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Research Paper

Angiotensin-II-induced Muscle Wasting is Mediated by 25-Hydroxycholesterol via GSK3β Signaling Pathway

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ABSTRACT

While angiotensin II (ang II) has been implicated in the pathogenesis of cardiac cachexia (CC), the molecules that mediate ang II's wasting effect have not been identified. It is known TNF-α level is increased in patients with CC, and TNF-α release is triggered by ang II. We therefore hypothesized that ang II induced muscle wasting is mediated by TNF-α. Ang II infusion led to skeletal muscle wasting in wild type (WT) but not in TNF alpha type 1 receptor knockout (TNFR1KO) mice, suggesting that ang II induced muscle loss is mediated by TNF-α through its type 1 receptor. Microarray analysis identified cholesterol 25-hydroxylase (Ch25h) as the down stream target of TNF-α. Intraperitoneal injection of 25-hydroxycholesterol (25-OHC), the product of Ch25h, resulted in muscle loss in C57BL/6 mice, accompanied by increased expression of atrogin-1, MuRF1 and suppression of IGF-1/Akt signaling pathway. The identification of 25-OHC as an inducer of muscle wasting has implications for the development of specific treatment strategies in preventing muscle loss.

1. Introduction

About 1/3 of patients with congestive heart failure suffer from cardiac cachexia (CC), which is characterized by muscle weakness and atrophy (Khawaja et al., 2014; Mancini et al., 1992; von Haehling et al., 2007). Patients with CC have a poor prognosis and there is no effective treatment for this condition, mainly due to the fact that mechanisms involved in CC remain largely unknown.

Clinical studies show that ang II is increased in patients with CC. We and others have shown that ang II infusion induces muscle wasting in rodents. However, ang II receptors are expressed at very low levels in adult skeletal muscle. Therefore, ang II induced muscle wasting may be mediated by other intermediate factors. It is well documented that TNF-α levels are increased in patients with CC (Cicoira et al., 2001; Doehner et al., 2007; Toth et al., 2006; Yende et al., 2006). In monocrotaline induced rat heart failure model, myocyte apoptosis and muscle atrophy are associated with increased levels of ang II and TNF-α (Dalla Libera et al., 2001). Blockade of ang II type 1 receptor with irbesartan prevented the increase of plasma TNF-α levels and inhibited muscle atrophy (Dalla Libera et al., 2001). Despite the correlation between ang II and TNF-α, no studies to date have examined this relationship directly to determine whether ang II induced muscle wasting is mediated by TNF-α.

TNF-α has two receptors, TNFR1 and TNFR2. Previous studies suggest that TNF-α induced muscle wasting is mediated by TNFR1 (Hardin et al., 2008; Llovera et al., 1998). We therefore took advantage of the TNFR1 knockout mouse model (TNFR1KO) to directly test the hypothesis that loss of TNFR1 would prevent ang II induced muscle wasting. We demonstrated that ang II induced muscle wasting is mediated by TNF-α through TNFR1. Furthermore, we identified Ch25h as the downstream target of TNF-α that mediate ang II's wasting effect. Ch25h

Abbreviations: ang II, angiotensin II; CC, cardiac cachexia; Ch25h, 25-hydroxylase; WT, wild type; TNFR1KO, TNF alpha type 1 receptor knockout; 25-OHC, 25-hydroxycholesterol; 20-OHC, 20α-hydroxycholesterol; 22-OHC, 22(R)-hydroxycholesterol; IR, ischemia reperfusion injury; CSA, cross sectional area.

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is an enzyme that convert cholesterol into 25-hydroxycholesterol (25-OHC), which has been shown to inhibit myogenesis via down regulation of IGF-1 signaling (Romanelli et al., 2009). We showed that intraperitoneal injection of 25-OHC resulted in muscle loss in C57BL/6 mice, accompanied by increased expression of atrogin-1, MuRF1 and suppression of IGF-1/Akt signaling and reduced phosphorylation of GSK3-beta. Our data suggest that ang II infusion activates TNF/25-OHC pathway, leading to increased expression of markers of proteolysis, apoptosis and impaired myogenesis through GSK3-beta activation.

2. Materials and Methods

2.1. Mice

Male C57BL/6 mice from Model Animal Research Center of Nanjing, and TNFR1 knockout (TNFR1KO) mice from Jackson Lab were maintained in a Special Pathogen Free animal facility at Soochow University. All animal experiments comply with the ARRIVE guidelines and are carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the manuscript has followed such guidelines. All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee of Soochow University.

2.2. Angiotensin II Infusion Model

Osmotic minipumps (ALZET model 1007D, ALZA Corp) were implanted to infuse angiotensin II at a rate of 500 ng/kg/min or diluent as described previously (Song et al., 2005). Seven days after implantation of the osmotic pumps, mice were anesthetized and muscles were removed, weighed, and snap-frozen and stored at −80 °C until processed.

2.3. Muscle Force Measurement

Mouse grip strength was measured daily for 3 consecutive days using a Grip Strength Meter (Ji-Nan Biotechnology, ShanDong, China). Each day, 5 grip strengths were assessed at 1 min intervals, and the average grip strength over 3 days was calculated.

