Receptor activator of nuclear factor-κB ligand is a novel inducer of myocardial inflammation

Sangmi Ock1†, Jihyun Ahn1†, Seok Hong Lee1, Hongryeol Park2, Jang Won Son1, Jae Gyun Oh3, Dong Kwon Yang3, Wang Soo Lee4, Ho-Shik Kim5, Jaerang Rho6, Goo Taeg Oh7, Evan Dale Abel8, Woo Jin Park3, Jeong-Ki Min9,10*, and Jaetaek Kim1*

1Division of Endocrinology and Metabolism, Department of Internal Medicine, College of Medicine, Chung-Ang University, Seoul 156-755, Republic of Korea; 2Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea; 3Global Research Laboratory and Department of Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea; 4Division of Cardiology, Department of Internal Medicine, College of Medicine, Chung-Ang University, Seoul, Republic of Korea; 5Department of Biochemistry, College of Medicine, Seoul, Republic of Korea; 6Department of Microbiology, College of Natural Sciences, Chungnam National University, Daejeon, Republic of Korea; 7Division of Life and Pharmaceutical Science, Ewha Womans University, Seoul, Republic of Korea; 8Division of Endocrinology, Metabolism and Diabetes, Program in Molecular Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA; 9Therapeutic Antibody Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-333, Republic of Korea; and 10Department of Biomolecular Science, University of Science and Technology, Daejeon, Republic of Korea

Received 31 August 2011; revised 23 January 2012; accepted 27 January 2012; online publish-ahead-of-print 1 February 2012

1. Introduction
Cardiovascular disease is the leading cause of mortality in the world, affecting more than 16 million people each year.1 Whether caused by myocardial infarction or chronic pressure overload (e.g. hypertension, aortic stenosis), cardiac remodelling develops in response to cardiac injury or abnormal haemodynamic stress. Cardiac remodelling is characterized by hypertrophy, contractile dysfunction, matrix metalloproteinase (MMP) accumulation, increased apoptosis, and abnormal myocardial energetics.2

† These authors contributed equally to this work.
* Corresponding author. Tel: +82 42 860 4137 (J.-K.M.)/+82 2 6299 1397 (J.K.); fax: +82 42 860 4594 (J.-K.M.)/+82 2 6299 1390 (J.K.), Email: jekmin@kribb.re.kr (J.-K.M.), jtkim@cau.ac.kr (J.K.)

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2012. For permissions please email: journals.permissions@oup.com.
Multiple lines of evidence indicate that chronic induction of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β, and IL-6 is associated with the onset and progression of cardiac remodelling.2–6 TNF-α is the most widely studied proinflammatory cytokine in cardiac pathophysiology. TNF-α promotes progressive left ventricular dysfunction and remodelling in animal models.7,8 Serum TNF-α has been shown to be elevated in patients with heart failure and correlates with mortality.9 IL-1 critically regulates the inflammatory response and is involved in the development of adverse remodelling by enhancing the expression of MMP following myocardial infarction.10

Receptor activator of nuclear factor (NF)-κB ligand (RANKL) is a member of the TNF superfamily that regulates bone remodelling and the immune system.11 RANKL activates its receptor RANK, which is expressed in osteoclasts, and promotes bone resorption.12 Recent evidence indicates a role for RANKL in cardiovascular pathophysiology. We have also previously demonstrated that RANKL stimulates inflammatory responses by increasing endothelial–leucocyte cell interaction and the expression of the cell adhesion molecules on vascular endothelial cells and increases endothelial permeability and induces angiogenesis in mice.13–15 Moreover, RANKL was detected in atherosclerotic plaques; it increased vascular smooth muscle cell calcification and the expression of tissue factor in macrophages.16–18 Besides its role in the vasculature, RANKL may play a role in the pathophysiology of heart failure. Expression of the RANKL decoy receptor, osteoprotegerin, RANK, and RANKL genes is increased in the ischaemic tissue in rat models of post-infarction heart failure. Furthermore, enhanced myocardial RANKL and RANK are seen in human heart failure.19 Helske et al.20 reported that RANKL staining but not mRNA was more intense in myocardial samples from patients with aortic stenosis. Despite this in vivo evidence implicating RANKL in heart failure, a direct role for RANKL in myocardial inflammation has not to our knowledge been reported previously. We hypothesized that RANKL generated from cardiomyocytes may specifically induce TNF-α, IL-1α, and IL-1β expression during myocardial remodelling. We report here that pressure overload stress induces RANKL and subsequent cytokines. Mechanistically, we documented that RANKL is required for up-regulation of proinflammatory cytokines via NF-κB-mediated autocrine signalling in cardiomyocytes.

