-stained sections of eWAT and scWAT. (C-D) Adipocyte size distribution determined from eWAT (C) and scWAT (D) histomorphometry and image analysis; insets (upper panels) show the mean adipocyte surface ± SEM ***p<0.001. $\chi 2$ test was performed for each histogram; ***p<0.0001, NOV-/- *vs* WT animals.

Figure 4: Expression of genes involved in adipocyte differentiation

HFD-fed mice for 16 wks (n=6-8 per group): (A) eWAT, (B) perirenal WAT (prWAT), (C) scWAT. Stroma-vascular fraction derived from 16 wks-HFD mice eWAT (D), scWAT (E) were cultured and subjected to adipocyte differentiation for 7 days. (F) 3T3-L1 cells transfected with control non-silencing siRNA (SC) or a specific siRNA against NOV (Si NOV); adipocyte differentiation was induced for 3 days. In the inset, the inhibition of NOV expression was evaluated on day 3 after adipocyte differentiation. Data represent the mean values of four measurements and correspond to a representative experiment performed at least three times with similar results. **p<0.01; ***p<0.001, NOV-/- or si NOV conditions *vs* control.

Figure 5: Expression of inflammatory genes and analysis of infiltrating immune cell populations

HFD-fed mice for 16 wks (n=6-8 per group): (A) eWAT, (B) perirenal WAT (prWAT), (C) scWAT. (D-E) Following 24 h of serum starvation, 3T3-L1 adipocytes were incubated either with (Ctr) or NOV (10 µg/mL) for 24 h and the level of CCL2 expression was evaluated in cells by RT-qPCR (D) or in the conditioned media by ELISA (E). Data represent the mean values ± SEM (n=4-9 experiments). *p<0.05, **p<0.01, *** p<0.001, KO mice or NOV-treated cells *vs* control. (F and G) Flow cytometry analysis of macrophages and lymphocytes

isolated from eWAT of WT and NOV-/- mice. (F) Percentages of CD11c+ (left panel), or CD206+ ^{high} (right panel) cells among CD11b+ F4/80+ macrophages. (G) Percentages of total CD8+ CD4+ or CD4-CD8- DN T cells (left panel) and CD4+Foxp3+ regulatory T cells or CD4+ Foxp3- conventional T cells (right panel) among stromal CD3+ lymphocytes. Data represent mean values ± SEM (n=4 per group).* p< 0.05. (H)

Figure 6: Expression of genes and proteins involved in energy expenditure

HFD-fed mice for 16 wks (n=6-8 per group): (A) BAT (B) eWAT ; (C and D) representative western blots of BAT and eWAT; (E and F) Bar graphs show fold change in UCP1 and PGC1α in KO (black bars) *vs* WT (open bars). The results are expressed as the means ± SEM. * p<0.05; ***p<0.001, NOV-/- *vs* WT mice.

Figure 7: Insulin signaling in siNOV-transfected 3T3-L1 adipocytes and in eWAT from HFD-fed mice

(A) Representative western blots of 3T3-L1 adipocytes transfected either with a control non silencing siRNA (Sc) or with two different specific siRNAs against NOV (si1 and si4) from day 3 to 6 after adipocyte differentiation. (B) Bar graphs show fold change in p-Akt/Akt and p-Erk/Erk in si1- and si4- *vs* sc- transfected cells. Values are the means ± SEM of 3 experiments. (C) NOV gene expression as determined in parallel cultures. *p<0.05; *** p<0.001, si1 or si4-treated *vs* sc-treated cells. (D) Representative western blots of eWAT from WT and KO mice following or not a 10 min exposure to insulin (0.5 Units). (E) Bar graphs show fold change in p-Akt/Akt. The results are expressed as the means ± SEM. ### p<0.001, NOV-/- *vs* WT mice; ***p<0.001, insulin-treated *vs* untreated animals.

Table 1: Organ weight and biological parameters in WT and KO mice under SD and HFD

		SD		HFD	
		WT	**KO**	**WT**	**KO**
Animals (n)		7	10	9	9
Organ weight (mg)/BW (g)					
	Liver	43.53±1.6	43.88±1.0	40.92±1.4	42.95±1.4
	Spleen	4.16±0.7	4.14±0.4	2.82±0.4	3.22±0.3
	Kidney	7.91±0.6	7.82±0.5	6.23±0.3	7.53±0.4*
	Pancreas	7.23±0.6	7.44±0.5	8.15±0.4	7.59±0.6
	Perirenal AT	2.88±0.6	3.44±0.6	9.44±1.1‡‡‡	4.84±0.9**
	Epididymal AT	6.45±0.9	5.90±0.9	22.81±1.71‡‡‡	13.19±1.4***‡‡‡
	Sub-cutaneous AT	6.71±0.8	7.72±0.7	16.74±2.2‡‡	9.13±1.7*
	Brown AT	4.1±0.7	3.83±0.6	6.56±0.7‡	4.8±0.5
Biological parameters					
	Glucose (mmol/l)	6.88±0.1	6.71±0.1	9.23±0.25‡‡‡	8.35±0.3*‡‡‡
	Insulin (mUI/L)	ND	ND	16.04±2.82	15.21±1.29
	Cholesterol (g/l)	0.748±0.05	0.683±0.02	1.79±0.1‡‡‡	1.51±0.1*‡‡‡
	Triglycerides (g/l)	0.481±0.03	0.472±0.03	2.65±0.1‡‡‡	2.35±0.1‡‡‡
	HDL-C (g/l)	0.726±0.06	0.715±0.054	1.55±0.1‡‡‡	1.29±0.1**‡‡‡
	LDL-C (g/l)	ND	ND	0.118±0.05	0.063±0.04
	Non Esterified Fatty Acids(µmol/l)	168±16	165±31	259±30‡	158±20**
	Leptin (ng/ml)	0.08±0.04	0.04±0.03	1.5±0.3‡‡‡	0.66±0.2*‡‡
	Adiponectin(mg/ml)	2.11±0.2	1.93±0.3	2.42±0.2	2.25±0.2

All these parameters were obtained at sacrifice after 16 wks of diet (SD or HFD). Results are expressed as means ± SEM.

*p<0.05; **p<0.01; ***p<0.001, KO *vs* WT mice (within the same diet).

‡ p<0.05; ‡‡p<0.01; ‡‡‡p<0.001, HFD *vs* SD mice (within the same genotype).

AT: Adipose tissue, BW: Body Weight, ND: not determined, SD: Standard Diet, HFD: High Fat Diet, HDL-C: HDL-cholesterol, LDL-C: LDL-cholesterol.