

Molecular mechanisms regarding potassium bromate-induced cardiac hypertrophy without apoptosis in H9c2 cells

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Abstract. Cardiac hypertrophy is commonly involved in cardiac injury. Oxidative stress can induce cardiac hypertrophy with apoptosis. Potassium bromate (KBrO₃) has been widely used as a food additive due to its oxidizing properties. In the present study, the rat-derived heart cell line H9c2 was used to investigate the effect of KBrO₃ on cell size. KBrO₃ increased cell size at concentrations <250 μ M, in a dose-dependent manner. Additionally, KBrO₃ also promoted the gene expression of two biomarkers of cardiac hypertrophy, brain/B-type natriuretic peptides (BNP) and β -Myosin Heavy Chain (β -MHC). However, apoptosis remained unobserved in these cells. Moreover, mediation of free radicals was investigated using a fluorescence assay, and it was observed that superoxide and reactive oxygen species (ROS) levels increased with KBrO₃. Effects of KBrO₃ were significantly reduced by tiron at concentrations sufficient to produce antioxidant-like action. Additionally, signals involved in cardiac hypertrophy such as calcineurin and nuclear factor of activated T-cells (NFAT) were also determined using western blot analysis. KBrO₃ increased the protein levels of both these molecules which were decreased by tiron in a dose-dependent manner.

Additionally, cyclosporine A attenuated the cardiac hypertrophy induced by KBrO₃ in H9c2 cells at concentrations effective to inhibit calcineurin, in addition to reducing mRNA levels of BNP or β -MHC. Finally, apoptosis was also identified in H9c2 cells incubated with KBrO₃ at concentrations >300 μ M. Collectively, these results provided a novel perspective that KBrO₃ induces cardiac hypertrophy without apoptosis at a low dose through the generation of ROS, activating the calcineurin/NFAT signaling pathway in H9c2 cells. Therefore, at a dose <250 μ M, KBrO₃ can be applied as an inducer of cardiac hypertrophy without apoptosis in H9c2 cells. KBrO₃ can also be developed as a tool to induce cardiac hypertrophy in animals.

Introduction

Cardiac hypertrophy, the first phase of cardiovascular disease, induces heart failure. However, cardiac hypertrophy is an important compensatory mechanism in response to physiological or pathological stimuli that involve regulation of cellular signaling mediators and transcript factors (1-3). Hypertrophic signals result in increased protein synthesis and increased cell cycle (4). Cardiac hypertrophy is characterized by cell enlargement, involving physiological and pathological hypertrophy (5). Pathological cardiac hypertrophy is often coupled with interstitial and perivascular fibrosis, in addition to apoptosis and the release of atrial natriuretic peptides (ANP) and brain/B-type natriuretic peptides (BNP). With the initiation of cardiac hypertrophy, concentric hypertrophy is the primary phenotype that highly resists after-load and is known as the adaptive phase. As the cardiac damage progresses, cell length increases, leading to increased hypertrophy (6). In cardiac hypertrophy, nuclear factor of activated T-cells (NFAT) is considered an important mediator of several signal-transduction pathways involved in the coordination of pathological stimulation (7). The role of reactive oxygen species (ROS) in the induction of cardiac hypertrophy has been demonstrated (8). However, oxidative stress induces apoptosis in addition to cardiac hypertrophy (9). Therefore, the development of a model of cardiac hypertrophy only without apoptosis is essential.

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Abbreviations: BNP, brain/B-type natriuretic peptides; NFAT, nuclear factor of activated T-cells; ROS, reactive oxygen species

Key words: potassium bromate, oxidative stress, tiron, hypertrophic signals, H9c2 cells

Potassium bromate (KBrO₃) has been widely used as a food additive and is also a by-product of disinfecting drinking water by ozonation (10). KBrO₃ is applied primarily due to its oxidizing properties and may cause lipid peroxidation and oxidative DNA damage in humans and other mammals (11). KBrO₃ is also known as a rodent carcinogen (12,13) and as a renal and/or neuro-toxicant in humans (14).

Free radicals produced by KBrO₃ are easily associated with cardiac injury, as the heart is very sensitive to ROS-induced damage (15). Thus, KBrO₃ has been classified as one of the cardiac toxins because lipid peroxidation increases with a significant reduction in cardiac antioxidant capacity (16). Recently, vanillin has been demonstrated as an antioxidant as it improves KBrO₃-induced cardiac injury in mice (17). However, the effect of KBrO₃ on cardiac hypertrophy remains undetermined.

In the present study, KBrO₃ was applied to the cardiac cell line H9c2, which is widely used to induce cardiac hypertrophy (18). The aim was to develop a novel and simple model of cardiac hypertrophy without apoptosis *in vitro*.

Materials and methods

Materials. Potassium bromate (KBrO₃), cyclosporine A, and antioxidant (tiron) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All other reagents were obtained from the supplier as indicated and were at least of analytical grade.

Cell culture. The H9c2 cells (cat. no. 60096; Bioresource Collection and Research Center, Hsinchu, Taiwan) were cultured as previously described (19). In brief, H9c2 cells were maintained in Dulbecco's modified Eagle's medium (pH 7.2; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Little Chalfont, UK). The H9c2 cells were plated at a density of 6,000 cells/cm² and were allowed to proliferate in the growth medium. Following plating, the medium was replaced on the second day. The next day, cells were incubated with testing agent(s) as described below.

