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The Effect of Rotative Stress on CAII, FAS, FASL, OSCAR, and TRAP Gene Expression in Osteoclasts

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ABSTRACT

This study was designed to explore the effects of rotative stress on carbonic anhydrase II (CAII), TNF receptor superfamily member 6 (FAS), FAS ligand (FASL), osteoclast-associated receptor (OSCAR), and tartrate-resistant acid phosphatase (TRAP) gene expression in osteoclasts. Osteoclasts were induced from RAW264.7 cells cultured in medium containing recombinant murine soluble receptor activator of NF-Kβ ligand (sRANKL). The mRNA and protein expression of CAII, FAS, FASL, OSCAR, and TRAP genes in osteoclasts was detected by RT-PCR and Western blot, respectively, after osteoclasts were loaded at various rotative stress strengths and times. No significant differences in mRNA and protein expression were observed between any of the control groups ($P > 0.05$). Importantly, rotative stress had a significant effect on the mRNA and protein expression of these genes ($P < 0.05$). We found a negative relationship between rotative stress strength and prolonged loading time and the expression of FAS/FASL genes in osteoclasts. In addition, there was a positive relationship between rotative stress strength and prolonged loading time and the expression of CAII, OSCAR, or TRAP genes in osteoclasts. Based on these results, rotative stress has a significant effect on CAII, FAS, FASL, OSCAR, and TRAP gene expression in osteoclasts. J. Cell. Biochem. 114: 388–397, 2013.

KEY WORDS: CARBONIC ANHYDRASE II; TNF RECEPTOR SUPERFAMILY MEMBER 6; FAS LIGAND; OSTEOCLAST-ASSOCIATED RECEPTOR; TARTRATE-RESISTANT ACID PHOSPHATASE; OSTEOCLASTS

A close relationship exists between bone remodeling and stress (Rodriguez et al., 2011; Hambli and Rieger, 2012; Li et al., 2012). After bone tissue, one kind of network system with three-dimension structures, is loaded stress, netlike bone trabecula appears deformation. A cross-linking reaction between synapses of bone cells by rotative liquid will trigger cellular functions.

Specific factors in osteoclasts play an important role in dynamic bone remodeling (Nguyen et al., 2011; Zhang et al., 2011, 2012; Agrawal et al., 2012; Fattore et al., 2012; Rumpler et al., 2012). When bone remodeling is abnormal, the number of osteoclasts and the activity of the factors increases, which results in increased bone resorption and the development of diseases, such as osteoporosis. In contrast, a reduction in the number and activity of osteoclasts results in a decrease in bone resorption and the development of diseases such as osteosclerosis.

During the course of a stress response in osteoclasts, several important cellular factors play a critical role in osteoclastic resorption (Barrow et al., 2011; Goettsch et al., 2011; Kitaura et al., 2011; Kohara et al., 2011; Böcker et al., 2012; Silva et al., 2012). A number of these genes have been the focus of more recent research studies, including tartrate-resistant acid phosphatase (TRAP), carbonic anhydrase II (CAII), osteoclast-associated receptor (OSCAR), and FAS/FASL. CAII synthesizes and secretes H^+, which promotes demineralization of sclerous tissues. As one type of cell with terminal differentiation, osteoclasts have the characteristic of undergoing rapid apoptosis. FAS/FASL are important in apoptosis and participate in cell differentiation, maturation, and death. Recently, some studies have indicated that the classical OPGL-RANK-OPG signal pathway is not the only mediator of osteoclastic resorption, and another important co-stimulatory molecule, OSCAR, also plays a role in

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osteoclast differentiation, maturation, migration, and resorption. In addition, TRAP is an enzyme that directly reflects osteoclast activity. A number of studies have explored osteoclast cytomechanics in the past, but our understanding of the mechanisms involved are limited because the culturing of osteoclasts is technically difficult and cell-loading devices are imperfect. Therefore, the action mechanism of the stress response in osteoclasts is still not clear. To date, there are no systematic studies that have explored the effects of rotative stress on CAII, FAS, FASL, OSCAR, and TRAP gene expression in osteoclasts.

In this study, we hypothesized that CAII, FAS, FASL, OSCAR, and TRAP would have a similar gene expression profile in osteoclasts under rotative stress. This idea was based on our previous study, in which the mRNA expression of ATP6al and ATP6i genes in osteoclasts had a uniform tendency under fluid shear stress (Hong et al., 2010). In this present study, the mRNA and protein expression of CAII, FAS, FASL, OSCAR, and TRAP genes in osteoclasts were measured by RT-PCR and Western blot after the cells were loaded with various rotative stresses produced by a new type of rotative machine designed by our osteoclast research team. The aim of this study was to determine the relationship between rotative stress and the expression of key genes with regard to the stress mechanism in osteoclasts. In future clinical work related to the cell biological relevance of the stress response, assessment will consider whether rotative stress will result in a lack of an anabolic effect in bone remodeling (Bonewald, 2007; Fahlgren et al., 2010; Johansson et al., 2011).

MATERIALS AND METHODS

IN VITRO OSTEOCLAST CULTURE AND VISUALIZATION OF CELLS

Fresh vials of RAW264.7 cells (ATCC: TIB-71, Chinese Academy of Medical Sciences) were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM; High Glucose, No.21013024; Gibco Corp.) until reaching 80% confluence, at which time the adherent cells were scraped away from the 25-cm^2 cell culture flask surface (No.353108; BD Falcon Corp.). These cells were gently adjusted to uniform cell suspension as an osteoclast culture.