2. Methods

2.1 Animals
Male C57BL6 mice of 8–10 weeks of age (25–30 g) were used in all experiments. All animal experiments were conducted with guidelines approved by the institutional animal care and use committee of the Chung-Ang University and Gwangju Institute of Science and Technology. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2 Study design for animal experiments
In the first experiment, 36 mice total, were subjected to sham (n = 12) or transverse aortic constriction (TAC) (n = 23) surgery. Mice were sacrificed 6 h, 3, or 17 days after sham or TAC surgery. In the second experiment, mice were randomly assigned to three groups: (i) sham plus saline (0.2 mL, oral gavage, n = 4), (ii) TAC plus saline (0.2 mL, oral gavage, n = 8), and (iii) TAC plus losartan (13.5 mg/kg/day, oral gavage, n = 8). Animals were treated for 7 days prior to harvesting hearts. Finally, to inhibit RANKL in vivo, we administered anti-mouse RANKL-neutralizing monoclonal antibody (RANKL mAb, 50 μg/25 g; Oriental Yeast, Tokyo, Japan) or control IgG2a (50 μg/25 g; BioLegend, San Diego, CA, USA) by single subcutaneous injection 18 h before TAC. Mice were randomly assigned to three groups: (i) sham plus control IgG2a (n = 4), (ii) TAC plus control IgG2a (n = 8), and (iii) TAC plus RANKL mAb (n = 7). Animals were sacrificed 7 days after surgery. The total post-surgical mortality was less than 10%. The final number of animals for each specific analysis is noted in the figures or their legends.

2.3 TAC surgery
The TAC surgery was performed as described previously.21 Animals were anaesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) administered via intraperitoneal injection. Mice were ventilated with a tidal volume of 0.1 mL and a respiratory rate of 120 b.p.m. (Harvard Apparatus, Holliston, MA, USA). The adequacy of anaesthesia was monitored during the surgical procedure by lack of withdrawal response to foot pinch. A longitudinal incision of 2–3 mm was made in the proximal sternum to allow visualization of the aortic arch. The transverse aortic arch was ligated between the innominate and left common carotid arteries with an overland 27-gauge needle. The needle was then immediately removed, leaving a discrete region of constriction.

2.4 Echocardiography
Mice were anaesthetized using 1.5% isoflurane and echocardiography was performed using the Vevo 770 System (VisualSonics Inc., Toronto, Ontario, Canada) with a 30 MHz transducer in the two-dimensional M-mode.

2.5 Tissue preparation and immunofluorescence staining
Following deep anaesthesia with isoflurane, mice were sacrificed by cervical dislocation. Hearts were fixed and cryosectioned and sections were incubated with the indicated primary antibodies, followed by the secondary antibodies. The slides were examined using a confocal microscope (LSM 510 META; Carl Zeiss, Jena, Germany). See Supplementary material online for further details.

2.6 Isolation of mouse cardiomyocytes
Cardiomyocytes were isolated from sham or TAC hearts as described previously.22 In brief, after heparin injection, animals were killed by cervical dislocation. The heart was rapidly excised and the aorta was retrogradely perfused with calcium-free Tyrode buffer. The enzymatic digestion was initiated by adding collagenase/hyaluronidase to the perfusion solution. The heart was then minced and cardiomyocytes dissociated in buffer with increasing calcium. See Supplementary material online for further details.

