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mTORC1 inhibition with rapamycin exacerbates adipose tissue inflammation in obese mice and dissociates macrophage phenotype from function

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ABSTRACT

Genetic- and diet-induced obesity and insulin resistance are associated with an increase in mechanistic target of rapamycin complex (mTORC) 1 activity in adipose tissue. We investigated herein the effects of pharmacological mTORC1 inhibition in the development of adipose tissue inflammation induced by high-fat diet (HFD) feeding, as well as in the polarization, metabolism and function of bone marrow-derived macrophages (BMDM). For this, C57BL/6J mice fed with a standard chow diet or a HFD (60% of calories from fat) and treated with either vehicle (0.1% Me_2SO, 0.2% methylcellulose) or rapamycin (2 mg/kg/day, gavage) during 30 days were evaluated for body weight, adiposity, glucose tolerance and adipose tissue inflammation. Although rapamycin did not affect the increase in body weight and adiposity, it exacerbated the glucose intolerance and adipose tissue inflammation induced by HFD feeding, as evidenced by the increased adipose tissue percentage of M1 macrophages, naive and activated cytotoxic T lymphocytes, and mRNA levels of proinflammatory molecules, such as TNF-α, IL-6 and MCP-1. In BMDM *in vitro*, pharmacological mTORC1 inhibition induced phosphorylation of NFκB p65 and spontaneous polarization of macrophages to a proinflammatory M1 profile, while it impaired M2 polarization induced by IL-4 + IL-13, glycolysis and phagocytosis. Altogether, these findings indicate that mTORC1 activity is an important determinant of adipose tissue inflammatory profile and macrophage plasticity, metabolism and function.

1. Introduction

Chronic low-intensity inflammation has been suggested as a major linking factor between visceral obesity and the development of diseases such as insulin resistance, diabetes, and metabolic syndrome (Hotamisligil, 2006). Mechanistically, obesity-associated inflammation seems to be triggered by a combination of metabolic endotoxemia as the result of an increased gut permeability to the component of gram-negative bacteria wall, namely lipopolysaccharide (LPS) (Cani et al., 2007), along with cell metabolic stress induced by chronic exposure to excessive amounts of nutrients (Hotamisligil and Erbay, 2008), both of which having as underlying cause the elevated intake of lipid-enriched hypercaloric diets.

Chronically elevated systemic levels of LPS and nutrients, such as saturated fatty acids, promote tissue insulin resistance in part through the activation of the toll-like receptor (TLR)-4, a pattern-recognition receptor that triggers, through IκB kinase (IKK)-nuclear

Abbreviations: 4E-BP, eIF4E binding protein; AUC, area under the curve; BMDM, bone marrow-derived macrophages; BSA, bovine serum albumin; CLEC10a, C-type lectin domain family 10 member A; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinases; FACS, Fluorescence-activated cell sorting; GLUT1, glucose transporter 1; GTT, glucose tolerance test; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFD, high-fat diet; IFN-γ, interferon γ; IKKB, IkB kinase beta; IL-10, interleukin-10; IL-13, interleukin-13; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; IRS, insulin receptor substrate; JNK, c-jun N-terminal kinase; KRB, krebs ringer bicarbonate; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MGL2, macrophage galactose *N*-acetyl-galactosamine specific lectin 2; mTOR, mechanistic target of rapamycin complex; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol 3,4,5 triphosphate; PPARγ, peroxisome proliferator-activated receptor gamma; PVDF, polyvinylidene difluoride; S6K, p70 ribosomal S6 kinase; TLR-4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TSC, tuberous sclerosis complex; UPR, unfolded protein response.

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factor-κB (NFκB), a potent proinflammatory response (Hotamisligil and Erbay, 2008; Tsukumo et al., 2007). One important signaling event downstream to TLR-4 that may contribute to the development of obesity-associated inflammation and insulin resistance is the activation of the Mechanistic Target Of Rapamycin (mTOR) complex 1 (mTORC1), a multiprotein complex composed by the serine/threonine kinase mTOR (catalytic core) and other regulatory proteins (Laplante and Sabatini, 2012). mTORC1, which is also activated by growth factors and amino acids, stimulates anabolic processes, such as protein and lipid synthesis and inhibits catabolic processes, such as autophagy (Laplante and Sabatini, 2012). More specifically, TLR-4 activates mTORC1 through the inhibitory phosphorylation of the mTORC1 upstream inhibitor tuberous sclerosis complex (TSC)-1 catalyzed by either IKKβ (Lee et al., 2007) or phosphoinositide 3 kinase (PI3K)-Akt (Martin et al., 2003). The latter follows the same canonical pathway induced by growth factors, in which PI3K activates Akt by raising levels of phosphatidylinositol 3,4,5 triphosphate (PIP_3), thus recruiting and activating phosphoinositide-dependent kinase (PDK)-1 and mTORC2 that, in turn, phosphorylate Akt at T308 and S473, respectively, enhancing its kinase activity (Laplante and Sabatini, 2012; Martin et al., 2003). Importantly, mTORC1, which is overactivated in several important metabolic organs, such as liver, skeletal muscle and adipose tissue in both genetic- and diet-induced obese rodents (Um et al., 2004; Khamzina et al., 2005), promotes insulin resistance by either activating its downstream target p70 ribosomal S6 kinase (S6K)-1 (Um et al., 2004; Khamzina et al., 2005) or indirectly activating, via endoplasmic reticulum (ER) stress and unfolded protein response (UPR), the c-JUN-N terminal kinase (JNK) (Urano et al., 2000; Ozcan et al., 2006), both kinases that catalyze the inhibitory serine phosphorylation of insulin receptor substrate (IRS)-1 impairing PI3K activation.