IN VITRO OSTEOCLAST CULTURES

RAW264.7 cell suspension was seeded into 35 mm culture dishes (No.430165; Corning Corp.) at a density of 1×10^4/dish. The osteoclast induction culture medium [α-minimal essential medium (MEM), No.41061-029; Gibco Corp.] contained 10% fetal bovine serum (FBS; No.16000-044; Gibco Corp.), 80 μg/ml recombinant murine soluble receptor activator of NF-Kβ ligand (sRANKL, No.315-11; Peprotech Corp.), 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium in each dish was changed every 3 days. On the sixth day of culture, mature osteoclasts were observed and photographed under an inverted microscope and photo system (No.IX51-A21PH, Olympus Corp., Japan).

TRAP STAINING FOR OSTEOCLAST IDENTIFICATION

Osteoclasts were stained according to the manufacturer's instructions of the TRAP Staining Kit (No.387A; Sigma Corp.).

ROTATIVE STRESS DEVICE DESIGNED BY OUR OSTEOCLAST RESEARCH TEAM

The rotative stress device was further developed by our osteoclast research team based on the liquid shear stress of a cell-loading system, which was used in our previous osteoclast study (patent number 200420034438, China). The generator mediated the rotation of culture dishes fixed onto the glass platform and cells were loaded based on relative centrifugal force (RCF). The formula $RCF_{max} = 1.118 \times 10^{-5} \times S^2 \times r$ was used to RCF_{max}, which represented the maximum RCF (g). The letter "S" and "r" represented the rotation speed (rpm) and radius (cm), respectively. The main components of the device included a medical-grade precise generator (No.TH37JB32-C; TianHeng Corp., China), a miniature speed regulator (No.US22A; GaoXin Corp., China), a quartz tube (No.JB; JinBo Corp., China), and a miniature intelligent digital displayer (No.SPB; XunPeng Corp., China). The power source used as 220 V and 50 Hz. A miniature speed regulator was used to regulate the rotation speed between 0 and 1,200 rpm. A miniature intelligent digital display provided a real-time display of speed. The loading objective(s) was fixed onto a glass platform (7 cm diameter, 8 mm thickness), and the generator put into quartz tube was 150 g (free-running speed for 0–1,000 rpm). The glass stand was able to support more weight (7 cm diameter, 10 mm thickness). The new type of rotative stress device was composed of two main parts. One main part included a–g components, and another main part included h–j components (Fig. 1).

EXPERIMENTAL GROUPS

Induced mature osteoclasts were loaded under low stress (30 rpm, 0.03 g), medium stress (60 rpm, 0.12 g), and high stress (120 rpm, 0.48 g). After osteoclasts from each group were loaded for 0 (control group), 1, 2, 4, and 8 h, the mRNA and protein expression of CAII, FAS, FASL, OSCAR, and TRAP were detected by RT-PCR and Western blot, respectively.

RT-PCR

The main reagents included an RNA extraction kit (TRIZOL, No.15596018; Invitrogen Corp.), Fermentas RevertAid™ First Strand cDNA Synthesis Kit (No.K1622; MBI Corp.), Taq DNA Polymerase (No.B1263; Promega Corp., China), GeneAmp PCR System (No.9700; ABI Corp.), gel imaging analytical system (No.2200; Alpha Corp.), and ultraviolet spectrophotometer (No.DU800; Beckman Coulter Corp.). Primers for the five indicated genes were designed using Primer Premier 5.0 software and then synthesized in the laboratory. Routine PCR procedures included RNA extraction, first-strand cDNA synthesis, and PCR amplification of the target gene fragment, gel electrophoresis, and imaging. Integrated optical density (IOD) of the electrophoresis strip was recorded for analysis. DNAse treatment of the samples was performed in this study. We also verified that the amplification was in the linear phase when the measurements were taken. The PCR reaction conditions were as follows: initial denaturation at 95°C for

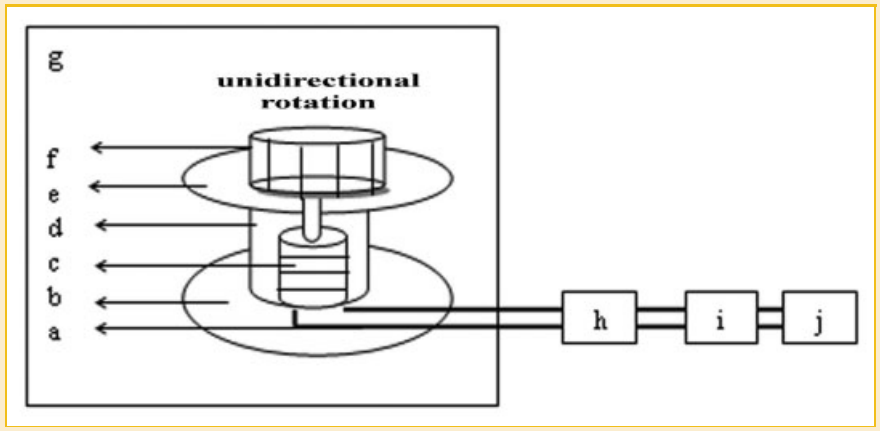

Fig. 1. Schematic diagram of a new-type of rotative stress device. The device was composed of the following parts: (a) means power line, (b) glass supporter, (c) generator, (d) silica glass cylinder, (e) objective table, (f) loading objective, (g) CO_2 incubator, (h) intelligent digital meter, (i) current transformer, and (j) power source. The dishes were fixed onto a glass platform, and the generator placed in the quartz tube provided the rotative force. Mature osteoclasts were used for stress loading experiments.