Experimental protocol. Briefly, H9c2 cells were incubated with KBrO₃ to identify the changes in cell size. Then, inhibitor was used to pretreat at 37°C for 60 min prior to the addition of KBrO₃. Solvent applied to dissolve the inhibitor was also pretreated in same manner at same volume and was termed the vehicle-treated group. Specific inhibitor(s) were applied to investigate the potential mechanism(s) of KBrO₃. Cyclosporine A (CsA) is a powerful immunosuppressant and has also been used to inhibit calcineurin (20). Therefore, as previously reported (21), CsA at an effective concentration (500 nM) was incubated with H9c2 cells at 37°C for 1 h prior to the treatment with KBrO₃. Additionally, tiron is a water-soluble and cell permeable antioxidant that scavenges superoxide (22,23). It was also applied as detailed above for CsA to investigate the role of free radicals in the effects of KBrO₃.

Measurement of cardiac hypertrophy. H9c2 cells at a density of 7.5x10³ cells/ml were arranged on a 24-well plate (Greiner Bio-One, Monroe, North Carolina, USA). Cells were starved

for 4 h in a serum-free medium prior to treatment with KBrO₃ at 37°C for 72 h. Briefly, following washing twice with cold PBS, the cells were fixed in 4% paraformaldehyde at room temperature for 15 min and washed with PBS containing 2% bovine serum albumin (GE Health Care Life Sciences) and 0.1% Triton X-100. Cells were stained with rhodamine phalloidin (Invitrogen; Thermo Fisher Scientific, Inc.) for 25 min at room temperature to identify the actin filaments and with DAPI (Abcam, Cambridge, UK) to stain the nucleus. An entire field of vision was characterized using a fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan) connected to an imaging system (DP2-BSW; Olympus Corporation). The cell sizes were magnified x200 and analyzed by the imaging system. Cell surface area size was determined and quantified by imaging to the complete boundary of the individual cells. The results were subsequently expressed as the percentage change of the surface area level of the cells, based on analysis using image J program, version 1.46 (National Institutes of Health, Bethesda, MD, USA; <http://imagej.nih.gov/ij/>) as described previously (24).

Identification of intracellular superoxide levels. Following the methods described in a previous study (25), H9c2 cells were seeded in 24-well plates at a density of 7.5x10³ cells/ml at 37°C overnight. Following starvation for 4 h in a serum-free medium, the cells were treated with 250 µM KBrO₃ at 37°C for an additional 72 h. For the detection of the intracellular superoxide levels, dihydroergotamine (10 µM) from Thermo Fisher Scientific Inc. was applied to react with intracellular superoxide ions at 37°C for 30 min following treatment with KBrO₃. An entire field of vision was characterized using a fluorescence microscope (IX71, Olympus Corporation) connected to an imaging system (DP2-BSW, Olympus Corporation). The results were subsequently expressed as a percentage of the intracellular superoxide levels in the cells based on the analysis using image J program, version 1.46 (National Institutes of Health), as described previously (24).

Determination of intracellular calcium. Changes in the intracellular calcium concentrations were also detected using a fluorescent probe, fura-2, as described previously (26). Fluorescence was continuously recorded by a fluorescence spectrofluorometer (F-2000; Hitachi, Ltd., Tokyo, Japan). The values of [Ca²⁺]_i were determined as described previously (26).

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). As previously described (24), Total RNA was extracted from H9c2 cells using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.). Total RNA (5 µg) was reverse-transcribed into cDNA with random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany). Analysis was carried out using LightCycler (Roche Diagnostics GmbH). PCR cycles were under the following conditions: Pretreatment at 95°C for 10 sec, 96°C for 10 sec, 60°C for 30 sec, 72°C for 1 sec, 40°C for 30 sec (45 cycles). β-actin was used as the control of the input RNA level. The primers used in RT-qPCR analysis were designed by Roche (Roche Diagnostics GmbH). The concentration of each PCR product was calculated relative to a corresponding standard curve. Analysis of relative gene expression data using

real-time quantitative PCR and the $2^{-\Delta\Delta C_q}$ method as described previously (27). The relative gene expression was subsequently indicated as the ratio of the target gene level to that of β -actin. The primers for BNP, β -Myosin Heavy Chain (β -MHC), and β -actin were:

BNP forward, 5'-GTCAGTCGCTTGGGCTGT-3' and reverse, 5'-CCAGAGCTGGGGAAAGAAG-3'; β -MHC forward, 5'-CATCCCCAATGAGACGAAGT-3' and reverse, 5'-GGG AAGCCCTTCTACAGAT-3'; β -actin forward, 5'-CTAAGG CCAACCGTGAAAAG-3' and reverse, 5'-GCCTGGATG GCTACGTACA-3'.

