Published in final edited form as:

J Mol Cell Cardiol. 2015 February ; 79: 287–294. doi:10.1016/j.yjmcc.2014.12.009.

RIP140 contributes to foam cell formation and atherosclerosis by regulating cholesterol homeostasis in macrophages

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Abstract

Atherosclerosis, a syndrome with abnormal arterial walls, is one of the major causes that lead to the development of various cardiovascular diseases. The key initiator of atherosclerosis is cholesterol accumulation. The uncontrolled cholesterol deposition, mainly involving low-density lipoprotein (LDL), causes atheroma plaque formation, which initiates chronic inflammation due to the recruitment of inflammatory cells such as macrophages. Macrophages scavenge excess peripheral cholesterol and transport intracellular cholesterol to high-density lipoprotein (HDL) for excretion or storage. Cholesterol-laden macrophage-derived foam cell formation is the main cause of atherogenesis. It is critical to understand the regulatory mechanism of cholesterol homeostasis in the macrophage in order to prevent foam cells formation and further develop novel therapeutic strategies against atherosclerosis. Here we identified a protein, RIP140 (receptor interacting protein 140), which enhances macrophage-derived foam cell formation by reducing expression of reverse cholesterol transport genes, A TP-binding membrane cassette transporter A-1 (ABCA1) and ATP-binding membrane cassette transporter G-1 (ABCG1). In animal models, we found that reducing RIP140 levels by crossing macrophage-specific RIP140 knockdown (M ϕ RIP140KD) mice with ApoE null mice effectively ameliorates high-cholesterol diet-induced atherosclerosis. Our data suggest that reducing RIP140 levels in macrophages significantly inhibits atherosclerosis, along with markers of inflammation and the number of macrophages in a western diet fed ApoE null mouse. This study provides a proof-of-concept for RIP140 as a risk biomarker of, and a therapeutic target for, atherosclerosis.

Keywords

RIP140; atherosclerosis; foam cell; reverse cholesterol transport

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Disclosure

Nothing to be disclosed.

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1. INTRODUCTION

Hallmarks of atherosclerosis are abnormal cholesterol metabolism and inflammation [1]. Macrophages are critically involved in cholesterol metabolism and inflammation in the progression of atherosclerosis [2]. Macrophages scavenge excess peripheral cholesterol by uptake of LDL, and transport intracellular cholesterol to high-density lipoprotein (HDL), which can be stored or excreted by the liver through reverse cholesterol transport (RCT) [3]. However, when cholesterol levels are pathologically elevated, cholesterol-laden macrophages become inflammatory and turn to active foam cells [2, 4]. Macrophage-derived foam cell formation marks the initiation of atherosclerosis. Cholesterol retention in the macrophage promotes foam cell formation. In macrophages, the ATP-binding membrane cassette transporter A-1 (ABCA1) and ATP-binding membrane cassette transporter G-1 (ABCG1) are the major transporters mediating RCT: ABCA1 regulates RCT to apolipoprotein A-I, and ABCG1 regulates RCT to mature HDL [5].

Receptor Interacting Protein 140 (RIP140) is a protein found in metabolic tissues, such as liver, muscle and adipose tissue [6, 7]. As a versatile co-regulator of various transcription factors, RIP140 regulates metabolism, such as fat accumulation in adipocytes, by affecting the expression of metabolic genes [8–11]. RIP140 also exerts various regulatory functions through its extensive post-translational modifications, including various forms of phosphorylation, lysine-acetylation, lysine methylation, arginine methylation, vitamin B6 conjugation, and ubiquitination, etc [12–16]. RIP140 is also known to be expressed in the monocyte-macrophage lineage and can regulate inflammatory responses [17–19]. Our recent study indicated that accumulation of intracellular cholesterol in the macrophage elevated RIP140 and that RIP140 expression was sufficient to enhance inflammatory cytokine production and the inflammatory potential of the macrophage [20].

