Cellular Signalling 27 (2015) 353–362

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

In vitro and *in silico* analysis of an inhibitory mechanism of osteoclastogenesis by salubrinal and guanabenz

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ARTICLE INFO

Article history:
Received 22 October 2014
Accepted 21 November 2014
Available online 27 November 2014

Keywords:
Osteoclasts
NFATc1
c-Fos
JunB
Salubrinal
Guanabenz

ABSTRACT

Inactivating bone-resorbing osteoclasts is a prime therapeutic strategy for the prevention of bone loss in patients with osteopenia and osteoporosis. Synthetic agents such as salubrinal and guanabenz, which attenuate stress to the endoplasmic reticulum, are reported to inhibit development of osteoclasts. However, the mechanism of their inhibitory action on osteoclasts is largely unknown. Using genome-wide expression profiles, we predicted key transcription factors that downregulated nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), a master transcription factor for osteoclastogenesis. Principal component analysis (PCA) predicted a list of transcription factors that were potentially responsible for reversing receptor activator of nuclear factor kappa-B ligand (RANKL)-driven stimulation of osteoclastogenesis. A partial silencing of NFATc1 allowed a selection of transcription factors that were likely to be located upstream of NFATc1. We validated the predicted transcription factors by focusing on two AP-1 transcription factors (c-Fos and JunB) using RAW264.7 pre-osteoclasts as well as primary bone marrow cells. As predicted, their mRNA and protein levels were elevated by RANKL, and the elevation was suppressed by salubrinal and guanabenz. A partial silencing of c-Fos or JunB by RNA interference decreased NFATc1 as well as tartrate-resistant acid phosphatase (TRAP) mRNA. Collectively, a systems-biology approach allows the prediction of a RANKL-salubrinal/guanabenz-NFATc1 regulatory axis, and *in vitro* assays validate an involvement of AP-1 transcription factors in suppression of osteoclastogenesis.

1. Introduction

The inhibition of de-phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) stimulates bone formation by osteoblasts [1–3] and suppresses bone resorption by osteoclasts [1,2,4]. This dual role of eIF2α signaling in bone remodeling presents a unique advantage for developing treatment of bone diseases such as osteoporosis, since few existing drugs are able to not only elevate bone formation but also prevent bone resorption [1,2]. It is reported that the stimulation of bone formation through eIF2α signaling is caused by translational activation of activating transcription factor 4 (ATF4) [1]. However, the regulatory mechanism for eIF2α-driven suppression of bone resorption has not been clarified.

Salubrinal and guanabenz are potent chemical agents for the inhibition of protein phosphatase 1 (PP1) that specifically de-phosphorylate eIF2α [5,6]. Through upregulating the phosphorylated level of eIF2α and reducing translational efficiency of most proteins except for a limited set of proteins, such ATF4, these agents attenuate stress to the endoplasmic reticulum [5,6]. Gene regulation by salubrinal and guanabenz, however, not only takes place at the level of translation but also at the level of transcription [2]. In osteoclasts, it has been shown that administration of salubrinal and guanabenz suppresses receptor activator of nuclear factor kappa-B ligand (RANKL)-driven activation of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [1,2], which is a master transcription factor of osteoclastogenesis [7].

Using genome-wide microarray expression analysis, the prime aim of this study was to determine an inhibitory mechanism of NFATc1 transcription by salubrinal and guanabenz. In order to predict potential transcription factors that downregulate RANKL-driven activation of NFATc1, we employed principal component analysis (PCA) [8]. PCA allowed us to evaluate the inhibitory effects of salubrinal and guanabenz through a mathematical procedure called singular value decomposition. When a principal component axis derived from singular value decomposition is aligned along a RANKL-salubrinal/guanabenz-NFATc1 regulatory axis, transcription factors that predominantly contribute to the suppression of osteoclastogenesis could emerge along a principal component axis. We predicted and validated transcription factors that regulate the RANKL-salubrinal/guanabenz-NFATc1 axis.

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http://dx.doi.org/10.1016/j.cellsig.2014.11.020
0898-6568/

Table 1
Real-time PCR primers used in this study.

Target	Forward primer	Backward primer
Cathepsin K	5′-CAGCTTCCCCAAGATGTGAT-3′	5′-AGCACCAACGAGAGGAGAAA-3′
NFATc1	5′-GGTGCTGTCTGGCCATAACT-3′	5′-GCGGAAAGGTGGTATCTCAA-3′
TRAP	5′-TCCTGGCTCAAAAAGCAGTT-3′	5′-ACATAGCCCACACCGTTCTC-3′
GAPDH	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′

2. Materials and methods

2.1. Cell culture

Mouse bone marrow cells isolated from long bones (femur and tibia) as well as RAW264.7 mouse pre-osteoclast cells were cultured in αMEM containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA) [9]. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.2. Osteoclastogenesis and TRAP (tartrate-resistant acid phosphatase) staining