2.7 Cell culture
Two- to 3-day-old Sprague–Dawley rats were killed by decapitation and neonatal rat cardiomyocytes (NRCMs) were prepared as described previously.23 In brief, following mechanical and enzymatic (collagenase/pancreatin) separation, the Percoll gradient-purified cardiomyocytes were plated on gelatin-coated plates in 10% horse serum, 3% foetal bovine serum, and 100 nM BrdU containing DMEM/M199 medium (Invitrogen, Carlsbad, CA, USA).
Figure 1  RANKL expression is induced by pressure overload in the heart. Mice were exposed to TAC for 6 h, 3, or 17 days. (A) Relative HW to BW (HW/BW) ratio 6 h, 3, or 17 days after sham or TAC surgery (n = 4 for sham and 6 h TAC, and n = 6 for 3-day TAC and 17-day TAC), HW/BW ratio of sham-operated mice was arbitrarily set as 1. Overall P < 0.001 by one-way ANOVA. **P < 0.01; ***P < 0.001 vs. sham by Tukey’s post hoc test. Expression of RANKL, TNF-α, IL-1α, and IL-1β mRNA in hearts 6 h (B), 3 days (C), or 17 days (D) after surgery. n = 3–6 per group. Results were normalized to GAPDH and mRNA level of sham hearts was arbitrarily set as 1. Representative RT–PCR are shown (left panel). *P < 0.05; **P < 0.01 vs. sham by Student’s t-test. #P < 0.05 vs. sham by the Mann–Whitney U-test. (E) Heart cryosections from 7-day sham or TAC mice were double-stained by immunofluorescence with anti-RANKL antibody (green) and anti-α-actinin antibody (red). Nuclei were stained with DAPI (blue). Merged images are shown (magnification, ×400). Scale bars are indicated. (F) RANKL immunofluorescence was quantified by measuring intracellular staining of RANKL (n = 4 for sham and TAC). **P < 0.01 vs. sham by the Mann–Whitney U-test. (G) RANKL mRNA expression in isolated cardiomyocytes from hearts of sham and TAC mice (n = 3 for sham, n = 4 for 7 days TAC). Representative RT–PCR are shown (left panel). Quantitative RT–PCR (right panel). Results were normalized to actin for quantitative RT–PCR and mRNA level of sham hearts was arbitrarily set as 1. *P < 0.05 vs. sham by Student’s t-test. (H) Immunostaining for TNF-α (green) is shown in the left panel, and staining for IL-1α (green) is shown in the right panel overlaid with DAPI-stained nuclei (blue) 7-day post-TAC (magnification, ×400). Scale bars are indicated.
Figure 2 Effects of Ang II receptor blockade on myocardial expression of RANKL and proinflammatory cytokines in TAC mice. mRNA (A) and protein levels (B) of RANKL after phosphate-buffered saline (PBS) or Ang II (1 μM) treatment for 24 h in NRCMs. Representative gel/blots are shown. Relative expression of RANKL was quantified by densitometry \( (n = 3–5 \text{ per group}) \). \( *P < 0.05 \) vs. PBS by Student’s t-test. (C–E) Mice were subjected to either sham or TAC surgery in the presence of saline (0.2 mL/day) or losartan (13.5 mg/kg/day) by oral gavage for 7 days. (C) HW/BW ratio \( (n = 4 \text{ for sham, } n = 5 \text{ for TAC + saline, and } n = 7 \text{ for TAC + losartan}) \). Overall \( P < 0.01 \) by the Kruskal–Wallis test. \( *P < 0.05 \) vs. sham; \( \#P < 0.05 \) vs. TAC + saline by a Mann–Whitney U-test with the Bonferroni correction. (D) Expression of RANKL, TNF-α, IL-1α, and IL-1β mRNA \( (n = 3–5 \text{ in each group}) \). Representative RT–PCR is shown (left panel). Overall \( P < 0.01 \) (RANKL), \( P < 0.001 \) (TNF-α), \( P < 0.01 \) (IL-1α), and \( P < 0.05 \) (IL-1β) by one-way ANOVA. \( \#P < 0.05 \); \( ***P < 0.001 \) vs. sham; and \( \#\#P < 0.01; \#\#\#P < 0.001 \) vs. TAC + saline by Tukey’s post hoc test. (E) Immunostaining for RANKL (green) and α-actinin (red) (upper panel), and TNF-α (green) or IL-1α (green) (lower panel). Nuclei were stained with DAPI (blue). Merged images are shown (magnification, \( \times 400 \)). Scale bars are indicated. (F) Attenuation of myocardial expression of proinflammatory cytokines by neutralization of RANKL in TAC mice. Expression of TNF-α, IL-1α, and IL-1β mRNA \( (n = 4–5 \text{ in each group}) \). Representative RT–PCR are shown (upper panel). Overall \( P < 0.001 \) (TNF-α) and \( P < 0.01 \) (IL-1α) by one-way ANOVA, and \( P < 0.05 \) (IL-1β) by the Kruskal–Wallis test. \( ***P < 0.01; \#\#P < 0.001 \) vs. sham + IgG2a, and \( \#\#\#P < 0.01; \#\#\#P < 0.001 \) vs. TAC + IgG2a by Tukey’s post hoc test. \( *P < 0.05 \) vs. sham + IgG2a and \( \#P < 0.05 \) vs. TAC + IgG2a by a Mann–Whitney U-test with the Bonferroni correction.
USA). Soluble RANKL (human CD8-conjugated form) was purified from insect cells, as described previously.24