2 min, followed by 32 cycles of denaturation at 95°C for 30 s, renaturation for 30 s, and extension at 72°C for 30 s. The annealing temperatures and primer sequences for the five genes are listed in Table I.

WESTERN BLOT

The main reagents were the following: tris–glycine buffer (No.28380; Piece Cor.), 10× Ponceau S (No.P0370; BioHao Corp., China), TBS buffer solution (No.0788-2PK; Amresco Corp.), 10× TBST solution (GMS12130; Genmed Corp.), confining liquid (No.PA106-01; TIANGEN Corp., China), PVDF membrane (No.IPVH00010; Millipore Cor.); gel imaging system (No.Gel-Doc2000; Bio-Rad Corp.), electrophoretic apparatus (No.HV164-5056; Bio-Rad Corp.). Primary antibodies included TRAP (N-17, No.sc-30832; Santa Cruz Biotechnology Inc.), OSCAR (M-17, No.sc-34237; Santa Cruz Biotechnology Inc.), CAII (G-2, No.sc-48351, Santa Cruz Biotechnology Inc.), FAS (5F9, No.sc-52394, Santa Cruz Biotechnology Inc.), FASL (101624, No.ab10512; Abcam Corp., UK). Cells were harvested, lysed in ice-cold cell lysis solution (Western lysis buffer, No. P0013; Beyotime Corp., China), and then centrifuged at 12,000 rpm. Protein was quantified with the BCA method, resolved by 8–12% SDS–PAGE, and then transferred onto PVDF membrane. The following steps included addition of antibodies, incubation, TBST washing and detection of proteins.

Primary antibodies were diluted 1:2,000, and the membranes were placed into western eluant (No. P0023C; Beyotime Corp.) three times for 5 min each. Secondary antibodies conjugated to horseradish peroxidase were diluted to 1:5,000 and washed three times for 5 min each. An electrochemiluminesence kit (No. P0019/P0020; Beyotime Corp.) was used to detected protein bands.

TABLE I. Annealing Temperature and Primer Sequence.

Primer name	Sequence (5′–3′)	Amplified length (bp)	Annealing temperature (°C)
Actin	F-CTAAGGCCAACCGTGAAA R-TGGAAGGTGGACAGTGAG	724	60
CAII	F-GATTGGACCTGCCTCACA R-ACACCTGGGTCTTGCTTT	507	54
FAS	F-AGGAGGGCAAGATAGATG R-CTGCGACATTCGGCTTTT	151	56
FASL	F-TGGAGCAGTCAGCGTCAG R-ACAGGTGGTGGTGGAGGTG	245	56
OSCAR	F-TGATTGGCACAGCAGGAG R-AAGGCACAGGAAGGAAATAGAG	272	55
TRAP	F-CTCCCACCCTGAGATTTG R-GTTTCCAGCCAGCACATA	263	57

STATISTICAL ANALYSIS

Experiments were performed in triplicate for each sample. Three measurements were made for each dish of this study. In total, 150 dishes were analyzed. Data were recorded as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to assess statistical differences using SPSS17.0 software (SPSS, Inc., Chicago, IL). A P-value <0.05 was considered statistically significant. In this study, the data were normally distributed. The least-significant difference (LSD) and linear correlation analyses were used.

RESULTS

IN VITRO OBSERVATION OF RAW264.7 CELLS IN CULTURE

After the RAW264.7 cells were seeded, the cells that were in suspension displayed a round or oval shape in the first stage. In addition, most of cells had 1–2 nuclei. After 2 days of culture, the cells grew rapidly and began to form colonies. In general, the cells occupied 80–90% of the flask surface area by the third day of culture.

IN VITRO OBSERVATION OF OSTEOCLAST CULTURES

Multinuclear cells became apparent after RAW264.7 cells had been induced with RANKL for 3 days, and more of these cells were

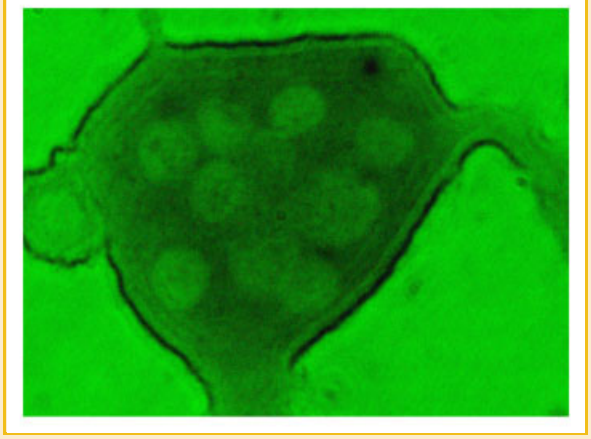

Fig. 2. In vitro osteoclast culture (400×). Osteoclast had an irregular shape and the nuclei were located in the middle of the cell body. Pseudopods extend out from the cytomembrane.