In this study, we provide novel data showing that RIP140 promotes foam cell formation by reducing cholesterol efflux. This process is mediated through the repression of ABCA1 and ABCG1. Further, cholesterol loading stimulates RIP140's post-translational modification that enhances its repressive activity. *In vitro*, augmenting RIP140 levels in the macrophage affects the efficiency of foam cell formation. Our preliminary study indicated that, fed a normal chow, mice with reduced RIP140 expression in macrophages, such as by macrophage specific RIP140 knockdown (m ϕ RIP140KD), exhibited no particular phenotypic changes. However, in ApoE null mice, lowering RIP140 levels specifically in the macrophage reduced the severity of western diet-induced atherosclerosis

2. MATERIALS AND METHODS

2.1. Animals

All studies were approved under University of Minnesota Institutional Animal Care and Use Committee. M ϕ RIP140KD transgenic mice were generated as described before[20], which carry a short hairpin RNA (shRNA)-expressing transgene driven by macrophage-specific human CD68 promoter. ApoE null mice were obtained from the Jackson Laboratory. Animals were maintained in the animal facilities in the University of Minnesota. Six-week-

old male mice were fed with a western diet (0.2% w/w total cholesterol, #D12079B, Research Diets, NJ, USA) for 16 weeks. Six mice were studied for each group.

2.2. Cell Culture and Chemicals

The mouse macrophage cell line was purchased from American Type Culture Collection. Cells were maintained as described previously. Mouse primary peritoneal macrophage was obtained as described[20]. Briefly, 4% thioglycollate was IP injected into mice. After 4 days, peritoneal macrophages were collected by peritoneal lavage. Cells were incubated with either 50ng/ml oxLDL or AcLDL (Intra Cel Resource, MD, USA) to induce foam cell formation. GW3965 hydrochloride (G6295, Sigma-Aldrich, MO, USA) at 5 μ M was used for LXR agonist treatment [21].

2.3. Histology

Aortic sinus tissues were collected after mice were sacrificed and perfused. Collected aortic sinus tissues were fixed in 4% paraformaldehyde overnight, transferred to 30% sucrose overnight and embedded in O.C.T. compound (Sakura Finetek, UK). Frozen sections were cut at 10 μ m thickness. Specific antibodies were applied for histological staining by using VECTASTAIN Elite ABC kit (PK-6101, Vector Labs, CA, USA) and DAB substrate kit (SK-4100, Vector Labs, CA, USA) according to manufacturer's instructions. RIP140 (ab42126), α -smooth muscle actin (α -SMA, ab5694) and Mac2 (ab2785) antibodies were purchased from Abcam (Cambridge, UK).

2.4. Sirius Red Staining

Tissue sections were air-dried and stained with 0.1% picro-sirius red solution, which was prepared by dissolving 500 mg sirius red (365548, Sigma-Aldrich, MO, USA) in 500 mL saturated picric acid (SP9200, Fisher Scientific, MA, USA), for 1 hour. Slides were washed with acidified water prepared by adding 5 mL acetic acid to 1 L ddH₂O, and images were acquired using microscope Zeiss (Axioplan 2 Upright).

2.5. Oil Red O Staining

Fixed cells or tissue sections were air-dried after incubated with 60% isopropanol at room temperature then stained with oil red O (O0625, Sigma-Aldrich, MO, USA) working solution. After 10 minutes staining, samples were washed with ddH₂O and images were acquired by microscope (Zeiss Axioplan 2 Upright). Oil red O was eluted by 100% isopropanol and measured optical density at 500 nm to quantitate staining. Oil red O stock solution was prepared by dissolving 350 mg oil red O in 100 mL isopropanol. Oil red O working solution was prepared by mixing 6 mL oil red O stock solution and 4 mL ddH₂O.

Aortae from ApoE null or ApoE null/RIP140 KD male mice were fixed in 4% formaldehyde. Staining of the aortae was done according to established procedure [22]. After rinsing in water, the aortae were placed in 60% Isopropanol for 2 minutes, followed by staining in 60% Oil Red O in water for 1 h at room temperature. The excess stain was removed by placing the tissues in isopropanol followed by water for a few seconds.

For quantification of plaque area, aortae were stained, aorta were cut open, pinned down and imaged under a 1X objective on the Zeiss Stereoscope for analysis. The red stained areas representing atherosclerotic plaques were measured using the AxioVision software. Data is represented as percent ratio of total plaque area over total vessel area.