2.8 Cell viability assay
NRCMs were incubated in the absence (control) or presence of various RANKL concentrations (0.1, 0.5, and 1 µg/mL). After 24 h, 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was added and incubated at 37°C for 4 h. The absorbance was measured at 570 nm. See Supplementary material online for details.

2.9 Measurement of cell size
NRCMs were incubated in the absence (control) or presence of 1 µg/mL RANKL for 24 h. Cells were fixed and the areas of at least 100 individual cells per condition were examined from four individual cell preparations using ImageJ software. See Supplementary material online for details.

2.10 Isolation of RNA and semi-quantitative or quantitative reverse transcriptase–polymerase chain reaction analysis
Total RNA was obtained from NRCMs using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX, USA). Different amounts of total RNA (0.5–2 µg) were amplified with reverse transcriptase–polymerase chain reaction (RT–PCR), and the correlation between the amount of RNA used and the level of PCR products obtained from target mRNAs and the internal standard (GAPDH or β-actin) mRNA was examined. See Supplementary material online for further details.

2.11 Western blot analysis
Heart tissues or cell lysates from NRCMs were subjected to SDS–PAGE and the protein bands in the gel were transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies, and the immunoreactive bands were visualized using a chemiluminescent substrate. Densitometric quantitation was achieved using an AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA). See Supplementary material online for further details.

2.12 Transfection of small interfering RNA
NRCMs were plated on 60 mm dishes at a density of 1 × 10⁶ cells and transfected (using Lipofectamine™ 2000, Invitrogen) with RANK siRNA (90 nM, MISSION siRNA, Sigma) or TRAF2 siRNA (200 nM, MISSION siRNA, Sigma) or TRAF6 siRNA (150 nM, MISSION siRNA, Sigma). The non-cognate control siRNA (Sigma) was used as a negative control. Approximately 72 h after transfection, cells were treated with RANKL for 3 h.

2.13 Enzyme-linked immunosorbent assay
The concentration of TNF-α in the culture supernatants was measured in triplicate using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instruction.