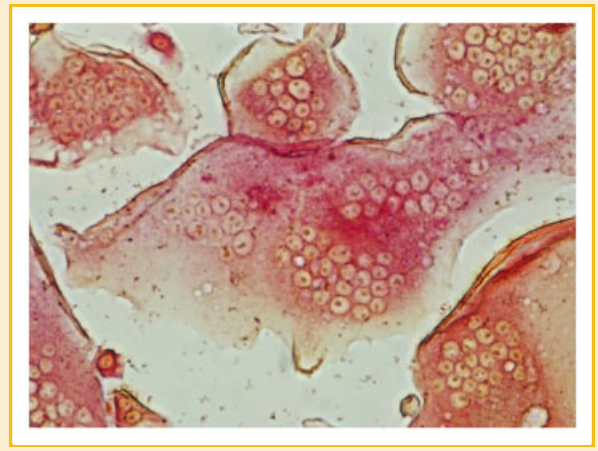

Fig. 3. TRAP staining of osteoclasts (400×). The cytoplasm had a rose pink staining with yellow nuclei.

observed by the fifth day. The number of multinuclear cells in the culture reached the peak by the sixth day, and cells with an irregular shape had a very large dimension. One representative cell demonstrated 50–80 nuclei (Fig. 2). Importantly, after the seventh day of culturing the cells in the presence of RANKL, apoptosis was observed in some cells.

TRAP STAINING OF OSTEOCLASTS

Cells were stained for TRAP and multinuclear positive osteoclasts were observed with a rose ink colored cytoplasm and light yellow nuclei (Fig. 3). In addition, the nucleoli were clearly observed in the cells.

CAII, FAS, FASL, OSCAR, AND TRAP mRNA EXPRESSION LEVELS IN OSTEOCLASTS

The CAII, FAS, FASL, OSCAR, and TRAP mRNA expression levels in osteoclasts subjected to various rotative stress loading strengths were measured (Table II). No significant differences in mRNA expression were observed between any of the control groups ($P > 0.05$), but importantly, rotative stress had a significant effect on the mRNA expression of these genes (Figs. 4 and 5; $P < 0.05$). Importantly, there was a negative relationship between rotative stress strength or prolonged loading time and the expression of FAS and FASL genes in osteoclasts. In contrast, a positive relationship between rotative stress strength or prolonged loading time and the

TABLE II. mRNA Expression of Five Genes in Osteoclasts Under the Influence of Various Rotative Stress Strengths in the Indicated Duration

		Time					
Stress strength	Gene	0 h	1 h	2 h	4 h	8 h	*P*-value
Low	CAII	6.29 ± 0.10	6.33 ± 0.07	6.58 ± 0.04	6.74 ± 0.06	6.84 ± 0.12	2.97E−05
Medium		6.26 ± 0.07	6.83 ± 0.04	6.86 ± 0.08	7.06 ± 0.04	7.29 ± 0.06	1.39E−08
High		6.24 ± 0.09	7.37 ± 0.08	7.48 ± 0.06	7.53 ± 0.10	7.64 ± 0.06	4.45E−09
	P	8.12E−01	3.97E−06	4.30E−06	2.67E−05	6.49E−05	
Low	FAS	8.16 ± 0.04	7.91 ± 0.02	7.81 ± 0.10	7.73 ± 0.12	7.49 ± 0.22	3.85E−03
Medium		8.10 ± 0.03	6.36 ± 0.10	6.20 ± 0.08	6.14 ± 0.09	6.06 ± 0.05	5.04E−11
High		8.04 ± 0.09	6.10 ± 0.05	5.84 ± 0.11	5.74 ± 0.09	5.64 ± 0.10	6.98E−11
	P	9.30E−02	1.02E−07	1.05E−06	5.5E−07	3.51E−05	
Low	FASL	7.28 ± 0.03	7.18 ± 0.26	7.06 ± 0.04	6.86 ± 0.11	6.78 ± 0.20	1.30E−02
Medium		7.24 ± 0.03	6.70 ± 0.02	6.67 ± 0.03	6.59 ± 0.04	6.46 ± 0.07	4.96E−09
High		7.22 ± 0.18	6.56 ± 0.07	6.47 ± 0.04	6.40 ± 0.02	6.22 ± 0.08	6.94E−09
	P	2.31E−01	6.274E−03	3.57E−06	6.30E−04	5.14E−03	
Low	OSCAR	6.32 ± 0.06	6.40 ± 0.02	6.46 ± 0.04	6.69 ± 0.07	6.80 ± 0.11	2.34E−05
Medium		6.26 ± 0.04	6.76 ± 0.05	7.21 ± 0.02	7.28 ± 0.02	7.42 ± 0.16	3.60E−08
High		6.22 ± 0.06	7.13 ± 0.11	7.36 ± 0.03	7.85 ± 0.06	8.01 ± 0.04	1.23E−10
	P	1.15E−E−01	4.94E−05	8.81E−08	6.45E−07	4.45E−05	
Low	TRAP	6.29 ± 0.02	6.40 ± 0.02	6.49 ± 0.02	6.65 ± 0.05	6.78 ± 0.05	6.51E−08
Medium		6.23 ± 0.06	7.06 ± 0.07	7.21 ± 0.03	7.28 ± 0.04	7.37 ± 0.03	2.07E−10
High		6.17 ± 0.06	7.23 ± 0.06	7.30 ± 0.06	7.41 ± 0.07	7.53 ± 0.08	8.77E−10
	P	5.57E−02	2.33E−06	3.51E−07	4.25E−06	6.56E−06	

Mean ± standard deviation; $\alpha = 0.05$.