Western blot analysis. Cells were harvested and washed with ice cold phosphate buffer solution, then homogenized in radio immunoprecipitation assay buffer which was prepared according to a previous method (24). Lysates were mixed and incubated on ice for 10 min. Cell debris was precipitated at 4°C for 10 min (16,099 x g). Protein concentrations were measured by bicinchoninic acid protein assay (Thermo Fisher Scientific, Inc.). Proteins were separated by 10% or 15% SDS-PAGE and electro-transferred onto a polyvinylidene fluoride membrane. Nonspecific binding was blocked by incubation in 5% nonfat milk at room temperature for 2 h. Membranes were subsequently incubated with the primary antibodies (at 1:1,000 dilution) at 4°C overnight, washed 3 times with TBST (0.05% Tween-20) and finally incubated with a HRP-conjugated secondary antibody at room temperature for 1 h. Protein bands were visualized using an chemiluminescence (ECL) kit (PerkinElmer, Inc., Waltham, MA, USA). The optical densities of the bands for calcineurin (18 kDa), NFAT3 (100 kDa), Histone H3 (15 kDa), and β -actin (43 kDa) were quantified using a laser densitometer (CHEM-400; Avegene Life Science, Taipei, Taiwan), as described in a previous study (28). The target antigens from the protein extracts were detected using primary antibodies specific for calcineurin (C0581; Sigma-Aldrich; Merck KGaA), NFAT3 (PA-1-021; Thermo Fisher Scientific, Inc.), or β -actin (A5441; Sigma-Aldrich; Merck KGaA) and Histone H3 (SC-8654; Cell Signaling Technology, Inc., Danvers, MA, USA). Additionally, the apoptotic markers such as Bcl2 (AB59348; Abcam, Cambridge, MA, USA), AIF (07-208; Merck KGaA) and Caspase-3 (9662S; Cell Signaling Technology, Inc.) were also determined.

Nuclear extraction. The extraction of the nuclear fraction was performed according to a previously described method (28), using a CNMCS Compartmental Protein Extraction Kit (BioChain Institute, Inc., Hayward, CA, USA). Briefly, H9c2 cells were collected and ice-cold lysis buffer (2 ml per 20 million cells) added to them. The cell mixture was passed through the needle base 50-90 times to disrupt the cell membranes and to release the nuclei from the cells. The degree of cell membrane disruption and the release of nuclei were monitored with a fluorescence microscope. The mixture was then centrifuged at 15,000 x g at 4°C for 20 min. The supernatant, which contained cytoplasmic proteins, was removed and was saved in a separate tube. The pellet was resuspended in ice-cold washing buffer (4 ml per 20 million cells), and the suspension was rotated at 4°C for 5 min, followed by centrifugation at 15,000 x g at 4°C for 20 min. The supernatant was

removed and ice-cold nuclear extraction buffer (1 ml per 20 million cells) was added to the pellet. Following rotation at 4°C for 20 min, the suspension was centrifuged at 15,000 x g at 4°C for 20 min. The supernatant, which contained nuclear proteins, was removed and saved for further use.

Cytotoxicity assay. Cell viability and survival were determined by an MTT assay (29). Briefly, 5,000 cells were plated in 24-well plates in triplicate and treated with concentrations between 250 and 5,000 μ M of KBrO₃ at 37°C for 72 h. Following treatment, the medium was removed and replaced with 500 μ l/well fresh medium, and 50 μ l MTT (final concentration 0.5 mg/ml) was added to each well. The plates were incubated at 37°C for 4 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. The formazan crystals were dissolved using a solution of 0.01 M HCl/SDS 10%. Finally, the absorbance in each individual well was determined at a wavelength of 595 nm, with the absorbance of the cells counted by a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Relative cell viabilities were calculated as percentages vs. control cells: Relative cell viability (%)=(absorbance of treated cells)/(absorbance of control cells) x100%.

Staining of live and dead cells. To image the live and dead cells, a LIVE/DEAD viability assay kit (Molecular Probes; Thermo Fisher Scientific, Inc.) was used, according to the manufacturer's protocol. H9c2 cells at a density of 5×10^4 cells/ml were incubated with two probes, calcein-AM (green color) and ethidium homodimer-1 (EtdD-1, bright red color), for intracellular esterase activity and plasma membrane integrity, respectively. Subsequently, specimens were observed under a fluorescence microscope (Olympus IX71; Olympus Corporation). The live cells are shown in green and the dead cells in red (magnification, x4). The images were analyzed using image J program, version 1.46 (National Institutes of Health). Each assay performed in triplicate.

Flow cytometry analysis. H9c2 apoptotic cell measurement was performed by flow cytometry with Annexin V-propidium iodide staining, as described previously (30), using a fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA). The Annexin reagent utilizes Annexin V to detect phosphatidylserine of apoptotic cells and a dead cell marker as an indicator of membrane stability. H9c2 cells were seeded in a dish (10 cm) at a density of 1×10^6 cells/well, and the experimental procedure was performed according to the manufacturer's protocol. Staining was analyzed by fluorescence-activated cell sorting on a flow cytometer (NOVO Cyte 3000; ACEA Biosciences, San Diego, CA., USA). Spectral compensation and data quantification were performed using NovoExpress software (2017 Version; ACEA Biosciences Inc., San Diego, CA, USA) that provided a variety of plots and gates for flow cytometry data analysis.

Statistical analysis. Data are presented as the mean \pm standard error from the indicated sample size (N) each performed in triplicate. One-way analysis of variance with post hoc Tukey test was used for multiple comparisons by SPSS

Table I. Effects of KBrO₃ on cultured cardiac cells.