2.14 Preparation of nuclear extracts and electrophoretic gel mobility shift assays
Nuclear proteins were isolated and subjected to electrophoretic gel mobility shift assays (EMSA) using 5′-biotin-labelled NF-κB double-strand

Figure 3 RANKL stimulates expression of TNF-α, IL-1α, and IL-1β in NRCMs. Concentration-dependent changes of mRNA levels of TNF-α (A), IL-1α (C), and IL-1β (D) after treatment with RANKL (0.1–1 µg/mL) for 3 h. (B) Measurement of concentration of TNF-α released in the medium using ELISA after treatment with RANKL (0.1–1 µg/mL) for 24 h. (E) NRCMs were treated with 1 µg/mL RANKL for 3 h in the absence or presence of actinomycin D (ActD). Relative levels of mRNA expression were normalized with respect to the levels of actin. n = 4–5, in triplicate. Overall P < 0.001 by one-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control by Tukey’s post hoc test.
oligonucleotide. Detection of the NF-κB-oligonucleotide complex was performed using a chemiluminescent EMSA kit. See Supplementary material online for further details.

2.15 Statistical analysis
Data are presented as the mean ± standard deviation. Statistical analysis was performed using either a Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s post hoc test once normality was demonstrated (Shapiro–Wilk test). Non-normally distributed data were analysed using either a Mann–Whitney U-test or a Kruskal–Wallis test followed by a Mann–Whitney U-test (adjusting the α-level by the Bonferroni inequality). A value of P < 0.05 was considered statistically significant.

3. Results
3.1 RANKL expression in mouse hearts is induced by pressure overload cardiac hypertrophy
To address whether RANKL might participate in the myocardial inflammation, we exposed mice to TAC. Heart weight (HW) normalized to body weight (BW) ratios significantly increased by 45% following 17 days of pressure overload (Figure 1A). Contractile function and cavity dimensions were preserved (see Supplementary material online, Table S1), indicating compensated cardiac hypertrophy. mRNA expression of RANKL, but not TNF-α, IL-1α, and IL-1β, was significantly increased at 6 h TAC (2.7-fold vs. sham; P < 0.01) (Figure 1B). However, after 3 days of TAC, expression levels of RANKL, TNF-α, and IL-1α were significantly increased (4.6-, 3.4-, and 7.5-fold vs. sham, respectively; P < 0.05) and IL-1β tended to increase (3.4-fold vs. sham) (Figure 1C). Expression levels of RANKL, IL-1α, and IL-1β were significantly increased after 17 days of TAC (Figure 1D). To verify that cardiomyocytes were the main source of RANKL in the pressure-overloaded myocardium, immunofluorescent staining was performed on sections of 7-day sham and TAC hearts (Figure 1E). RANKL immunoreactivity was significantly increased on cardiomyocytes as indicated by co-staining for sarcomeric α-actinin in sections of TAC compared with sham hearts (P < 0.01) (Figure 1F). Quantitative RT–PCR analysis of isolated mouse cardiomyocytes showed increased RANKL mRNA expression in 7 days TAC compared with sham hearts, confirming that RANKL was from cardiomyocytes (P < 0.05) (Figure 1G). Both TNF-α and IL-1α were strongly stained in 7-day TAC myocardium (Figure 1H).

Next, we examined whether angiotensin II (Ang II), which is known to induce cardiomyocyte hypertrophy, increases RANKL expression in NRCMs. Exposure of NRCMs to 1 μM Ang II significantly increased RANKL mRNA and protein levels (Figure 2A and B). Given that Ang II receptor blockade prevents cardiac hypertrophy with pressure overload in mice, we administered the Ang II receptor blocker, losartan, to TAC mice to determine whether this would attenuate RANKL induction and the subsequent induction of proinflammatory cytokines. As expected, TAC for 7 days resulted in a significant increase in the HW/BW ratio, which was attenuated by treatment with losartan (Figure 2C). Interestingly, treatment of TAC mice with losartan significantly reduced expression of RANKL, TNF-α, IL-1α, and IL-1β mRNA compared with the TAC plus saline group (Figure 2D). Notably, the amount of positive immunostaining for RANKL, TNF-α, and IL-1α was significantly reduced in TAC plus losartan.
compared with TAC plus saline hearts (Figure 2E). Next, to examine whether RANKL is necessary for the up-regulation of proinflammatory cytokines, we inhibited RANKL in TAC hearts by injection of RANK mAb, as described previously.27 Neutralization of RANKL significantly reduced expression of TNF-α, IL-1α, and IL-1β mRNA in the TAC plus RANKL mAb group compared with the TAC plus IgG2a group (Figure 2F). These results suggest that RANKL generated from cardiomyocytes is required for up-regulation of proinflammatory cytokines in pressure overload hearts.

