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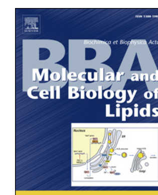

Rapamycin negatively impacts insulin signaling, glucose uptake and uncoupling protein-1 in brown adipocytes

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ABSTRACT

New onset diabetes after transplantation (NODAT) is a metabolic disorder that affects 40% of patients on immunosuppressive agent (IA) treatment, such as rapamycin (also known as sirolimus). IAs negatively modulate insulin action in peripheral tissues including skeletal muscle, liver and white fat. However, the effects of IAs on insulin sensitivity and thermogenesis in brown adipose tissue (BAT) have not been investigated. We have analyzed the impact of rapamycin on insulin signaling, thermogenic gene-expression and mitochondrial respiration in BAT. Treatment of brown adipocytes with rapamycin for 16 h significantly decreased insulin receptor substrate 1 (IRS1) protein expression and insulin-mediated protein kinase B (Akt) phosphorylation. Consequently, both insulin-induced glucose transporter 4 (GLUT4) translocation to the plasma membrane and glucose uptake were decreased. Early activation of the N-terminal Janus activated kinase (JNK) was also observed, thereby increasing IRS1 Ser 307 phosphorylation. These effects of rapamycin on insulin signaling in brown adipocytes were partly prevented by a JNK inhibitor. In vivo treatment of rats with rapamycin for three weeks abolished insulin-mediated Akt phosphorylation in BAT. Rapamycin also inhibited norepinephrine (NE)-induced lipolysis, the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and uncoupling protein (UCP)-1 in brown adipocytes. Importantly, basal mitochondrial respiration, proton leak and maximal respiratory capacity were significantly decreased in brown adipocytes treated with rapamycin. In conclusion, we demonstrate, for the first time the important role of brown adipocytes as target cells of rapamycin, suggesting that insulin resistance in BAT might play a major role in NODAT development.

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1. Introduction

Immunosuppressive therapy accounts for the remarkable success attained in organ transplant survival. On the other hand, some immunosuppressive agents (IAs) have shown several metabolic side effects such as hypertension, dyslipidemia and new onset diabetes after transplant (NODAT) [1]. NODAT has been defined as a heterogeneous condition of abnormal glucose tolerance with variable onset, duration and severity, with similar diagnosis criteria as type 2 diabetes mellitus (T2D). It is also referred to as post-transplantation diabetes mellitus (PTDM) sharing characteristics with T2D, but the pathophysiology may differ [2], recommending particular diagnosis and attention [3].

Abbreviations: NODAT, new onset diabetes after transplantation; IAs, immunosuppressive agents; BAT, brown adipose tissue; Akt, protein kinase B; mTOR, mammalian target of rapamycin; GLUT4, glucose transporter 4; IRS, insulin receptor substrate; JNK, N-terminal Janus activated kinase; NE, norepinephrine; UCP-1, uncoupling protein-1; Ser, serine; Thr, threonine.

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Rapamycin, the mammalian target of rapamycin (mTOR) inhibitor, has been used in clinical practice as an alternative to classical IAs such as steroids, mycophenolate mofetil and calcineurin inhibitors to minimize their serious side-effects, including nephrotoxicity, hypertension, dyslipidemia, insulin resistance and NODAT [4–6], increasing the risk of cardiovascular disease [7]. Despite its higher immunosuppressive capacity, rapamycin is also associated with several severe side effects; however, the molecular mechanisms remain to be elucidated [8–9].

Studies from our group and others have shown that rapamycin reduces insulin sensitivity in peripheral tissues, including liver and muscle in rodents and white adipose tissue (WAT) in both human and rodents [10–14]. Although the precise mechanisms remain to be elucidated, rapamycin treatment impairs lipolysis, insulin-stimulated glucose uptake and inhibits insulin signaling in white adipocytes, playing a major role in rapamycin-induced insulin resistance [10,13,15–16]. However, no studies have yet been performed to address the effects of rapamycin in brown adipose tissue (BAT).

Despite having been considered an irrelevant tissue in adult humans for a long time, consistent results have now revealed the metabolic relevance of BAT, in particular its important role in the regulation of thermogenesis and energy homeostasis [17–18]. BAT contains uncoupling protein-1 (UCP-1), which uncouples oxidative phosphorylation releasing energy stored in the mitochondrial proton electrochemical gradient as heat in response to catecholamines. In addition, BAT is a major site for lipid metabolism, fatty acids being the main fuel to maintain the thermogenic capacity of the tissue [19]. High metabolic activity from BAT has been identified mainly in the cervical and supraclavicular areas in adult humans by ^{18}F-deoxyglucose (FDG)-positron emission tomography (PET)-computed tomography (CT) [20–27]. These studies demonstrate that BAT is not only present but it is indeed active in adult humans with significant importance in whole-body glucose homeostasis when activated. In addition, subcutaneous white adipocytes can gain a browning phenotype after severe adrenergic stress [28].

Growing evidence shows that impaired BAT function is also involved in the development of insulin resistance. In particular, BAT lipoatrophy can induce visceral adiposity, severe glucose intolerance, high postprandial glucose levels and a defect in acute insulin secretion [29–30]. Moreover, these effects might be linked to mTOR, as suggested using adipocyte-specific mTOR-knockout mice with both mTORC1 and mTORC2 complexes deleted [31]. This study showed that ablation of mTOR, a critical regulator of adipogenesis and systemic energy metabolism, decreased both BAT and WAT mass and caused insulin resistance in mice.

