SIRT1 inactivation induces inflammation through the dysregulation of autophagy in human THP-1 cells

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ABSTRACT

Inflammation plays a crucial role in atherosclerosis. Monocytes/macrophages are some of the cells involved in the inflammatory process in atherogenesis. Autophagy exerts a protective effect against cellular stresses like inflammation, and it is regulated by nutrient-sensing pathways. The nutrient-sensing pathway includes SIRT1, a NAD$^+$-dependent histone deacetylase, which is implicated in the regulation of a variety of cellular processes including inflammation and autophagy. The mechanism through which the dysfunction of SIRT1 contributes to the regulation of inflammation in relation to autophagy in monocytes/macrophages is unclear. In the present study, we demonstrate that treatment with 2-[(2-Hydroxynaphthalen-1-ylmethylene)amino]-*N*-(1-phenethyl)benzamide (Sirtinol), a chemical inhibitor of SIRT1, induces the overexpression of inflammation-related genes such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 through nuclear factor (NF)-κB signaling activation, which is associated with autophagy dysfunction, as shown through p62/Sqstm1 accumulation and decreased expression of light chain (LC) 3 II in THP-1 cells. The autophagy inhibitor, 3-methyladenine, also induces inflammation-related NF-κB activation. In p62/Sqstm1 knockdown cells, Sirtinol-induced inflammation through NF-κB activation is blocked. In addition, inhibition of SIRT1 is involved in the activation of the mammalian target of rapamycin (mTOR) pathway and is implicated in decreased 5′-AMP activated kinase (AMPK) activation, leading to the impairment of autophagy. The mTOR inhibitor, rapamycin, abolishes Sirtinol-induced inflammation and NF-κB activation associated with p62/Sqstm1 accumulation. In summary, SIRT1 inactivation induces inflammation through NF-κB activation and dysregulates autophagy via nutrient-sensing pathways such as the mTOR and AMPK pathways, in THP-1 cells.

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1. Introduction

Cardiovascular disease (CVD) is the most frequent cause of death in patients with diabetes mellitus [1] and metabolic syndrome [2], and atherosclerosis is a major cause of CVD. Atherosclerosis has been identified as an inflammatory disease, and the underlying pathology is characterized by persistent inflammation of the vascular tissue [3]. Systemic and vascular micro-inflammation is related to insulin resistance in diabetes or metabolic syndrome, and monocytes/macrophages are key players in the inflammatory pathogenesis of atherosclerosis. Activation of monocytes in the circulation and infiltrating macrophages in the atherosclerotic lesion releases various inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 [4]. Therefore, studying the regulatory mechanisms of inflammation in monocytes/macrophages is important for preventing cardiovascular diseases.

Autophagy, a lysosomal degradation pathway, plays a crucial role in removing protein aggregates as well as damaged organelles for the maintenance of intracellular homeostasis during various stress conditions [5]. Suppression of basal homeostatic autophagy may cause age-related diseases including atherosclerosis. Recent reports show that autophagy in macrophages may produce protective effects against advanced atherosclerosis [6], and the impairment of autophagy in macrophages leads to the inflammation, resulting in the progression of atherosclerosis [7]. Autophagy is regulated by nutrient-sensing pathways including SIRT1, mammalian target of rapamycin (mTOR) and 5′-AMP activated kinase (AMPK) [5]. The dysregulation of nutrient-sensing pathways and the subsequent impairment of cellular functions are involved in the pathogenesis of insulin resistance and vascular dysfunction. Among the nutrient-sensing pathways, SIRT1 dysfunction is observed in various tissues and cell types in animals models of insulin resistance [8–11]. In addition, de Kreutzenberg et al. reported that insulin resistance negatively affects both SIRT1 gene and protein expression in the peripheral blood monocytes of humans with

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metabolic syndrome [12]. SIRT1, a NAD$^+$-dependent deacetylase, is a homologue of silent information regulator (Sir2), which was initially identified from studies in aging yeast [13]. Homologues of Sir2 in higher eukaryotes are known as sirtuins, and there are seven sirtuins in mammals. Numerous reports show that SIRT1 activation may improve or retard age-related diseases such as diabetes and cardiovascular disease by regulating a variety of cellular processes including decreasing inflammation and inducing autophagy [14]. Therefore, we hypothesize that the dysregulation of SIRT1 plays a role in impairing autophagy, resulting in the dysregulation of the maintenance of accumulated molecules, with subsequent effects on inflammation in monocytes/macrophage. In addition, we hypothesize that decreased autophagy may lead to insulin resistance and atherosclerosis.

In the present study, we demonstrate that SIRT1 inactivation impairs starvation-induced autophagy leading to the accumulation of p62/Sqstm1 and resulting in inflammation through NF-κB activation. In addition, inhibition of SIRT1 contributes to the regulation of nutrient-sensing pathways by the activation of mTOR and the inhibiting AMPK activation, thereby causing the suppression of autophagy.