### 3.2 RANKL stimulates expression of TNF-α, IL-1α, and IL-1β

To identify the molecular mechanisms mediating RANKL-induced cytokine expression, we cultured NRCMs. We first examined whether the RANKL receptor, RANK, is expressed in NRCMs. RT–PCR and immunoblot analysis confirmed robust expression of RANK in NRCMs (see Supplementary material online, Figure S1). To determine whether RANKL induces expression of proinflammatory cytokines such as TNF-α, IL-1α, and IL-1β in NRCMs, we performed semi-quantitative RT–PCR analysis. As shown in Figure 3A, C, and D, RANKL stimulated the induction of TNF-α, IL-1α, and IL-1β mRNA expression in a concentration-dependent manner (11-, five-, and 21-fold increases for TNF-α, IL-1α, and IL-1β, respectively) that corresponded with increased TNF-α production by ELISA (Figure 3B). A statistically significant increase in TNF-α, IL-1α, and IL-1β mRNA occurred at a concentration of 0.5 μg/mL with maximal stimulation observed in response to 1 μg/mL RANKL. All subsequent experiments were performed using 1 μg/mL RANKL.

The RANKL-induced increase in proinflammatory cytokines was completely abolished by pre-treatment with actinomycin D, indicating that...
these changes resulted from increased transcription rather than by stabilizing pre-existing mRNA (Figure 3E). NRCMs treated with 0.1–1 μg/mL RANKL showed similar viability compared with untreated control cells (see Supplementary material online, Figure S2A), indicating that RANKL produces proinflammatory cytokines without inducing cell death in NRCMs. To evaluate whether RANKL induces cardiomyocyte hypertrophy, we treated NRCMs with RANKL and measured cell size. Cell size was unaffected in RANKL-treated cells, indicating that RANKL signalling is not involved in cell size regulation (see Supplementary material online, Figure S2B).

3.3 RANKL induces NF-κB activation in NRCMs

When RANK binds RANK, multiple intracellular signalling pathways can be activated, including extracellular signal-regulated kinase (ERK), Akt, p38 mitogen-activated protein kinase (p38 MAPK), and NF-κB.28 As shown in Figure 4A–C, RANKL transiently increased ERK, Akt, and p38 MAPK phosphorylation. In contrast, inhibitor of κB (IkB)α phosphorylation on serine 32, which is a critical regulatory site that leads to the degradation of IkBα with subsequent nuclear translocation of NF-κB,29 exhibited sustained activation for up to 60 min (Figure 4D). Next, we examined DNA-binding activity of NF-κB in RANKL-treated cells. Consistent with IkBα phosphorylation, RANKL induced strong DNA-binding activity of NF-κB after treatment of cells for 30–90 min, which was competed by an excess of unlabelled oligonucleotides (Figure 4E). In addition, IkB kinase (IKK) inhibitor completely suppressed RANKL-induced TNF-α, IL-1α, and IL-1β mRNA expression, suggesting that NF-κB mediates RANKL-induced proinflammatory cytokine expression (Figure 4F).