2.4. In Vivo Delivery of Hydroxycholesterols

25-Hydroxycholesterol (25-OHC, Sigma, Cat# H1015), 20α-hydroxycholesterol (20-OHC, Sigma, Cat# H6378) and 22(R)-hydroxycholesterol (22-OHC, Sigma, Cat# H9384), dissolved in 0.3% ethanol, were injected to C57BL/6 mice daily for 7 days (40 μg/mouse, i.p.). Control mice were injected with 0.3% ethanol.

2.4.1. Tibialis Anterior (TA) Muscle Injection of Lentivirus Overexpressing Ch25h

The mice were anesthetized. Each TA muscle was injected with 50 μl of concentrated viral preparations (7.0×10^7 transducing units/ml). The contralaterol TA muscles were injected with lentivirus carrying empty vector.

2.4.2. GSK3β Inhibitor TDZD-8 Treatment

Two hours before ang II infusion, the mice were injected with either TDZD-8 (Sigma, Cat# T8325, 5 mg/kg, 10% DMSO in PBS) or PBS containing 10% DMSO.

2.4.3. C2C12 Cell Culture

The mouse myoblast cell line C2C12 (ATCC Cat# CRL-1772, RRID:CVCL_0188) from American Type Culture Collection (Manassas, VA) was cultured in DMEM (ThermoFisher Scientific) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. To induce differentiation, DMEM containing 2% horse serum (Gibco, Cat# 16050122) was added to C2C12 cells when the density of cells reached 70–80%.

2.4.4. Satellite Cell Isolation

TA muscles were dissected, minced and digested with Collagenase D and Dispase type II (Roche) (Liu et al., 2016). After digestion, the cells were resuspended in DMEM containing 1% glutamine and 10% FBS and plated on collagen coated dishes. Differentiation was induced by culturing the cells in DMEM containing 1% glutamine and 2% horse serum.

2.4.5. Myotube Calculation

Microscopic images of myotubes were captured using a digital camera mounted on a Leica microscope (DFC310 FX). Myotube diameter and length measurements were obtained using ImageJ software. Three short-axis measurements were taken along the length of a given myotube and averaged. The results were averaged from three independent experiments.

2.4.6. Construction of Lentivirus Overexpressing TNF-α and Ch25h

The LV5 plasmid (GenePharma, Shanghai, China) containing mouse TNF-α or Ch25h cDNA and packaging plasmids ΔR8.74, VSV-G and Rev were co-transfected into 293T cells as described previously (Zhou et al., 2014). The packaging plasmids were provided by Dr. Yun Zhao, Soochow University.

2.4.7. Microarray Analysis

C2C12 myoblasts were cultured in DMEM containing 10% FBS and then treated with TNF-α (50 ng/ml) for 24 h. In separate experiments, C2C12 cells were cultured in DMEM containing 2% horse serum for 4 days and then treated with TNF-α (50 ng/ml) for 24 h. Total RNA was extracted from C2C12 cells using Trizol reagent. RNA samples were labeled and hybridized to Affymetrix Mouse Gene 2.0 ST Array, and analyzed by Shanghai Biotechnology Corporation. The microarray data have been submitted to the NCBI Archive, under accession number GSE92671.

2.4.8. Western Blot

Proteins extracted from gastrocnemius muscles were separated by SDS-PAGE and blotted to PVDF membranes (Millipore, ISEQ00010). The following antibodies were from Cell Signaling Technology: cleaved caspase-3 (Cat# 9664 RRID:AB_2070042), GAPDH (Cat# 2118S RRID:AB_10698756), Akt (Cat# 4691S RRID:AB_915783), p-Akt (Cat#

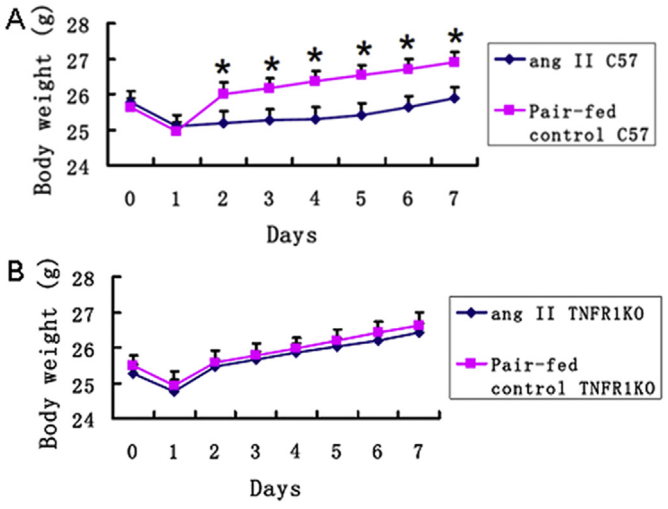

Fig. 1. TNFR1 knockout prevents ang II induced muscle wasting in mice. Changes in body weight of wild type C57BL/6 (A) and TNFR1KO mice (B) after ang II infusion for 7 days. n = 7 mice/group. P: pair-fed, sham infused mice, A: ang II infused mice. *$P < 0.01$.