Tissue Inhibitor of Metalloproteinase 3 Deficiency Causes Hepatic Steatosis and Adipose Tissue Inflammation in Mice

ROSSELLA MENGHINI,[*] STEFANO MENINI,[‡] ROBERTA AMORUSO,[*] LOREDANA FIORENTINO,[*] VIVIANA CASAGRANDE,[*] VALERIA MARZANO,[*,§] FEDERICA TORNEI,[*] PIERFRANCESCO BERTUCCI,[‖] CARLA IACOBINI,[‡] MATTEO SERINO,[*] OTTAVIA PORZIO,[*,‖] MARTA L. HRIBAL,[¶] FRANCO FOLLI,[#] RAMA KHOKHA,[**] ANDREA URBANI,[§,‡‡,§§] RENATO LAURO,[*,‖] GIUSEPPE PUGLIESE,[‡] and MASSIMO FEDERICI[*,‖‖]

[*]Department of Internal Medicine, University of Rome "Tor Vergata," Rome, Italy; [‡]Department of Clinical Sciences, "La Sapienza" University, Rome, Italy; [§]Laboratory of Proteomics, European Brain Research Institute/Santa Lucia Foundation, Rome, Italy; [‖]Department of Laboratory Medicine, [‖‖]Center for Atherosclerosis, "Policlinico Tor Vergata" University Hospital, Rome, Italy; [¶]University of Magna Graecia, Catanzaro, Italy; [#]Division of Diabetes, University of Texas Health and Science Center, San Antonio, Texas; [**]Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada; [‡‡]Centro Studi sull'Invecchiamento, University Foundation "G. D'Annunzio," Chieti, Italy; and the [§§]Department of Biomedical Science, University "G. D'Annunzio," Chieti, Italy

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Background & Aims: **Obesity-driven, low-grade inflammation affects systemic metabolic function and can lead to insulin resistance, hepatic steatosis, and atherosclerosis. Decreased expression of tissue inhibitor of metalloproteinase 3 (Timp3) is a catalyst for insulin resistance and inflammation. Timp3 is a natural inhibitor of matrix metalloproteinases, tumor necrosis factor-α–converting enzyme (TACE), and vascular endothelial growth factor receptor 2, and therefore could affect signaling processes involved in inflammation and angiogenesis.** ***Methods:*** **We assessed the effects of Timp3 on inflammation, tissue remodeling, and intermediary metabolism in mice, under conditions of environmental stress (high-fat diet), genetic predisposition to insulin resistance (insulin receptor [Insr] haploinsufficiency), and varying levels of inflammation (Timp3 or Tace deficiencies). Metabolic tests, immunohistochemistry, real-time polymerase chain reaction, and immunoblotting were used to compare data from wild-type, $Insr^{+/-}$, $Timp3^{-/-}$, $Insr^{+/-}Timp3^{-/-}$, and $Insr^{+/-}Tace^{+/-}$ mice placed on high-fat diets for 10 weeks.** ***Results:*** **$Insr^{+/-}Timp3^{-/-}$ mice showed a higher degree of adipose and hepatic inflammation compared with wild-type, $Insr^{+/-}$, $Timp3^{-/-}$, and $Insr^{+/-}Tace^{+/-}$ mice. In particular, the $Insr^{+/-}Timp3^{-/-}$ mice developed macrovesicular steatosis and features of severe nonalcoholic fatty liver disease, including lobular and periportal inflammation, hepatocellular ballooning, and perisinusoidal fibrosis. These were associated with increased expression of inflammatory and steatosis markers, including suppressor of cytokine signaling 3 and stearoyl CoA desaturase 1, in both liver and adipose tissue. Interestingly, $Insr^{+/-}Tace^{+/-}$ mice had a nearly opposite phenotype.** ***Conclusions:*** **Timp3, possibly through its regulation of TACE, appears to have a role in the pathogenesis of fatty liver disease associated with obesity.**