Contents	Control	KBrO ₃ (100 μ M)	KBrO ₃ (200 μ M)	KBrO ₃ (250 μ M)
Relative area level (fold change)	1.00 \pm 0.07	1.46 \pm 0.06	2.07 \pm 0.11 ^a	2.74 \pm 0.09 ^b
Relative mRNA level of BNP/ β -Actin	1.00 \pm 0.00	1.19 \pm 0.03	1.60 \pm 0.05 ^a	2.09 \pm 0.07 ^b
Relative mRNA levels of β -MHC/ β -Actin	1.00 \pm 0.00	1.13 \pm 0.05	1.54 \pm 0.05 ^a	1.99 \pm 0.01 ^b
Ratio of Bcl2/ β -Actin protein	0.74 \pm 0.08	0.74 \pm 0.09	0.73 \pm 0.09	0.73 \pm 0.07
Ratio of Caspase-3/ β -Actin protein	0.63 \pm 0.09	0.62 \pm 0.08	0.61 \pm 0.07	0.60 \pm 0.07
Ratio of AIF/ β -Actin protein	0.70 \pm 0.10	0.70 \pm 0.09	0.67 \pm 0.08	0.68 \pm 0.08

Data are presented as the mean \pm standard error (n=6). ^aP<0.05 or ^bP<0.001 vs. the non-treated control. AIF, apoptosis-inducing factor; Bcl, B-cell lymphoma; β -MHC, β -Myosin Heavy Chain; BNP, brain/Btype natriuretic peptides; KBrO₃, potassium bromate.

version 16.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of KBrO₃ on H9c2 cell size. In a preliminary experiment, H9c2 cells were incubated with KBrO₃ (150 μ M) for 24, 48, 72 and 84 h. KBrO₃ increased the H9c2 cell size gradually, with the maximal response observed at 72 h following treatment (data not shown). Therefore, H9c2 cells were incubated with KBrO₃ at concentrations of 150 μ M for 72 h. However, different from high glucose, cellular osmolality was not influenced by KBrO₃ in the preliminary experiments. As demonstrated in Fig. 1A, KBrO₃ increased the H9c2 cell size in a dose-dependent manner as shown in Table I. Additionally, the mRNA expression levels of the biomarkers of cardiac hypertrophy, such as BNP and β -MHC were also increased by KBrO₃ in the same manner, as demonstrated in Table I. However, KBrO₃ did not modify the cell number of H9c2 cells even at the highest dose (control group: 8.2 \times 10⁴ cells/cm²; KBrO₃ 250 μ M group: 7.8 \times 10⁴ cells/cm²). Notably, apoptosis was not identified in H9c2 cells incubated with KBrO₃ at the effective concentrations as demonstrated in Fig. 1B and Table I as the levels of apoptosis-associated markers remain unchanged.

Oxidative stress and KBrO₃-induced cardiac hypertrophy in H9c2 cells. The changes in two oxidative biomarkers, ROS and superoxide, were investigated following KBrO₃-induced cardiac hypertrophy in H9c2 cells. As demonstrated in Fig. 2, KBrO₃ notably increased levels of both oxidative biomarkers, and these were reversed by tiron at effective concentrations.

Effect of antioxidant on KBrO₃-induced cardiac hypertrophy in H9c2 cells. H9c2 cells were treated with the antioxidant tiron to investigate the mediation of oxidative stress following the KBrO₃-induced cardiac hypertrophy. Following pretreatment with tiron for 30 min, cardiac hypertrophy induced by KBrO₃ was reduced in a dose dependent manner (Fig. 3A). Additionally, the expression levels of the two biomarkers BNP and β -MHC were also similarly reduced by tiron, as demonstrated in Table II.

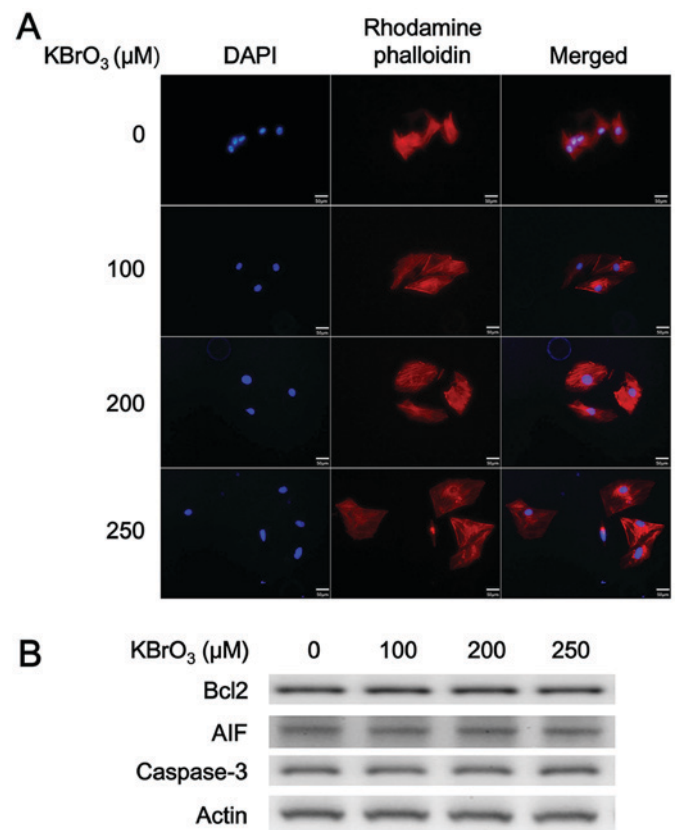


Figure 1. Effects of KBrO₃ on the cardiac cell line H9c2. Cells incubated with KBrO₃ at the indicated concentrations were used to assay (A) cell size and (B) changes in levels of various proteins associated with apoptosis. AIF, apoptosis-inducing factor; Bcl2, B-cell lymphoma; KBrO₃, potassium bromate.