2. Material and methods

2.1. Materials

The anti-phospho-AMPK (Thr172), anti-light chain (LC) 3, anti-phospho-mTOR, anti-mTOR, anti-phospho-p70 S6 kinase (Thr421/Ser424), anti-p70 S6 kinase, anti-phospho-NF-κB (p65), and anti-NF-kB (p65) antibodies were obtained from Cell Signaling (Beverly, MA, USA). The p62/Sqstm1 antibodies were purchased from Medical & Biological Laboratories (Nagoya, Japan). 2-[(2-Hydroxynaphthalen-1-ylmethylene)amino]-N-(1-phenethyl)benzamide (Sirtinol) was from Calbiochem (San Diego, CA, USA). Horseradish peroxidase-linked anti-rabbit, anti-mouse secondary antibodies and RIPA buffer were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RPMI1640 medium and fetal bovine serum were obtained from American Type Culture Collection (Rockville, MD, USA). 6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (Ex-527), Rapamycin, 3-methyladenine (3-MA) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. THP-1 cell culture

THP-1 cells (American Type Culture Collection, Rockville, MD) were cultured in Falcon flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 10 mM HEPES and 1500 mg/L sodium bicarbonate, and they were maintained at 37 °C in a 5% CO$_2$ incubator. After serum starvation for 24 h, cells were incubated with 10 μM Sirtinol, 10 μM EX-527, 5 mM or 10 mM 3-MA for 5, 10 or 20 h at 37 °C. In some experiments, cells were pre-incubated with 100 ng/ml of rapamycin for 3 h, and then incubated with 10 μM Sirtinol. Prior to the assays, the culture medium was centrifuged at 1000g for 10 min, the supernatant was discarded, and the cells were used for the assays described below.

2.3. Western blotting

The samples were homogenized in ice-cold RIPA buffer. Total protein samples from the cells were used for Western blot analysis. These samples were separated on 5–20% gradient SDS-PAGE gels and transferred to polyvinylidene difluoride fmembranes (Immobilon; Millipore, Bedford, MA, USA). After blocking with 5% milk, the membranes were incubated overnight with the previously

mentioned antibodies at 4 °C. The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies, and the bands were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

2.4. Quantitative RT-PCR

The isolation of total RNA from the cells, cDNA synthesis by reverse transcription, and quantitative real-time PCR were performed as described previously [15]. Analytical data were normalized by using 18S as an internal control. The PCR primer sets are listed below. TNF-α: sense (5′–3′) CCCAGGCAGTCAGATCATCTTC, anti-sense (5′–3′) AGCTGCCCCTCAGCTTGA. IL-6: sense (5′–3′) AAGCCAGAGCTGTG CAGATGAGTA, anti-sense (5′–3′) TGTCCTGCAGCCACTGGTTC, 18S: sense (5′–3′) ACTCAACACGGGAAACCTCA, anti-sense (5′–3′) AACCA GACAAATCGCTCCAC.

2.5. RNA interference

The siRNA for human p62/Sqstm1 (Accell SMARTpool) and a negative control (Accell non-targeting siRNA) were purchased from Thermo Scientific (Fremont, CA). THP-1 cells were seeded onto a forty-eight-well plate and were transfected with p62/Sqstm1 siRNA using Accell delivery media. After 72 h, the cells were exposed to Sirtinol for 10 or 20 h, and the levels of TNF-α mRNA expression or p62/Sqstm1 and phospho-NF-kB (p65) expression were determined by quantitative RT-PCT or Western blotting.

2.6. Statistics

The data are expressed as the mean ± standard deviation (S.D.). The Tukey multiple-comparison test was used to determine the significance of pairwise differences among three or more groups. Statistical significance between two groups was determined using the unpaired t-test. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Inhibition of SIRT1 decreases autophagy in THP-1 cells

We first investigated the effects of SIRT1 inhibition on starvation-induced autophagy by immunoblotting for p62/Sqstm1 and LC3 II. Treatment with Sirtinol, a chemical inhibitor of sirtuins such as SIRT1, significantly increased the p62/Sqstm1 levels and decreased the LC3 II levels when compared to the control (Fig. 1A). Moreover, treatment with EX-527, a different SIRT1 chemical inhibitor, also decreased the starvation-induced autophagy (Fig. 1B). These data indicate that inhibition of SIRT1 decreases starvation-induced autophagy in THP-1 cells.

3.2. Inhibition of SIRT1 increases inflammation in THP-1 cells

We examined whether inhibition of SIRT1 induces inflammation in THP-1 cells. The activation of transcription factor NF-κB (p65) plays a crucial role in inflammation-related gene expression in the pathogenesis of atherosclerosis [16]. Therefore, NF-κB activation was assessed by immunoblotting for the phosphorylated species of NF-κB (p65). Treatment with Sirtinol significantly increased NF-κB (p65)-phosphorylation in THP-1 cells (Fig. 1C) and induced the overexpression of inflammation-related genes such as TNF-α and IL-6 when compared to the control cells (Fig. 1D).