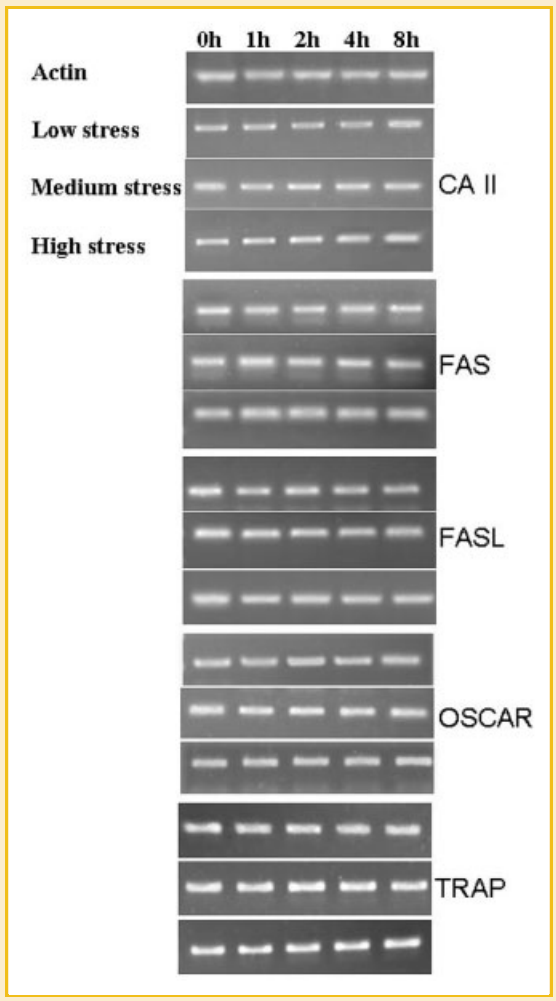

Fig. 4. RT-PCR of five genes in osteoclasts under the influence of various rotative stresses. Lanes 1–5: 0, 1, 2, 4, and 8 of stress time, respectively. Each gene was assessed from cells under low stress, medium stress, and high stress, respectively.

expression of CAII, OSCAR, or TRAP genes in osteoclasts was also observed.

CAII, FAS, FASL, OSCAR, AND TRAP PROTEIN EXPRESSION IN OSTEOCLASTS

We next assessed the protein expression levels of CAII, FAS, FASL, OSCAR, and TRAP in osteoclasts under various rotative stress conditions (Table III). Similar to the RT-PCR results, rotative stress affected the protein expression of these five genes significantly ($P < 0.05$), and a negative relationship was identified between rotative stress strength or prolonged loading time and FAS/FASL protein expression (Figs. 6 and 7). In contrast, however, there was a positive relationship between rotative stress and the protein expression of CAII, OSCAR, and TRAP genes (Figs. 6 and 7).

TREND OF MRAN AND PROTEIN EXPRESSION OF CAII, FAS, FASL, OSCAR, AND TRAP GENES IN OSTEOCLASTS EXPOSED TO ROTATIVE STRESS

The trend of gene expression of CAII, FAS, FASL, OSCAR, and TRAP was next assessed in osteoclasts under various rotative stresses and time durations. The expression of CAII, OSCAR, and TRAP genes tended to be elevated in osteoclasts from any of the strength groups, whereas FAS and FASL expression tended to be down-regulated under the same conditions. Similar findings were observed in cells exposed to rotative stress for the time points indicated.

DISCUSSION

Previously described cell loading devices have had flaws that have prevented fully accurate assessments of the effects of rotative stress on cells (Colombo et al., 2011; Schätti et al., 2011). In the past, these types of studies are critically important in dynamic bone remodeling. Several studies have shown that various stress parameters, such as strain, pressure, flow, and perturbation, can affect gene expression (Duncan and Turner, 1995; Aguirre et al., 2006; Kanzaki et al., 2006; Kim et al., 2006; Mehrotra et al., 2006; Penolazzi et al., 2006; Nakao et al., 2007). However, the equipment currently available for assessing these parameters has limitations. The pressure load parameter is measured by assessing the atmosphere in a closed container containing a cell culture under pressure. However, sealed apparatuses restrict long-term cell culture studies. Strain load is determined by measuring the deformation of elastic membrane. However, cell observation and gene detection are often difficult in these types of experiments. The flow load is measured by assessing the fluid medium against cells, but complicated manipulations can easily contaminate cultured cells. The perturbation load is measured by assessing the medium that is stirred with a vibrating stick, but similar to flow load measurements, the cell culture can be easily contaminated. Based on the characteristics of these loading devices and the limitations of current devices, our osteoclast research team designed and manufactured a new type of rotative loading device for this study. Our newly designed rotative loading device has several advantages, including a smaller dimension, lighter weight, easy manipulation, complete sterility, and long-time loading ability. The findings of this study showed that the device could overcome the challenges of other devices and provide high-quality data.

Although some studies have explored the cytomechanics of osteoclasts, the currently available data are limited (Cho et al., 2010; Kameo et al., 2011; Lecuit et al., 2011; Juffer et al., 2012). In this present study, we determined the following: (1) there was a negative relationship between rotative stress strength and prolonged loading time and FAS/FASL gene expression in osteoclasts; and (2) there was a positive relationship between rotative stress strength and prolonged loading time and CAII, OSCAR, and TRAP gene expression in osteoclasts. Therefore, we found that three of these genes were up-regulated in response to rotative stress, while two o of the genes were down-regulated. Sanuki et al. (2010) previously found that compressive force could increase TRAP-positive osteoclast differentiation by increasing M-CSF production and