Cellular signaling pathway and KBrO₃-induced cardiac hypertrophy in H9c2 cells. Cardiac hypertrophy is controlled simultaneously by stimulatory (prohypertrophic) and counter-regulatory (antihypertrophic) mechanisms via the calcineurin-NFAT signaling pathway (31). Therefore, the changes in the signaling pathway were investigated using western blotting. As demonstrated in Fig. 3, both calcineurin (Fig. 3B) and NFAT3 expression (Fig. 3C) levels were elevated by KBrO₃ at the concentration sufficient to induce cardiac hypertrophy in H9c2 cells. However, this action of KBrO₃ was

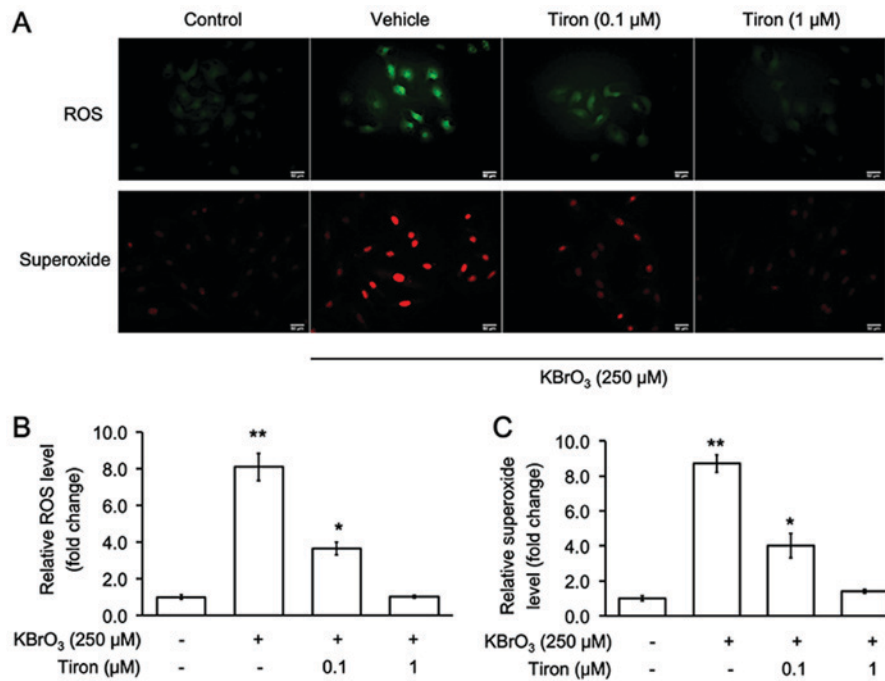


Figure 2. Free radicals induced by KBrO₃ in H9c2 cells. (A) Representative images demonstrating the formation of free radicals, such as ROS and superoxide, induced by KBrO₃ at the indicated concentration. The antioxidant tiron at the indicated concentrations reduced the effect of KBrO₃ in a dose dependent manner, compared with the vehicle-treated control (vehicle was the solvent used to dissolve tiron). (B) Quantification of ROS and (C) quantification of superoxide levels. Data are presented as the mean \pm standard error (n=6). *P<0.05 or **P<0.01 vs. the non-treated control. KBrO₃, potassium bromate; ROS, reactive oxygen species.

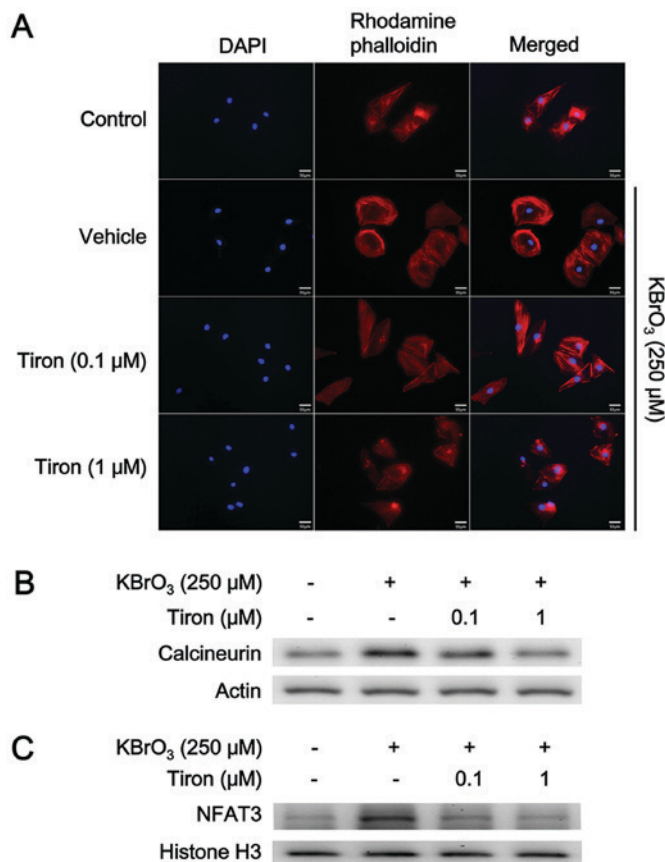


Figure 3. The antioxidant tiron attenuates KBrO₃-induced cardiac hypertrophy in H9c2 cells. Cells incubated with KBrO₃ at the indicated concentration were used to investigate (A) the cell size and the changes in hypertrophic signals, such as (B) calcineurin and (C) NFAT3. KBrO₃, potassium bromate; NFAT3, nuclear factor of activated T-cells.

reduced by tiron in a dose dependent manner (Fig. 3B and C; Table II). Moreover, the cellular calcium levels were measured and it was demonstrated that KBrO₃ increased cellular calcium levels which were then attenuated by tiron, as demonstrated in Table II.