While many studies have evaluated the effect of IAs, in particular rapamycin, in WAT, no studies have tested the effects of rapamycin in BAT. Therefore, the goal of this work was to analyze the impact of rapamycin in insulin sensitivity, thermogenic gene expression and mitochondrial respiration in brown adipocytes.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture reagents were from Invitrogen (Carlsbad, CA, USA). Insulin, NE and CL316243 were from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). The anti-Large T antigen (LTAg) antibody was kindly provided by J. de Caprio (Dana Farber Cancer Institute, Boston, MA). Anti-phospho JNK (Thr 183/Tyr 185) (#4668), anti-phospho HSL (Ser 660) (#4126) and anti-Akt (#9272) antibodies were from Cell Signaling Technology (MA, USA). Rapamycin, anti-phospho IR (Tyr 1162/Tyr 1163) (sc-25103-R), anti-IR (sc-711), anti-JNK (sc-571), anti-phospho Akt1/2/3 (Ser 473) (sc-7985-R), anti-phospho Akt1/2/3 (Thr 308) (sc-16646-R), anti-phospho S6K1 (Thr 389) (sc-11759) and anti-caveolin-1 (sc-894) antibodies were from Santa Cruz (Palo Alto, CA, USA). Anti-IRS1 (06-248) and anti-phospho IRS1 (Ser 307) (07-247) antibodies were from Merck Millipore (Merck KGaA, Darmstadt, Germany). The anti-IRS2 antibody was a gift of M. White (Boston Children's Hospital, Boston, USA). The anti-GLUT-4 (AB 1346) antibody was purchased from Chemicon (Chemicon International, CA, USA). The anti-UCP-1 antibody was purchased from Abcam (Cambridge, UK). SP600125 was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

2.2. Generation of an immortalized brown preadipocyte cell line from suckling rats

Brown preadipocytes were isolated from the interscapular BAT of 20-day old suckling Sprague-Dawley rats as previously described [32]. Briefly, after digestion with collagenase A, mature brown adipocytes were allowed to float and then discarded. The infranatant was filtered through a 25 μm silk filter and then centrifuged. Brown preadipocytes were seeded in DMEM supplemented with 10% neonatal calf serum (NCS), 3 nM insulin, 10 mM HEPES, 50 IU penicillin, 50 μg/ml streptomycin and 15 μM ascorbic acid. Precursor cells proliferate actively under these conditions. Once brown preadipocytes reached 50–60% confluence, these cells were infected with retroviral particles encoding SV40 Large T antigen. For this, viral Bosc-23 packaging cells were transfected at 70% confluence by calcium phosphate coprecipitation with 3 μg/6 cm-dish of the puromycin-resistance retroviral vector pBabe encoding attenuated SV40 Large T antigen (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA). Then, primary brown preadipocytes were infected with polybrene (4 μg/ml)-supplemented virus for 48 h followed by selection with puromycin (1 μg/ml) for a further 2 weeks. Five different pools of immortalized cells, generated in independent infections, were expanded.

2.3. Differentiation of brown preadipocytes

For differentiation, immortalized brown preadipocytes were grown in DMEM supplemented with 20% fetal calf serum (FCS), 20 nM insulin and 1 nM triiodothyronine (T3) (differentiation medium, DM) until reaching confluence as previously described [33]. Next, the cells were cultured for two days in induction medium (IM) consisting of differentiation medium supplemented with 0.5 μM dexamethasone, 0.125 μM indomethacin and 0.5 mM isobutyl-methyl-xanthine (IBMX). Then, cells were cultured in DM until day 6 that exhibited a fully differentiated phenotype with numerous multilocular lipid droplets in their cytoplasm. To evaluate lipid droplets accumulation, Oil Red O staining was performed. Differentiated brown adipocytes were fixed in 10% paraformaldehyde for 30 min at room temperature. Then, cells were washed with PBS and subsequently rinsed in 60% isopropanol for 5 min to facilitate the staining of neutral lipids and stained with filtered Oil Red O working solution (0.3% Oil Red O in isopropanol) for 15 min. The excess of Oil Red O was removed by rinsing the culture dishes with water for several times.

2.4. Preparation of protein extracts and Western blot

Cells were scraped off in ice-cold PBS, pelleted by centrifugation at 4000 ×g for 10 min at 4 °C and resuspended in buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM disodium pyrophosphate, 50 mM NaF, 100 μM Na3VO4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin pH 7.6 (lysis buffer). Cell lysates were clarified by centrifugation at 12,000 ×g for 10 min at 4 °C. Protein content was determined by the Bradford method, using the Bio-Rad reagent and BSA as the standard. After SDS-PAGE, proteins were transferred to Immobilon membranes (Merck-Millipore), blocked using 5% non-fat dried milk or 3% bovine serum albumin (BSA) in 10 mM Tris-HCl, 150 mM NaCl pH 7.5, and incubated overnight with the antibodies indicated in 0.05% Tween-20, 10 mM Tris-HCl, 150 mM NaCl pH 7.5. Immunoreactive bands were visualized using the ECL Western blotting protocol (Merck-Millipore).

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