2.6. Reverse Transcription and Quantitative PCR

RNA was isolated and purified by Trizol reagent followed by manufacturer's instruction. Reverse transcription was conducted by using High-Capacity cDNA Reverse Transcription Kit containing RNase Inhibitor (Applied Biosystems). Quantitative PCR (qPCR) was carried out as described previously[20]. Primer information is available upon request. Each expression analysis was performed triplicate and normalized to b-actin.

2.7. Cholesterol Fluorometric and Triglyceride Colorimetric Assays

Cholesterol fluorometric and triglyceride colorimetric assays were performed using commercial kits according to the manufacturer's instruction (10007640 and 10010303, Cayman, MI, USA). Plasma were isolated using EDTA-coated micro-container (365974, BD, NJ, USA). Intracellular cholesterol concentration was normalized to protein concentration.

2.8. Cholesterol Efflux Assay

Cholesterol efflux assay was followed and modified by established procedure[23]. Briefly, cells were incubated with 50ng/ml AcLDL and 10 μ ci of H³-cholesterol (NET139001MC, Perkin Elmer, MA, USA) overnight to induce foam cell formation. Cells were then incubated with Apo A-I (SRP4693, Sigma-Aldrich, MO, USA) or HDL (L8039, Sigma-Aldrich, MO, USA) in DMEM medium with 0.2% cholesterol free BSA for 6 hours. Culture medium were collected after 14000 rpm to remove cells. Cells were lysed by 0.1N NaOH. Radioactivity in medium and cells was measured by scintillation counter. Cholesterol efflux rate was calculated as (medium cpm)/(medium + cell cpm) $\times$ 100%.

2.9. Western Blotting

Protein isolation and Western blotting were performed as described before[10]. Antibodies purchased from Abcam (Cambridge, UK) are: RIP140 (ab42126), ABCA1 (ab18180), ABCG1 (ab52617). Antibodies purchased from Santa Cruz Biotechnology (TX, USA) are: b-actin (SC-47778), ERK1 (SC-93), ERK2 (SC-153). Antibodies obtained from Cell Signaling Technology (MA, USA) is phospho-ERK1/2 (9101).

2.10. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as described[24] using the RIP140 antibody (ab42126, Abcam, Cambridge, UK). Primers for LXR-binding promoter regions of ABCA1 and ABCG1 in qPCR were: 5'-GGGGAAAGAGGGAGAGAACAG-3'/5'-GAATTACTGGTTTTTGCCGC-3' (for ABCA1) and 5'-TGCCCTGTAGTAACCTCTGTA-3'/5'-TCATGTGCGACTCCTCCCA-3' (ABCG1) [25].

2.11. Statistical Analysis

Experiments were carried out at least twice and results were presented as means $\pm$ SD. Comparisons between groups were made by unpaired two-tailed Student's t-tests, or 1-way and 2-way ANOVA. P values of 0.05 were considered statistically significant.

3. Results

3.1. RIP140 promotes modified LDL induced macrophage foam cell formation

To examine the effects of RIP140 on macrophage-derived foam cell formation, we altered the expression level of RIP140 in primary peritoneal macrophage by infection with lentivirus encoding RIP140 expression vector or RIP140-specific shRNA. Two modified forms of LDL, oxidized LDL (oxLDL) and acetylated-LDL (AcLDL), were applied to cells to induce foam cell formation. Using oil red O staining to detect foam cell formation, we found that macrophages with elevated RIP140 levels were more susceptible to modified LDL-induced foam cell formation (Fig. 1A upper panel, S1A), while macrophages with lowered RIP140 levels were more resistant to this induction (Fig. 1B upper panel, S1B). Quantification of intracellular total cholesterol (TC), free cholesterol (FC) and esterified cholesterol (CE) further validates our finding (Fig. 1A lower panel, 1B lower panel). Data from the macrophage cell line Raw 264.7 showed consistent results (data not shown).