Adipose tissue strongly contributes to obesity-associated inflammation by developing a local inflammatory process characterized by enhanced tissue recruitment of innate and adaptive immune cells, higher leukocyte polarization to a proinflammatory M1 phenotype along with an elevated secretion of proinflammatory cytokines by both adipocytes and leukocytes (Hotamisligil, 2006). Among adipose tissue-residing leukocytes, macrophages, which in obesity account for approximately 50% of the cells composing adipose tissue (Weisberg et al., 2003), were shown to play a central causative role in the development of obesity and associated complications. Indeed, genetic or pharmacological macrophage depletion attenuates diet-induced obesity, inflammation and insulin resistance (Weisberg et al., 2003; Bu et al., 2013). Both mTORC1 and 2 are important regulators of macrophage phenotypic plasticity, metabolism and function, therefore influencing innate and adaptive immune responses (Weichhart et al., 2015; Weichhart and Saemann, 2009). In a previous study, we have shown that myeloid cell deficiency of mTORC2 achieved by Rictor deletion, which acts as upstream activator of mTORC1, promotes the spontaneous polarization of macrophages to classically activated M1 proinflammatory phenotype (Festuccia et al., 2014a). Similar M1 polarization was also seen upon treatment of human monocytes *in vitro* (Mercalli et al., 2013) and in mice *in vivo* following treatment with the mTORC1 partial inhibitor rapamycin (Makki et al., 2014).

Taking into account that mTORC1 is activated by proinflammatory mediators, is overactivated in the adipose tissue upon obesity and regulates macrophage phenotype and function, we raised the hypothesis that this complex may be involved in the development of adipose tissue chronic low-grade inflammation. To test this, mice fed with a high-fat diet (HFD) were treated since the beginning of the dietary regimen with mTORC1 inhibitor rapamycin by gavage and evaluated for adipose tissue inflammation. Being macrophages important determinants of adipose tissue metabolism and inflammatory profile, we also investigated whether pharmacological mTOR inhibition affects macrophage phenotype, metabolism and function in such a manner that could contribute to the major phenotypes seen in adipose tissue *in vivo* upon rapamycin treatment.

2. Methods

2.1. Animals

Animal procedures were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of São Paulo, Brazil (Protocol 098/2010, CEUA). C57BL/6J mice (Jackson Laboratories) were housed at 25 ± 2 °C with a light/dark cycle of 12 h/12 h, fed with a standard chow diet (NUVILAB CR-1®-Sogorb Inc., São Paulo, Brazil; 63% carbohydrates, 25% protein and 12% fat, %Kcal) or high-fat diet (20% carbohydrates, 20% protein, 60% fat, %Kcal), described in details before (Belchior et al., 2015), and treated with either vehicle (0.1% Me_2SO, 0.2% methylcellulose) or rapamycin (2 mg/ kg/day) by gavage during 30 days. Body weight and food intake were measured weekly.

2.2. Intraperitoneal glucose tolerance test (GTT)

Mice were food deprived for 6 h and injected intraperitoneally with glucose (1 g/ kg). Tail-vein blood glycemia were determined before and 15, 30, 45, 60 and 90 min after glucose injection, using a OneTouch Johnson & Johnson glucometer.

2.3. Adipose tissue-resident leukocytes

Epididymal adipose depot was harvested, minced and digested in DMEM high-glucose containing 20 mM HEPES, 2% BSA, 1.0 mg/mL collagenase II (Sigma Chemical, St. Louis, MO, USA), pH 7.4 for 45 min at 37 °C with orbital agitation (150 rpm). Samples were then filtered in a 100 μm filter to remove undigested tissue and debris, centrifuged at 1500 rpm for 10 min and cleaned from floating mature adipocytes. Cell pellet containing the stromal-vascular fraction was re-suspended in red blood cell lysis buffer for 3 min, centrifuged, filtered at 70 μm, resuspended in PBS containing 2% fetal bovine serum and destined for FACS analysis as described below.

2.4. Peritoneal-derived macrophages

After euthanasia, mice peritoneal cavity was washed with DMEM in sterile conditions. Cells were collected, centrifuged and the pellet was resuspended in red blood cell lysis buffer for 5 min. Next, cells were centrifuged, resuspended in DMEM, 10% FBS, 1% pen/strep and plated in 6-well plates. Plates were incubated at 37 °C for 60 min to allow macrophages to adhere, washed with PBS and scraped in western blotting lysis buffer.

2.5. Bone marrow-derived macrophages (BMDM)

Mouse tibia and femur were harvested, cleaned from adherent tissue, immersed in 70% ethanol for 4 min and transferred to sterile PBS. Bone extremities were cut and the marrows were flushed with PBS, centrifuged at 1500 rpm, 4 °C and incubated for 5 min with red blood cell lysis buffer. Cells were centrifuged, resuspended in DMEM containing 15% fetal bovine serum, 1% pen/strep and 30% L929 medium, filtered and plated at density of 0.5×10^6 cells per well in six-well plates. Medium was changed after 3 days. After six days, medium was replaced and cells were treated with DMSO (vehicle, 0.1%), rapamycin (100 nM) or torin 1 (250 nM) 30 min before the addition of LPS (50 ng/mL) plus interferon (IFN)-γ (5 ng/mL, Peprotech, Rock Hill, NJ, USA) for M1 polarization or