Obesity, a chronic disorder with increased incidence and prevalence in Western countries, is believed to induce a systemic low-grade inflammatory state predisposing to diabetes, vascular diseases, and cancer, arising from deregulated release of free fatty acids and inflammatory cytokines from adipose tissue.[1] Free fatty acids and proinflammatory cytokines activate serine-threonine kinases such as c-Jun-N-terminal kinase (JNK) and protein kinase C θ, which perturb glucose homeostasis by blunting insulin-receptor–dependent signaling pathways in peripheral tissues including liver.[1–3]

One major metabolic consequence of increased lipid efflux to the liver and obesity-driven inflammation is hepatic steatosis, which is sustained by alteration in both inflammatory and metabolic pathways.[4–6] Pathways and mechanisms linking obesity to hepatic steatosis are not completely elucidated but involve expression of monocyte chemotactic protein-1 (MCP-1) and its receptor C-C motif chemokine receptor-2.[7,8]

We recently identified a modifier gene for insulin resistance and inflammation in mice, tissue inhibitor of metalloproteinase 3 (Timp3).[9] Timp3 acts pericellularly to modulate several cell membrane enzymes and receptors.[10,11] Among Timp3 substrates, tumor necrosis factor (TNF)-α–converting enzyme (TACE, also named *a disintegrin and metalloproteinase domain 17* or *ADAM17*) regulates TNF receptors, epidermal growth factor receptor (EGFR), and interleukin 6 receptor signaling pathways, putting Timp3 at the cross-road of inflammatory and

Abbreviations used in this paper: EGFR, epidermal growth factor receptor; HFD, high-fat diet; Insr, insulin receptor; JNK, Jun-N-terminal kinase; MCP-1, monocyte chemotactic protein-1; NAFLD, nonalcoholic fatty liver disease; NO, nitric oxide; PPARγ, peroxisome proliferator-activated receptor γ; SCD1, stearoyl CoA desaturase 1; SOCS-3, suppressor of cytokine signaling 3; TACE, tumor necrosis factor-α–converting enzyme; Timp3, tissue inhibitor of metalloproteinase 3; TNF, tumor necrosis factor; WAT, white adipose tissue; WT, wild-type.

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fibrosis signals.[10] However, via its inhibitory properties on matrix metalloproteinase-2 and matrix metalloproteinase-9 as well as vascular endothelial growth factor receptor 2, Timp3 may affect tissue remodeling and angiogenesis.[11–14] Moreover, we have shown that TACE deficiency confers partial protection from high-fat diet (HFD)-induced insulin resistance,[12] with positive effects on liver ability to resist inflammatory effects increased by HFD, particularly TNF-α–mediated insulin resistance.

Here, we test and exploit the effects of the signaling switch properties of Timp3 connecting inflammation, tissue remodeling, and intermediary metabolism. We show that combination of genetic manipulation and environmental stress (HFD) impacts on inflammation, steatosis, and fibrosis, resulting in a phenotype with characteristics similar to nonalcoholic fatty liver disease (NAFLD).[4–6,13,14]

Methods

Animal Models and Analytic Procedures

Insulin receptor $(Insr)^{+/-}Timp3^{-/-}$ and $Insr^{+/-}Tace^{+/-}$ were obtained breeding $Insr^{+/-}$, $Timp3^{-/-}$, and $TACE^{+/-}$ mice on a C57/BL6 background, as previously described.[9,12] Metabolic testing procedures have been described previously.[9,12] Briefly, for glucose tolerance tests animals were fasted for 16 hours and injected with 2 g/kg body weight of glucose into the peritoneal cavity; insulin tolerance tests were performed by injection of 0.75 U/kg body weight of human regular insulin (Novo Nordisk/AS Bagsvaerd, Denmark) into the peritoneal cavity of animals fasted for 6 hours. Blood glucose concentrations were determined by using an automated Onetouch Lifescan Glucometer (Milpitas, CA). Hormones and adipokines levels were measured using commercial kits: insulin (Mercodia, Uppsala, Sweden), adiponectin (R&D Systems), and leptin (R&D Systems, Minneapolis, MN). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using a Modular P analyzer (Roche SpA, Monza, Italy). Individually caged mice from all groups were fed an HFD (60% of calories from fat, code D12492; Research Diets, New Brunswick, NJ) or chow (standard chow, 10% calories from fat, code 4RF18; GLP Mucedola Srl, Settimo Milanese, Italy) for 10 weeks after weaning as indicated. Studies were performed only in male mice. Animal studies were approved by the University of Tor Vergata Animal Care and Use Committee.