3.4 RANKL increases TNF-α, IL-1α, and IL-1β mRNA expression via RANK–TRAF2/TRAF6–PLC–PKC–NF-κB pathways

To determine whether RANKL signals by binding to RANK, siRNA interference was used to knock down RANK expression in NRCMs. RANK siRNA transfection effectively reduced the mRNA and protein levels of RANK relative to controls (Figure 5A). RANKL-induced DNA-binding activity of NF-κB was significantly inhibited in NRCMs treated with RANK siRNA (Figure 5B). Furthermore, RANKL-mediated expression of TNF-α, IL-1α, and IL-1β mRNA was significantly attenuated (Figure 5C). RANK is known to interact with members of the TNF receptor-associated factor (TRAF) family to mediate signalling events initiated by RANKL.30 Of the six known mammalian TRAFs, TRAF2 and TRAF6 are important for NF-κB activation.28,31 To determine the participation of TRAFs in RANKL signalling to induce proinflammatory cytokines, siRNA interference was used to knock down TRAF2 and TRAF6 expression in NRCMs. As shown in Figure 5D and E, transfection with TRAF2 or TRAF6 siRNA effectively reduced the mRNA and protein levels of TRAF2 or TRAF6 relative to controls and RANKL-mediated expression of TNF-α, IL-1α, and IL-1β mRNA expression was significantly attenuated. These results clearly demonstrate a requirement for TRAF2 and TRAF6 in mediating RANKL/RANK signalling that stimulates expression of these proinflammatory cytokines in NRCMs. TRAFs then initiate signalling cascades via activation of downstream MAPKs (ERK, p38 MAPK) and Akt to promote RANKL-induced cell survival, differentiation, and inflammation.28 To determine whether these signalling pathways are involved in RANKL-induced proinflammatory cytokine expression, we utilized specific pharmacological
inhibitors of the ERK (PD98059), p38 MAPK (SB203580), and phosphoinositide 3-kinases (PI3K) (LY294002). We established the effects of appropriate concentrations of these inhibitors by measuring phosphorylation and determined the optimal concentration of inhibitors (data not shown). Thus, 10 μM of PD98059 and SB203580 and 20 μM of LY294002 inhibited the ERK, p38 MAPK, and Akt pathways, respectively. We treated NRCMs with RANKL in the presence or absence of these signalling inhibitors and examined expression of TNF-α, IL-1α, and IL-1β mRNA. As shown in Figure 5F, these inhibitors had no significant inhibitory effects on RANKL-induced cytokine expression. We previously reported that in endothelial cells, RANKL induces NF-κB activation and cell adhesion molecules expression via signalling cascades involving PLC–PKC. Therefore, we further examined whether PLC–PKC signalling pathways are involved in RANKL-mediated inflammatory responses in NRCMs. The PLC inhibitor (U73122) and the PKC inhibitor (chelerythrine chloride; CTC) substantially inhibited RANKL-induced TNF-α, IL-1α, and IL-1β mRNA expression (Figure 5G). Consistently, RANKL-induced phosphorylation of IkBα and DNA-binding activity of NF-κB were significantly inhibited in NRCMs treated with these inhibitors (Figure 5H and I). These results document that RANKL increased TNF-α, IL-1α, and IL-1β mRNA expression in NRCMs via a pathway that involves the sequential activation of RANK–TRAF2/TRAF6–PLC–PKC and NF-κB (Figure 6).

4. Discussion

In the present study, we clearly demonstrated that RANKL is a novel component of the cardiac response to pressure overload. The initial increase in RANKL at 6 h of TAC precedes the induction of endogenous cytokines which is consistent with the hypothesis that RANKL could induce cytokine expression in the heart. These correlations were directly supported by experiments using RANKL mAb. Although TAC is commonly used to model pressure overload and heart failure, acute pressure overload could be associated with ischaemia. Thus, the possibility exists that ischaemia could induce RANKL. This limitation of the present studies will be addressed in the future using models of ischaemia/reperfusion. Furthermore, we found that Ang II stimulated RANKL expression and treatment of TAC mice with losartan, significantly attenuated cardiac hypertrophy, along with decreased expression of RANKL, TNF-α, IL-1α, and IL-1β. Collectively, our data suggest that cardiomyocytes up-regulate RANKL followed by increased cytokine expression in pressure-overloaded hearts.