3.2. RIP140 suppresses reverse cholesterol transport (RCT) by repressing the expression of ABC transporters

ABC transporters mediated active RCT is critical to the removal of accumulated cholesterol from macrophages. To determine how RIP140 regulated LDL-induced macrophage-derived foam cell formation, we examined the expression of ABCA1 and ABCG1. The data show that elevating RIP140 repressed ABCA1 and ABCG1 expression (Fig. 2A), whereas lowering RIP140 enhanced ABCA1 and ABCG1 expression (Fig. 2B). We further validated whether RIP140 altered the function of ABCA1 and ABCG1 in mice by employing transgenic murine lines with macrophage specific RIP140 knockdown (M ϕ RIP140KD)[20]. Peritoneal macrophages were isolated from wild type and M ϕ RIP140KD mice, and cholesterol efflux functional assays were performed. Figure 2C shows that M ϕ RIP140KD macrophages exhibited an increased cholesterol efflux rate to Apo A-I, which indicates the function of ABCA1, and an increased cholesterol efflux rates to HDL, which indicates the function of ABCG1. In sum, specific knockdown of RIP140 in primary macrophages increases cholesterol efflux, while increasing RIP140 expression in primary macrophages decreases cholesterol efflux.

3.3. Oxidized LDL modifies RIP140 on lysine acetylation to repress LXR and suppress the expression of ABCA1 and ABCG1

Various post-translational modifications on RIP140 regulate its versatile functions. We previously reported that extracellular-signal-related kinase 2 (ERK2) activation stimulates its Tyr204 phosphorylation, leads to its lysine acetylation and enhances its gene repressive activity[12]. We thus examined whether RIP140 could be post-translationally modified by modified forms of LDL. The data show that oxLDL indeed enhanced lysine acetylation on RIP140 and promoted its HDAC3 recruitment – a hallmark of its gene repressive activity

(Fig. 3A). We further employed RIP140 K158/287Q mutant (mimicking its constitutive lysine acetylation, CP) and K158/287A mutant (mimicking a lack of lysine acetylation, CN) in Raw 264.7 cells to determine the effects of these post-translational modifications on RIP140 function[12]. It appears that cells with RIP140 CP mutant expressed less ABCA1 and ABCG1 (Fig. 3B), indicating that lysine acetylated RIP140 possesses more repressive activity on ABCA1 and ABCG1 genes. Liver X receptor (LXR) is a key transcriptional factor to regulate ABCA1 and ABCG1 expression, and RIP140 can be a co-regulator for LXR[26]. We employed ChIP to examine the occupancy of RIP140 on these gene regulatory regions. The data show that oxLDL indeed enhanced the binding of RIP140 to LXR response element on the promoter of ABCA1 and ABCG1 (Fig. 3C). To further validate RIP140 indeed acted to suppress LXR activation of ABCA1 and ABCG1 expression, we tested the effects of LXR agonist, GW3965 hydrochloride. As shown in Fig. 3D, LXR agonist activated the expression of ABCA1 and ABCG1, but both their basal and induced expression levels were dramatically reduced when RIP140 level was elevated by over-expression. All together, these results validate that lysine acetylated RIP140 represses the expression of ABCA1 and ABCG1 via co-regulating (co-repressing) LXR on the promoters of these genes.

3.4. Specific RIP140 knockdown in the macrophage attenuates western diet-induced atherosclerosis

To assess the function of RIP140 in diseases where foam cells play a key role, we employed a well-established atherosclerosis animal model, ApoE null mice[27]. We crossed the M ϕ RIP140KD mice, where RIP140 in the macrophage populations was specifically silenced, with the ApoE null mice to generate macrophage RIP140 knockdown in the ApoE null background (ApoE null/RIP140KD). Right panel of fig. 4A shows that the macrophage RIP140 expression level in these ApoE null/RIP140KD mice was reduced as compared to the control ApoE null mice. The ApoE null and ApoE null/RIP140KD mice were fed with a western diet for 16 weeks to induce atherosclerosis. The body weight and plasma concentrations of triglyceride (TG) and cholesterol after a normal diet or western diet feeding were not affected by RIP140 knockdown in the macrophage (Figure S2), therefore we only focused on the phenotype of animals with western diet feeding. However, the lesion size in the aortic sinus of ApoE null/RIP140KD mice was reduced as compared to the ApoE null mice ($p < 0.05$) (Fig. 4A, left and middle panel, Fig. S3). In addition, plaque composition was analyzed in histological staining for major constituents of atherosclerotic plaques: α -smooth muscle actin (α -SMA) for smooth muscle cells (SMC), MAC2 for macrophages, and sirius red for collagen (Fig. 4B–E). The expression of RIP140 was monitored in the plaque areas (Fig. 4D). Quantification of staining indicates that fewer macrophages and fewer collagens were found in the plaque areas of aortic sinus of ApoE null/RIP140KD mice, but no apparent change was detected for SMC (Fig. 4B–E). As compared to the ApoE null mice, the ApoE null/RIP140KD mice expressed significantly lower levels of pro-inflammatory markers [tumor necrosis factor- α *Tnf- α* , interleukin 1- β *Il1- β* , interleukin 6 *Il6*] and Monocyte Chemoattractant Protein-1 (*Mcp-1*) in the aortic root (Fig. 4F). Furthermore, ApoE null/RIP140KD mice expressed significantly lower levels of endothelial adhesion molecules [intracellular adhesion molecule 1 (*Icam1*) and vascular cell