Bone marrow cells were plated at 1.2×10^5 and 1.0×10^6 cells into 12-well or 60 mm dishes, respectively, and cultured with 10 ng/ml M-CSF (macrophage colony-stimulating factor; PeproTech, Rocky Hills, NC, USA) for 3 days. The surface-attached cells were used as osteoclast precursors. These precursors were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech). RAW264.7 cells were plated at 1.0×10^5 cells into a 60 mm dish and cultured with 20 or 50 ng/ml RANKL in the presence and absence of salubrinal or guanabenz (R&D

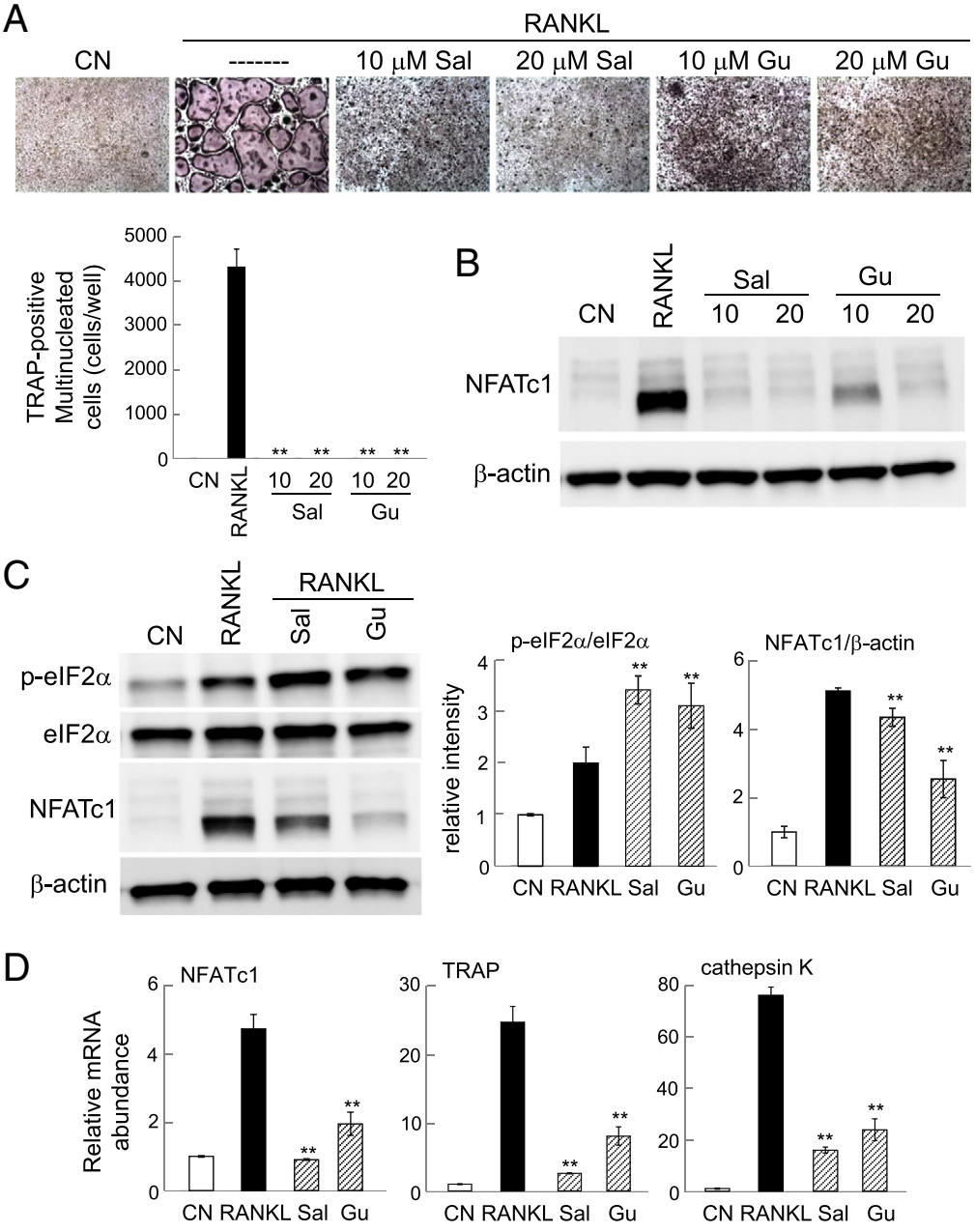

Fig. 1. Inhibitory effects of salubrinal and guanabenz on development of osteoclasts. (A) Dose-dependent suppression of TRAP-positive multinucleated osteoclasts by salubrinal and guanabenz in bone marrow cells. Note that the double asterisk indicates $p < 0.01$. (B) Salubrinal and guanabenz-driven inhibition of NFATc1 on day 2 in bone marrow cells. (C) Elevation of p-eIF2α and reduction of NFATc1 by salubrinal and guanabenz on day 1 in RAW264.7 cells. (D) Salubrinal- and guanabenz-induced reduction of mRNA expression levels of NFATc1, TRAP, and cathepsin K on days 1 in bone marrow cells.