Cyclosporine A inhibits KBrO₃-induced cardiac hypertrophy in H9c2 cells. Cyclosporine A (CsA) is a powerful immunosuppressant and has also been used to inhibit calcineurin (20). The cell size of H9c2 and mRNA expression levels of BNP and β -MHC were measured. As illustrated in Fig. 4, CsA inhibited KBrO₃-induced cardiac hypertrophy in H9c2 cells. Therefore, mediation of the calcineurin-NFAT signaling pathway in KBrO₃-induced cardiac hypertrophy was identified.

Characterization of KBrO₃-induced apoptosis and damage in H9c2 cells. In a previous study, apoptosis was induced by H₂O₂ at concentrations higher than that used to induce cardiac hypertrophy in cardiac cells (32). Therefore, the possible toxic effect of KBrO₃ on H9c2 cells was investigated. As demonstrated in Fig. 5A, KBrO₃ at high concentrations may damage H9c2 cells and cell death was observed in H9c2 cells treated with KBrO₃ >1 mM, in a dose-dependent manner. Epifluorescent staining of cells following treatment with KBrO₃ illustrated mostly apoptotic/necrotic cells with orange red fluorescent nuclei (Fig. 5B) while cells with green fluorescent nuclei were considered as an indicator of live cells, indicating that cells underwent apoptosis at high doses of KBrO₃. Additionally, Annexin V-FITC staining was used. As demonstrated in Fig. 5C and D, apoptosis induced by KBrO₃ at high doses was clearly identified using the flow cytometry.

Table II. Cardiac hypertrophy induced by KBrO₃ is reduced by the antioxidant Tiron.

Contents	Control	Vehicle + KBrO ₃ (250 μ M)	Tiron (0.1 μ M) + KBrO ₃ (250 μ M)	Tiron (1 μ M) + KBrO ₃ (250 μ M)
Relative area level (fold change)	1.00 \pm 0.08	2.56 \pm 0.12 ^b	1.62 \pm 0.10 ^a	1.02 \pm 0.09
Relative mRNA level of BNP/ β -Actin	1.00 \pm 0.00	1.96 \pm 0.04 ^b	1.48 \pm 0.03 ^a	1.22 \pm 0.05
Relative mRNA level of β -MHC/ β -Actin	1.00 \pm 0.00	1.91 \pm 0.06 ^b	1.49 \pm 0.05 ^a	1.20 \pm 0.04
[Ca ²⁺] _i nM	176.30 \pm 4.06	327.40 \pm 11.22 ^b	257.32 \pm 13.28 ^a	185.69 \pm 9.58
Ratio of calcineurin/ β -Actin protein	0.31 \pm 0.04	0.81 \pm 0.05 ^b	0.49 \pm 0.04 ^a	0.29 \pm 0.03
Ratio of NFAT3/Histone H3 protein	0.45 \pm 0.02	0.87 \pm 0.05 ^b	0.63 \pm 0.02 ^a	0.49 \pm 0.04

Data are presented as the mean \pm standard error (n=6) (Vehicle the solvent used to dissolve tiron). ^aP<0.05 or ^bP<0.01 vs. the non-treated control. β -MHC, β -Myosin Heavy Chain; BNP, brain/B-type natriuretic peptides; KBrO₃, potassium bromate.