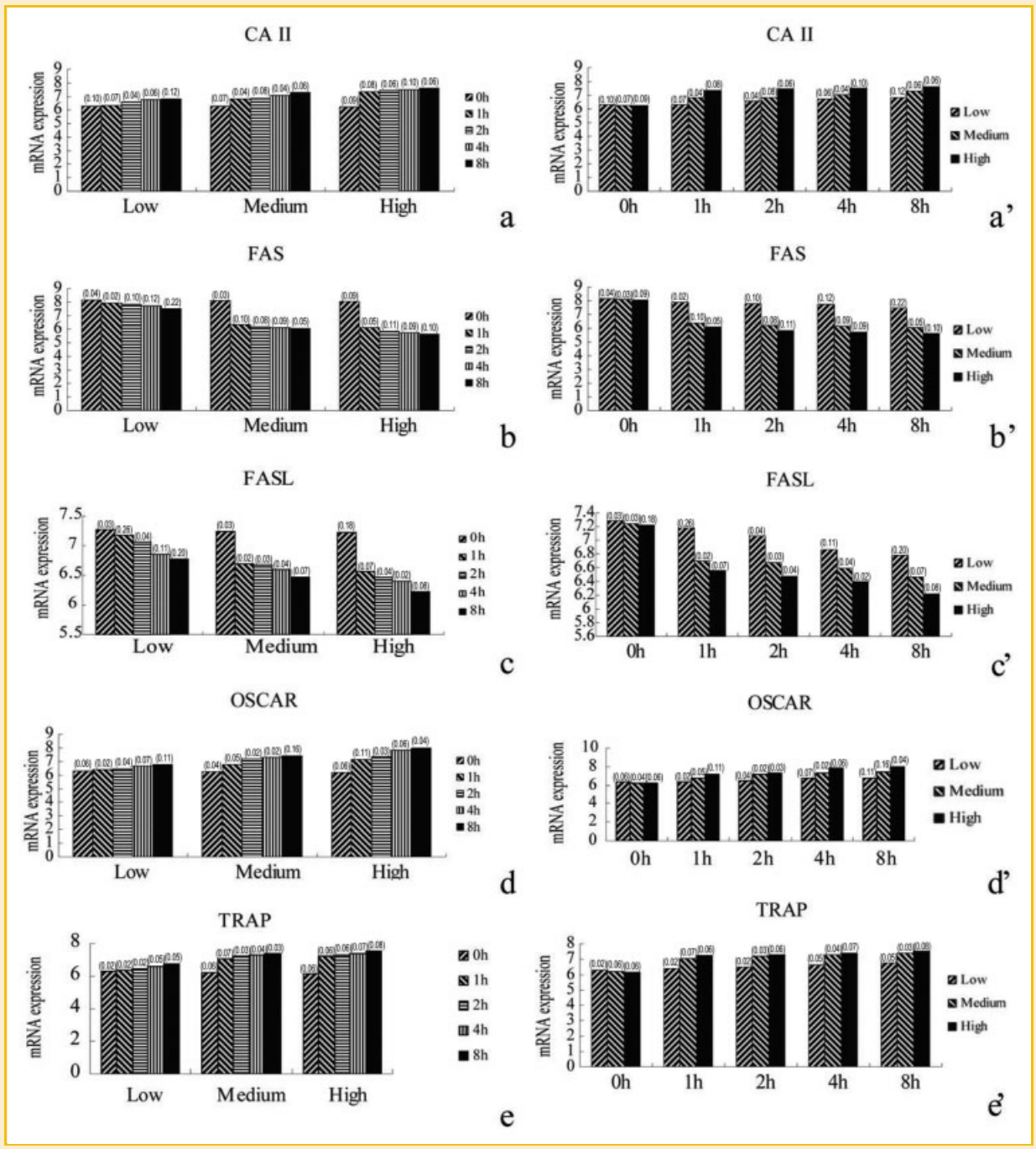

Fig. 5. Histogram of mRNA expression of five genes in osteoclasts under the influence of various rotative stress strengths and action times. a–e: mRNA expression of CAII, FAS, FASL, OSCAR, and TRAP genes, respectively. The X axis shows the various loading strength groups. a′–e′: mRNA expression of CAII, FAS, FASL, OSCAR, and TRAP genes, respectively. The X axis indicates that various load times.

decreasing osteoprotegerin (OPG) production through prostaglandin (PGE) (2) in osteoblasts. Another finding also indicated that TRAP-positive osteoclasts formed due to static mechanical compression-mediated up-regulation of RANKL expression in synovial cells (Ichimiya et al., 2007). In addition, prolonged hypoxia induced expression of TRAP at low-to-moderate levels, because hypoxia was found to evoke a stress signal through an autocrine mechanism (Srinivasan and Avadhani, 2007). Together, these results are in agreement with the findings of this study.

It has also been shown that TRAP expression in osteoclasts can be enhanced through various mechanisms (Yu et al., 2009). In our previous study, we found that fluid shear stress up-regulated CAII (Zhang et al., 2006). In the current study, we found that OSCAR was also up-regulated under similar conditions. Our results were also in

TABLE III. Protein Expression of Five Genes in Osteoclasts Under the Influence of Various Rotative Stress Strengths in the Indicated Duration