Western Blots

Western blots were performed on total tissue homogenates and nuclear and cytosolic extracts prepared as previously described.[9,12,15,16] The following antibodies were used: anti-phospho Thr172 AMP-activated protein kinase (AMPK) (Cell Signaling Technology Inc, Danvers, MA) and total AMPK (UBI Upstate Biotechnology Inc, Waltham, MA), anti-phosphoTyr1068 EGFR and total EGFR (Santa Cruz, Santa Cruz, CA), anti-phosphoSer180/Ser181 IkB kinase (IKK)α/β and total IKK (Cell Signaling Technology Inc), anti-phosphoThr183/Tyr185 JNK and total JNK (Cell Signaling Technology Inc), anti-phospho Ser473 Akt and total Akt (Cell Signaling Technology Inc), anti-phosphoSer256 FoxO1 (Cell Signaling Technology Inc) and total FoxO1 (Santa Cruz), FoxA2 (Santa Cruz), Timp3 (Santa Cruz), Socs-3 (Santa Cruz), p65 (Abcam Inc, Cambridge, MA), histone 1 (Santa Cruz), topoisomerase I (Santa Cruz), and tubulin (Santa Cruz).

Insulin Signaling Studies

Insulin signaling studies with phospho-specific antibodies were performed as previously described.[9,12] Briefly, experiments were performed in overnight-fasted, 8- to 12-week-old mice. Animals were anesthetized by the intraperitoneal administration of sodium pentobarbital (65 mg/kg), and human insulin (5 U) was injected through the inferior vena cava. Liver and epididymal fat were removed 1 and 4 minutes after insulin injection, respectively, and homogenized as previously described.[9,12]

Gene Expression Analysis

Total RNA was isolated from fresh adipose tissues and liver using Trizol reagents (Invitrogen Corp, Eugene, OR). A total of 2 μg total RNA was reverse-transcribed into complementary DNA (cDNA) using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction was performed on individual samples from adult mice using an ABI PRISM 7700 System and TaqMan reagents (Applied Biosystems). Each reaction was performed in triplicate using standard reaction conditions. Calculations were performed by a comparative cycle threshold method: the starting copy number of test samples was determined in comparison with the known copy number of the calibrator sample (delta-delta Cycle threshold). The relative gene copy number was calculated as 2^{-ddCt}as previously described.[9,12] The list of Applied Biosystems primers for the 62 genes studied is listed in supplementary Table 1 (see supplementary material online at www.gastrojournal.org).

Adipose Tissue Histologic Analysis

Epididymal fat was obtained from 4-month-old mice, specimens were fixed in 10% paraformaldehyde, and embedded in paraffin. Consecutive sections (10 μm) then were mounted on slides and stained with H&E. MCP-1 and F4/80 immunostaining were performed using Abcam antibodies following the manufacturer's instructions. Adipose cell size was calculated with National Institutes of Health (Bethesda, MD) Image 1.62 software by manual tracing of at least 500 adipocytes for each genotype.

Histology and Quantification of Liver Lesions

Formalin-fixed liver tissue was processed, and 5-μm–thick paraffin sections were stained with H&E and Masson's trichrome for histologic analysis. For Oil Red