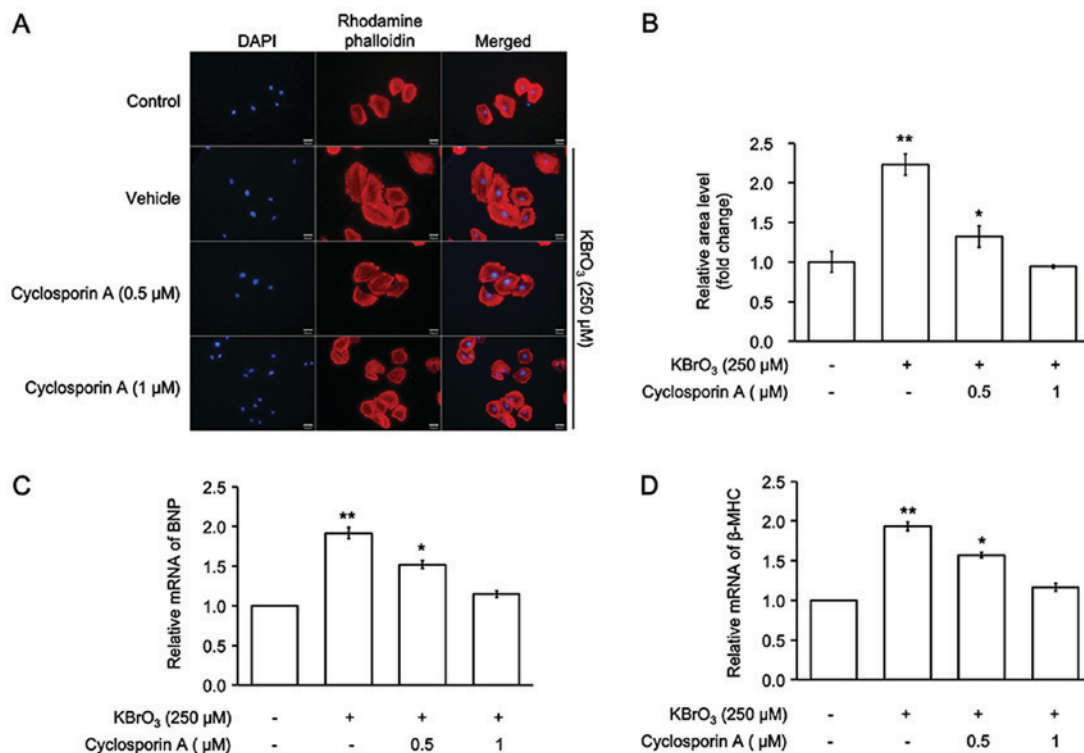


Figure 4. Cyclosporine A, a calcineurin inhibitor, attenuates KBrO₃-induced cardiac hypertrophy in H9c2 cells. Cells incubated with KBrO₃ were used to investigate cardiac hypertrophy, via (A and B) cell size changes and the mRNA expression levels of the hypertrophic indicators, such as (C) BNP and (D) β -MHC (Vehicle was the solvent used to dissolve cyclosporine A). Data are presented as the mean \pm standard error (n=6). *P<0.05 or **P<0.01 vs. the non-treated control. β -MHC, β -Myosin Heavy Chain; BNP, brain/B-type natriuretic peptides; KBrO₃, potassium bromate.

Discussion

In the present study, KBrO₃ at concentrations <250 μ M induced cardiac hypertrophy without apoptosis in a cultured cell line. Additionally, it was demonstrated that free radicals and/or oxidative stress were mediated by the effect of KBrO₃, which is fully consistent with previous reports that KBrO₃ induces damage via oxidative stress (10-12). Cardiac injury induced by KBrO₃ has also been observed in rats (15,16), and histological evidence demonstrates that KBrO₃ induces cardiac apoptosis and/or necrosis in rodents (33). However, cardiac hypertrophy induced by KBrO₃ has not been previously examined.

The direct effect of KBrO₃ on cardiac cells resulted in an increase in the size of H9c2 cells, which was demonstrated

using visual identification and a parallel increase in biomarkers of cardiac hypertrophy. Increased expression levels of ANP or BNP in plasma levels are widely used as reliable indicators of cardiac hypertrophy in the clinic (34). In the present study, the mRNA expression level of BNP in H9c2 cells was increased by KBrO₃ in a dose-dependent manner. This was in addition to another indicator β -MHC, which has been described previously (35). Similar to natriuretic peptides, β -MHC or α -skeletal actin has also been used as a biomarker of pathological hypertrophy (36). In the present study, KBrO₃, similar to its role in increasing the expression of BNP, also increased the expression of β -MHC. Furthermore, KBrO₃ increased the size of H9c2 cells (hypertrophy) but did not increase cell numbers (hyperplasia).

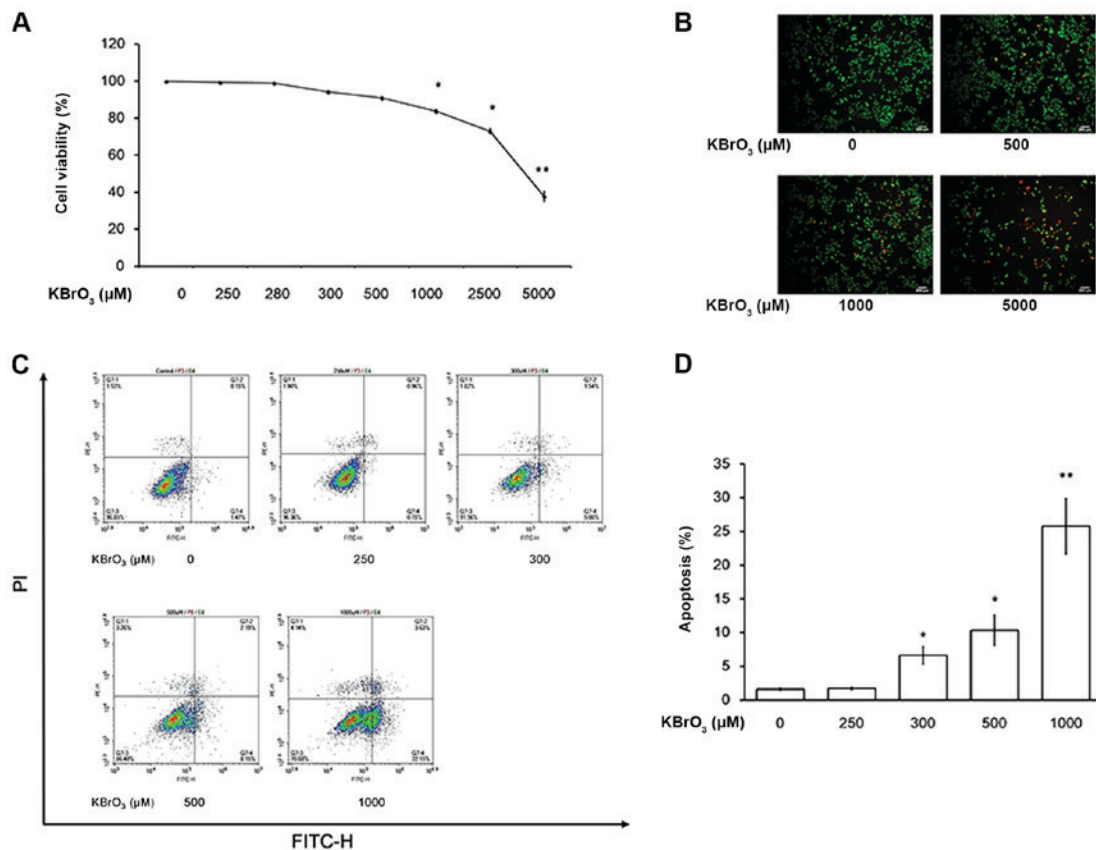


Figure 5. Cell death is induced by KBrO₃ at high concentrations in H9c2 cells. (A) KBrO₃ damaged cardiac cells at concentrations >1 mM. (B) Cell death measured using a LIVE/DEAD viability assay kit. (C) Apoptosis was measured by Annexin V-FITC analysis and (D) its quantification indicated that apoptosis occurred in H9c2 cells incubated with KBrO₃ at concentrations >300 μM. *P<0.05 or **P<0.01 vs. the non-treated control (at dose 0). KBrO₃, potassium bromate; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Therefore, KBrO₃ induced cardiac hypertrophy in H9c2 cells.