Stress strength	Gene	Time 0 h	1 h	2 h	4 h	8 h	*P*-value
Low	CAII						7.13E−07
Medium		95.12 ± 3.94	141.60 ± 1.52	165.26 ± 2.64	194.59 ± 7.21	211.36 ± 1.63	5.75E−11
High		92.97 ± 4.69	216.51 ± 0.54	246.01 ± 2.90	259.35 ± 0.82	261.27 ± 1.30	9.83E−15
	P	1.91E−01	8.92E−11	1.38E−09	5.88E−08	4.24E−10	
Low	FAS	5.23 ± 0.16	5.12 ± 0.05	4.69 ± 0.09	4.47 ± 0.06	4.15 ± 0.05	2.18E−07
Medium		5.14 ± 0.10	4.10 ± 0.04	2.16 ± 0.04	1.14 ± 0.03	0.89 ± 0.03	9.13E−16
High		5.12 ± 0.08	3.09 ± 0.03	2.07 ± 0.07	1.09 ± 0.03	0.82 ± 0.03	1.16E−15
	P	4.99E−01	2.04E−09	1.06E−08	1.10E−10	3.95E−11	
Low	FASL	4.56 ± 0.09	4.29 ± 0.03	3.73 ± 0.05	3.58 ± 0.03	3.40 ± 0.10	4.57E−09
Medium		4.51 ± 0.09	4.18 ± 0.07	3.70 ± 0.04	2.55 ± 0.05	1.50 ± 0.02	1.36E−13
High		4.44 ± 0.19	3.34 ± 0.09	2.67 ± 0.05	1.72 ± 0.04	1.35 ± 0.04	2.82E−11
	P	5.47E−01	4.93E−06	2.16E−07	3.66E−09	2.66E−08	
Low	OSCAR	0.82 ± 0.06	1.06 ± 0.03	1.13 ± 0.06	1.54 ± 0.05	1.96 ± 0.09	7.07E−09
Medium		0.75 ± 0.10	1.09 ± 0.03	1.28 ± 0.03	1.60 ± 0.08	2.38 ± 0.17	1.48E−08
High		0.65 ± 0.09	1.94 ± 0.08	2.02 ± 0.08	2.14 ± 0.09	3.27 ± 0.16	6.87E−10
	P	1.56E−01	1.11E−06	2.91E−06	1.17E−04	7.60E−05	
Low	TRAP	18.95 ± 0.83	22.46 ± 0.82	24.11 ± 0.51	40.85 ± 0.44	49.95 ± 1.25	1.78E−12
Medium		18.35 ± 0.82	47.24 ± 0.42	50.51 ± 0.60	56.15 ± 1.07	60.65 ± 0.33	4.28E−14
High		18.13 ± 0.91	77.65 ± 1.60	105.76 ± 3.91	111.13 ± 1.87	113.86 ± 0.31	3.77E−13
	P	5.22E−01	3.31E−09	2.82E−08	1.62E−09	1.29E−10	

Mean ± standard deviation; $\alpha = 0.05$.

agreement with a previous study showing that the apoptosis rate of osteoclasts increased from 24% to 41% after being subjected to tensional force for 2 days, with a subsequent decrease due to the up-regulation of TGF-b1/OPG (Kobayashi et al., 2000). The FAS/FASL genes associated with apoptosis took on a down-regulated expression tendency in this present study.

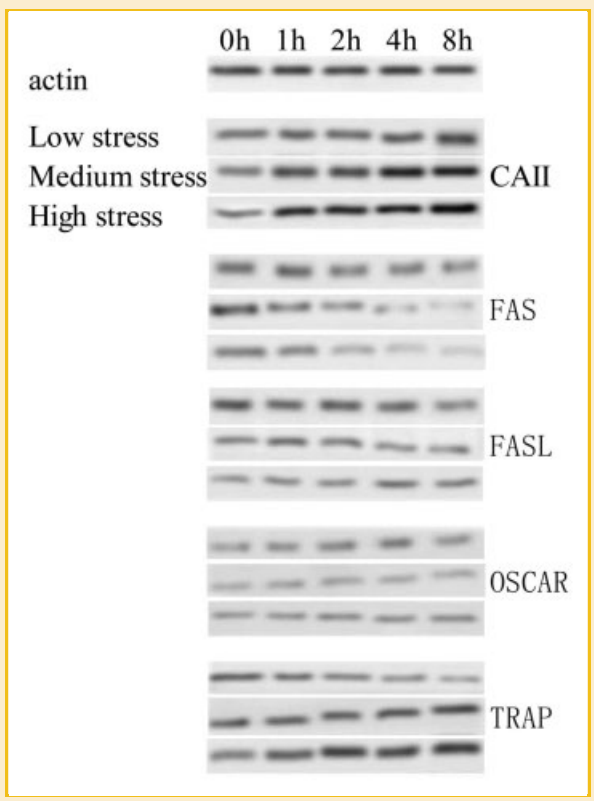

Fig. 6. Western blots of five genes in osteoclasts under the influence of various rotative stresses. Lanes 1–5: 0, 1, 2, 4, and 8 h of stress time, respectively. Each gene was loaded with low stress, medium stress, and high stress, respectively.

We found changes in gene expression with regard to bone modeling. The expression of CAII, OSCAR, and TRAP genes tended to be elevated when osteoclasts were loaded with various stress strength and times, whereas FAS and FASL expression tended to be down-regulated under the same conditions. The up-regulation of the former gene may be due to their involvement as markers of osteoclasts. For example, osteopetrosis may occur due to inhibition of CAII gene function, and high expression of the OSCAR gene is observed during the accurate stage of rheumarthritis. In addition, TRAP staining is generally considered as an indispensable means for osteoclastic resorption. Based on these relationships, the three genes had a similar expression pattern. However, FAS and FASL are factors that are associated with osteoclast apoptosis and deceasing bone resorption. Importantly, FAS/FASL have different functions than CAII, OSCAR, and TRAP, which were underscored in this study.