In our study, we showed that RANKL stimulates expression of TNF-α, IL-1α, and IL-1β in cultured cardiomyocytes. The concentrations of RANKL used in this study were similar to those reported previously. To date, the underlying signalling pathways by which RANKL regulates TNF-α, IL-1α, and IL-1β gene expression is not known. The presence of RANK in NRCMs does not necessarily mean that RANKL signals through RANK. First, we try to determine whether RANKL signals through binding to RANK using RANK siRNA. Treatment with RANK siRNA inhibited not only RANKL-induced DNA-binding activity of NF-κB but also expression of TNF-α, IL-1α, and IL-1β mRNA, confirming RANKL signalling through binding to RANK. TRAFs such as TRAF2 and TRAF6 were identified to associate with the cytoplasmic tail of RANK to activate multiple intracellular signalling pathways. RANK has three binding domains for TRAFs. Each of these have a different binding affinity for either TRAF2, TRAF5, or TRAF6, which relays the RANK stimulation signal and activates downstream pathways including NF-κB, and c-Jun N-terminal kinase (JNK). In TRAF2 or TRAF6 knockdown NRCMs, RANKL-induced TNF-α, IL-1α, and IL-1β mRNA expression were significantly suppressed. Therefore, it is plausible that TRAF2 and TRAF6 are essential adaptor proteins for linking RANKL to downstream signalling, a finding which is consistent with a previous report.

Myocardial inflammation occurs during the development of cardiac hypertrophy and heart failure and involves proinflammatory signalling pathways, such as NF-κB. In our study, RANKL induced strong DNA-binding activity of NF-κB and the IKK inhibitor suppressed RANKL-induced TNF-α, IL-1α, and IL-1β mRNA expression, suggesting that NF-κB is critically involved in regulating the expression of TNF-α, IL-1α, and IL-1β. Consistent with these results, we previously reported that NF-κB is involved in RANKL-induced intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression in endothelial cells. The question arises as to the identity of downstream kinases that link this adaptor to NF-κB activation. RANKL is known to induce multiple downstream pathways, such as Akt, MAPK (ERK, p38 MAPK, JNK), and PLC–inositol(1,4,5)trisphosphate [Ins(1,4,5)P3] pathways. Ultimately, RANKL leads to activation of transcription factors, including NFATc1, NF-κB, and AP-1. We have previously shown that ERK and JNK activation are necessary events for RANKL-induced tissue factor expression in macrophages. Kim et al. showed that RANKL regulates endothelial cell survival through the PI3K–Akt pathway. As expected, RANKL activated phosphorylation of ERK, p38 MAPK, and Akt in NRCMs. However, inhibition of ERK, p38 MAPK, or Akt did not suppress RANKL-induced TNF-α, IL-1α, and IL-1β expression, suggesting that Akt or MAPK signalling is not involved in this process. Activation of PLC increases Ins(1,4,5)P3 and diacylglycerol concentrations, which activate intracellular calcium mobilization and serine–threonine protein kinases, such as PKC. In the present study, RANKL-mediated TNF-α, IL-1α, and IL-1β expression was totally abolished in the presence of the PLC or the PKC inhibitor. In addition, RANKL-induced DNA-binding activity of NF-κB was suppressed by PLC or PKC inhibitors, which suggests that this effect is mediated by the PLC–PKC–NF-κB signalling pathway.

In conclusion, we have shown to the best of our knowledge for the first time that pressure overload stress induces RANKL production in the heart, which induces proinflammatory cytokines in cardiomyocytes via an NF-κB-mediated mechanism. Thus, this study adds strong support that RANKL promotes inflammation in the remodeling heart by an autocrine mechanism. As such, the RANKL/RANK system might be a potential target for reducing adverse myocardial remodelling in failing hearts.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: None declared.

Funding

This work was supported by a grant from the Korea Healthcare technology R&D Project (A101749), Ministry of Health & Welfare, Republic of Korea, to W.J.P and J.K.
References


3. Abe J. Role of PKCs and NF-kappaB activation in myocardial inflammation: enemy or ally? J Mol Cell Cardiol 2007;43:404–408.


6. Torre-Amione G. Immune activation in chronic heart failure. Am J Cardiol 2005;95:3C–8C.