adhesion molecule 1 (*Vcam1*), as well as extracellular matrix [collagen 1 (*Col1*) and collagen 3 (*Col3*)] in the aortic root (Fig. 4F).

Taken together, these data support that reducing RIP140 levels in the macrophage can significantly inhibit the progression of atherosclerosis.

4. Discussion

Cholesterol imbalance, especially in the macrophage, is a major culprit triggering atherosclerosis. Macrophages can engulf excess cholesterol via specific receptors and also exert reverse cholesterol transport to expel cholesterol[28, 29]. It has been suggested that reducing cholesterol retention in the macrophage can ameliorate atherosclerosis. The current study provides a proof of concept in animal models of atherosclerosis and identifies a new target, RIP140, for augmenting this disease. The study also determines the mechanism that RIP140 represses the expression of ABCA1 and ABCG1 to reduce RCT and that cholesterol can alter RIP140's post-translational modification that further escalates its RCT-repressing effects. This current study also shows that reduction in macrophage's RIP140 level lowers the expression levels of pro-inflammatory cytokines. This is in line with our previous report that RIP140 activates pro-inflammatory cytokine production by serving as a coactivator of NF- κ B [20]. Thus, lowering the level of RIP140 in the macrophage can reduce macrophage-derived foam cell formation and contribute to reduced inflammation, ultimately this helps to ameliorate atherosclerosis and dampen monocyte/macrophage accumulation in the plaque. Given that RIP140 affects steroid receptor inhibition, it would be interesting to see whether macrophage specific reduction in RIP140 level would similarly reduce foam cell formation and inflammation in female animals.

Studies have indicated that LDL triggers p42/44 MAPK (ERK1/2) activation[30, 31]. We previously reported that activated ERK1/2 stimulates RIP140 phosphorylation[12]. This current study shows that oxLDL activates ERK1/2, which can stimulate RIP140's phosphorylation and subsequently induce its lysine-acetylation to enhance its transcriptional co-repressive activity, which is in line with previous observations, and validates that fat contents in the cell can affect RIP140's biological activity. In the macrophage, RIP140 functions to repress the expression of ABCA1 and ABCG1 by co-repressing LXR-target genes such as ABCA1 and ABCG1. Thus, lowering RIP140 levels or altering RIP140 modification, such as blocking its phosphorylation or lysine-acetylation, can reduce its repressive effects on ABCA1 and ABCG1, and can be beneficial. *In vivo* animal study provides strong evidence that reducing RIP140 levels in the macrophage could be a therapeutic strategy for atherosclerosis.

We previously reported that MiR-33 targets RIP140's 3'UTR to repress its expression[18]. Other studies showed that MiR-33 also reduces the expression of ABCA1 and ABCG1[32, 33]. It has also been shown that Mir-33 deficiency raised HDL to increase cholesterol efflux[34]. Thus, while MiR-33 can effectively reduce RIP140 levels, it may not be a therapeutically viable strategy in the case of atherosclerosis because of its broad effects. Currently, there is no specific compound that can directly repress RIP140 levels or augment its post-translational modifications. Further worthy studies are needed such as to screen for

compounds that can specifically target RIP140 to reduce its expression level or to augment its post-translational modifications. Furthermore, RIP140 can also serve as a disease maker, such as in assessing the risk or progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank P-C Ho's technical assistance in the beginning of this project. We also thank Lillehei Heart Institute's core histology laboratory.