Loading strength and the time of stress are critical parameters, and strength that is too high or for too long can result in the disruption of cells from the flask and subsequent death. Therefore, we decided to use loading strengths that provided low stress (30 rpm, 0.03 g), medium stress (60 rpm, 0.12 g), and high stress (120 rpm, 0.48 g) after several pretests. In addition, we decided on loading times of as 0 (control group), 1, 2, 4, and 8 h. Our previous study found that gene expression in osteoclasts occurs in a rhythm based on time, and osteoclasts had the highest mRNA expression after maturing for 24 h (Zhang et al., 2011). Importantly, however, the cells began to undergo apoptosis after 48 h due to terminal differentiation (Zhang et al., 2011). Therefore, based on these previous findings, we chose 8 h as the longest loading time, and found that apoptosis did not occur, which allowed for proper assessment of the gene expression of osteoclasts undergoing rotative stress.

In summary, based on the findings of this study as well as previous studies, we conclude that: (1) functions of signaling factors

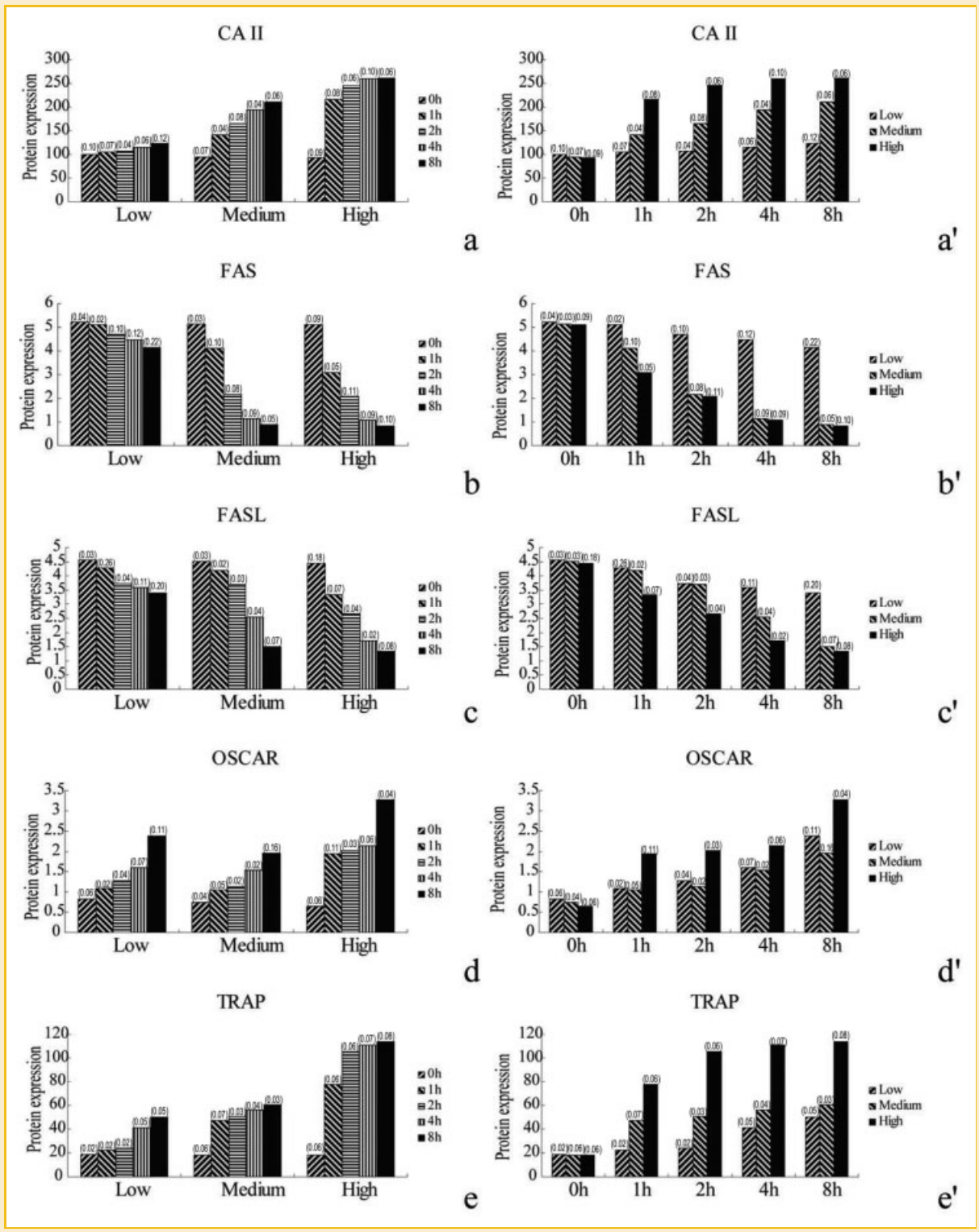

Fig. 7. Histogram of protein expression of five genes in osteoclasts under the influence of various rotative stress strengths and action times. a–e: mRNA expression of CAII, FAS, FASL, OSCAR, and TRAP genes, respectively. The X axis shows the various loading strength groups. a′–e′: mRNA expression of CAII, FAS, FASL, OSCAR, and TRAP genes, respectively. The X axis indicates that various load times.

may be induced by stress; (2) there was an adjustment in the signaling pathway because of similar stress patterns (Suzuki et al., 1996; Lee and Kim, 2003; Kawaguchi et al., 2004; Nakamura et al., 2005; Tanaka et al., 2006).

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