ROS produced by KBrO₃ are proposed as the primary mediators in tissue damage (11,16). Thus, the changes in ROS and superoxide levels were investigated. Similar to hyperglycemia-induced cardiac damage (24), KBrO₃ increased ROS and superoxide levels in H9c2 cells. Additionally, the effect of tiron, which is a water-soluble and cell permeable antioxidant that scavenges superoxide (22,23), reduced the effect of KBrO₃ in a dose-dependent manner. Tiron alleviates cardiac damage through a marked reduction in oxidative stress (37,38). In the present study, tiron inhibited the effects of KBrO₃ including the induction of cardiac hypertrophy and formation of ROS in H9c2 cells.

H₂O₂ induces cardiac hypertrophy only at concentrations <50 μM in cardiomyocytes isolated from rats (32). Furthermore, KBrO₃ induced disorder at concentrations <250 μM in the cultured H9c2 cell line. Similar to the effects of H₂O₂, KBrO₃ induced apoptosis and/or necrosis in H9c2 cells at high concentrations (>300 μM). Therefore, the effective concentration of KBrO₃ is limited within this range for the induction of cardiac hypertrophy in H9c2 cells.

The role of the calcineurin signaling pathway was also investigated in KBrO₃-induced cardiac hypertrophy. Calcineurin may dephosphorylate NFAT3, leading to nuclear translocation (31). Subsequently, the nuclear NFAT3 participates in the promotion of hypertrophic gene expression including that of BNP and β-MHC to induce cardiac

hypertrophy (6,7). Additionally, ROS increases cellular calcium (39). In the present study, it was demonstrated that KBrO₃ significantly increased the calcium level, which may promote the calcineurin and NFAT3 signaling pathway in H9c2 cells. This conclusion is also supported by the evidence from cyclosporine A, which at a concentration effective to inhibit calcineurin, attenuated the KBrO₃-induced cardiac hypertrophy. Notably, these effects of KBrO₃ were also reduced by tiron in a dose-dependent manner. Therefore, KBrO₃ likely increases hypertrophic signaling via elevation of ROS in H9c2 cells, but this needs to investigate in the future.

The mechanism(s) for oxidative stress-induced cardiac hypertrophy remains unclear. Many parameters have been examined (40), including osteopontin (41). However, most of these reports have investigated hyperglycemia-induced cardiac damage, particularly apoptosis (40). Hyperglycemia induces cardiac injury primarily through ROS generation (38). However, the inflammatory parameters are also involved in diabetic cardiomyopathy, particularly in the *in vivo* models (42). Therefore, the difference in cardiac hypertrophy between hyperglycemia and KBrO₃ should be clarified in the future.

In cardiac research, oxidative stress or ROS-induced cardiac hypertrophy is widely induced by H₂O₂ in H9c2 cells (43). Additionally, 4-hydroxy-2-nonenal (44) or lactosylceramide (45) has also been applied to induce cardiac hypertrophy via ROS in a way similar to that of KBrO₃.

Therefore, KBrO₃ could be used as an inducer of cardiac hypertrophy in cells. In rats, KBrO₃ at a dose of 1.2 g/kg for 4 weeks produces damages without mortality (46), and in Sprague-Dawley rats, KBrO₃ at a dose of 20 mg/kg for 4 weeks induces cardiac injury (47). Generally, KBrO₃ at a dose of 20-30 mg/kg damages cardiac tissues in rats (16). The histological evidence also demonstrates that KBrO₃ at 20 mg/kg twice weekly for 4 weeks induces cardiac fibrosis in rats (33). In a clinic, nine cases of accidental KBrO₃ poisoning in a bakery demonstrated hematemesis and renal failure as the possible adverse outcomes (48). Although the cardiac injury seems not so critical in the human response to KBrO₃, further investigations on human cardiac cells are required. Moreover, characterization of the dose of KBrO₃ effective to induce cardiac hypertrophy in animals is also essential in the future. The role of signaling pathways need to be investigated using small interfering or short hairpin RNA techniques, in order to understand the damage resulting from KBrO₃ in cardiomyocytes. Additionally, the effects of potassium or bromate on cardiac hypertrophy remain unclear. Further investigations are required to evaluate the pharmacokinetics and/or pharmacodynamics of KBrO₃.

In conclusion, in the present study, KBrO₃ at concentrations <250 μ M induced cardiac hypertrophy in H9c2 cells without apoptosis mainly through free radicals to increase cellular calcium levels and so activate the calcineurin and NFAT3 signaling pathway. Therefore, KBrO₃ may be applied to develop a novel cell model of cardiac hypertrophy for future research.

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Availability of data and materials

The datasets used and analyzed during the present study are available from corresponding author on reasonable request.

Author's contributions

SK, KC and JC designed experiments. YL, YC and WL performed the experiments and analyzed data. SK, YL and KC drafted the manuscript. All authors discussed, revised, and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they do not have any competing interests.

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