Source of Funding

This work was supported by DK54733, DK60521, DK54733-11S, the Dean's Commitment, the Distinguished McKnight Professorship of University of Minnesota (LNW), 1R21HL104596 (JH) and Lillehei Heart Institute's pilot grant support (LNW and JH).

Abbreviations

LDL	low density lipoprotein
HDL	high density lipoprotein
RIP140	receptor interacting protein 140
ABCA1	ATP-binding membrane cassette transporter A-1
ABCG1	ATP-binding membrane cassette transporter G-1
RCT	reverse cholesterol transport
oxLDL	oxidized low density lipoprotein
AcLDL	acetylated-low density lipoprotein
ApoE	apolipoprotein E
ApoA-I	apolipoprotein A-I
LXR	liver X receptor
ERK2	extracellular-signal-related kinase 2

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Highlights

- RIP140 promotes macrophage-derived foam cell formation.
- RIP140 suppresses LXR-regulated expression of ABCA1 and ABCG1
- Lowering RIP140 level in macrophage ameliorates atherosclerosis.

Fig. 1. RIP140 enhances oxidized LDL-promoted cholesterol accumulation in macrophages
 (A) Upper panel: intracellular cholesterol accumulation was assessed by oil red O staining in primary peritoneal macrophages overexpressing RIP140. Lower panel: Quantification of TC, FC and CE. (B) Upper panel: intracellular cholesterol accumulation was assessed by oil red O staining in primary peritoneal macrophages with knockdown of RIP140. Lower panel: Quantification of TC, FC and CE. Data were presented as fold change. The concentration from the control group was arbitrarily set as 1, *P<0.05. Experiments were performed for three times.

Fig. 2. RIP140 suppresses reverse cholesterol transport in macrophages

(A) ABCA1 and ABCG1 protein and mRNA levels of primary peritoneal macrophages with overexpression of RIP140. (B) ABCA1 and ABCG1 protein and mRNA levels of primary peritoneal macrophages with knockdown of RIP140. (C) Macrophages from M ϕ RIP140KD mice exported more cholesterol to ApoA-I, via ABCA1, and HDL, via ABCG1. Data were presented as mean $\pm$ SD, *P<0.05. Experiments were performed three times.

Fig. 3. Oxidized LDL modifies RIP140 on lysine acetylation to repress LXR

(A) Activation of ERK1/2, and lysine-acetylation of RIP140 and its interaction with HDAC. OxLDL induced ERK1/2 activation to promote lysine-acetylation on RIP140 and its interaction with HDAC3. (B) The effect of RIP140 mutants on ABCA1 and ABCG1 expression. CP, RIP140 mutant mimicking constitutive lysine-acetylation; CN, RIP140 negative mutation for lysine-acetylation; EV, control vehicle only. (C) CHIP assay. Binding of RIP140 to LXR response element on ABCA1 and ABCG1 promoter area was enhanced by oxLDL treatment. (D) Effects of LXR agonist on mRNA levels of ABCA1 and ABCG1 in Raw 264.7 macrophages overexpressing RIP140. Data were presented as mean±SD, *P<0.05. Experiments were performed three times.

Fig. 4. RIP140 knockdown in macrophage ameliorates atherosclerosis in ApoE null mice
 (A) Oil red O staining of aortic sinus (left panel) and quantitative relative lesion size (middle panel) after 16 weeks western diet feeding. Average lesion size of ApoE null mice was set as 1. Relative macrophagic RIP140 mRNA expression level is shown on the right panel. (B) Mac2 staining of plaques in aorta sinus area. (C) Quantification of macrophage infiltration areas. (D) α -SMA (α -smooth muscle actin), RIP140 and sirius red staining of plaques in aorta sinus areas. (E) Quantification of SMC (smooth muscle cells) and collagen areas. (F) mRNA levels of *Tnf- α* , *Il1- β* , *Il6*, *MCP-1*, *Icam1*, *Vcam1*, *Col1* and *Col3* in aortic root. Data show results from ApoE null mice crossed with M ϕ RIP140KD mice. Experiments were performed two times.