Proteomic analysis reveals KRIT1 as a modulator for the antioxidant effects of valproic acid in human bone-marrow mesenchymal stromal cells

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Abstract
Valproic acid (VPA) protects human bone marrow-mesenchymal stromal cells (hBM-MSCs) against oxidative stress and improves their migratory ability through increasing the secretion of trophic factors. This suggests that VPA may be an excellent candidate for improving stem cell function. However, the molecular mechanisms of VPA in BM-MSCs are not known. In this study, we used a proteomic approach to investigate VPA-associated targets under oxidative stress conditions. Krev/Rap1 interaction Trapped-1 (KRIT1), a modulator for the homeostasis of intracellular reactive oxygen species (ROS), was identified as a target protein by two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) analyses. The up-regulation of KRIT1 and its target proteins (SOD2 and FoxO1) with VPA treatment of hBM-MSCs was revealed by qPCR and immunoblot analysis. Damage from oxidative stress was reduced in VPA-pretreated BM-MSCs, which was also confirmed by qPCR and immunoblot analysis. In addition, increased in intracellular ROS by H2O2 were also reduced by VPA pretreatment in BM-MSCs. This suggests that VPA reduces intracellular ROS level by the modulation of KRIT1 and its correlated proteins, FoxO1, SOD2, and cyclin D1. Thus, this study is the first to provide evidence that VPA modulates KRIT1 and intracellular ROS in BM-MSCs.

Keywords
Antioxidant effect, bone marrow-mesenchymal stromal cells (BM-MSCs), KRIT1, mesenchymal stromal cells (MSCs), proteomic analysis, valproic acid (VPA)

Introduction
Bone marrow-mesenchymal stromal cells (BM-MSCs) possess the capacity to differentiate into several cell types (Caplan, 2007; Pittenger et al., 1999) including neuron-like cells (Woodbury et al., 2000) and also secret diverse trophic factors (Caplan, 2006; Cho et al., 2010b; Nagai et al., 2007). In stem cell therapy, these capacities enable the engrafted stem cells to recover and protect the damaged tissues from apoptosis and inflammation (Chen et al., 2003; Cho et al., 2010a). These effects promise an increase in the beneficial efficacy of stem cell therapies (Cho et al., 2010a). However, most engrafted stem cells die within a week after transplantation, which severely limits therapeutic efficacy (Fan et al., 2012). This suggests that combined treatment with a cell protective reagent might provide better efficacy in stem cell therapy.

The KRIT1 gene is associated with cerebral cavernous malformations (CCMs) (Riant et al., 2010) (vascular lesions of the central nervous system), leading to seizures, focal neurological deficits and fatal intracerebral hemorrhage (Del Curling et al., 1991; Otten et al., 1989). Three CCM genes - CCM1/KRIT1, CCM2/MGC4607, and CCM3/PDCD10, have been identified so far (Cavalcanti et al., 2012). More than 40% of familial CCM patients are affected with mutations in KRIT1, most mutations causing the truncation of the KRIT1 protein (Revencu & Vikkula, 2006).

KRIT1 plays a crucial role in molecular mechanisms involved in the maintenance of intracellular reactive oxygen species (ROS) homeostasis to prevent oxidative cellular damage. Cells deficient in KRIT1 have a significant increase in intracellular ROS levels (Goitre et al., 2010). These cells also exhibit changes in the expression of the antioxidant protein SOD2 and the transcriptional factor FoxO1 (Goitre et al., 2010). Recent yeast two-hybrid screening led to the discovery of the Kelch family protein Nd1-L as a novel protein that interacts with KRIT1 (Guazzi et al., 2012).

Valproic acid (VPA), a known histone deacetylase (HDAC) inhibitor (Miller et al., 2003), has been clinically...
used as an anticonvulsant for the treatment of epilepsy (Grosshans et al., 1980; Rogawski & Loscher, 2004), bipolar disorder (Drevets, 2000; Rajkowska, 2000), and migraine headaches (Shahien et al., 2011) over the past four decades. Our previous study showed that an optimal dose and time of VPA treatment prevented the cell death induced by oxidative stress and improved the migratory ability of human BM-MSCs (Cho et al., 2012). Thus VPA-pretreated MSCs may have a better chance to survive engraftment, improving the efficacy of stem cell therapy. However, the molecular mechanisms of VPA on BM-MSCs are still unknown.

In the present study, we used proteomic techniques to investigate the differentially expressed proteins in VPA-pretreated hBM-MSCs under H2O2-induced oxidative stress conditions. Here, we report that VPA modulates intracellular ROS levels through the modulation of KRIT1 in hBM-MSCs.

Materials and methods

Cell culture

hBM-MSCs were purchased from Lonza (Walkersville, MD) and cultured in T75 flasks (Nunc, Penfield, NY) according to the supplier’s recommendations. Cells were cultivated in DMEM low glucose medium (GIBCO, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and penicillin (100 U/ml)/streptomycin (100 µg/ml). Cells were maintained in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO2. Sixth-passage MSCs were used for experiments.

Protein sample preparation and two-dimensional gel electrophoresis analysis

hBM-MSCs were incubated with/without 200 µg/ml VPA for 12 hours (ctrl- and VPA-MSCs) and then treated with 1.5 mM H2O2 for 30 min (H2O2- and VPA-H2O2-MSCs). The MSCs were washed with PBS and collected by scraping. Collected cells were harvested by centrifugation (400 × g, 5 min, 4°C) and resuspended in 1 ml ice-cold TE buffer (50 mM Tris base, 5 mM EDTA-Na, pH 8.5). Cells were re-harvested, the supernatant discarded, and the cell pellets stored in a −70°C deep-freezer until their use.

The cells were extracted with a lysis solution composed of 7 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1 mM benzamidine by agitation for an hour at room temperature. After centrifugation at 15 000 × g for an hour at 15°C, soluble fractions were collected and measured by the Bradford method (Bradford, 1976).

The total proteins from soluble fractions were subject to two-dimensional gel electrophoresis (2DE) according to procedures previously described by Jung (Jung et al., 2008). IPG dry strips (4-10 NL IPG, 24 cm, Genomine, Korea) were equilibrated for 12–16 hours with the lysis solution (7 M urea, 2 M thiourea, 2% CHAP, 1% DTT, 2% pharmalyte, 1 mM benzamidine) and then 500 µg of the proteins were loaded. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EGS 3500 XL power supply (Amersham Biosciences) following the manufacturer’s instruction. After IEF, gel electrophoresis was carried out in SDS–PAGE gels (20 x 24 cm, 10–16%) and stained with Coomassie blue.

Quantitative analysis of the digitized images was carried out using the PDQuest (version 7.0, BioRad, Hercules, CA) software according to the protocols provided by the manufacturer. The quantitative intensity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression changes (over two-fold) compared with ctrl- and VPA-treated MSCs.

In-gel digestion, MALDI-TOF, and database searches

Protein spots were enzymatically digested in-gel, similar to a method previously described by Shevchenko (Shevchenko et al., 1996), using a modified porcine trypsin (Promega, Madison, WI). The peptide analysis was performed using an Ultraflex matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). In brief, the peptides were evaporated with an N2 laser at 337 nm using a delayed extraction approach and accelerated with a 20 kV injection pulse for the TOF analysis. Each spectrum represents the cumulative average of 300 laser shots. The ProFound search program, developed by Rockefeller University (http://prowl.rocketefeller.edu/prowl-cgi/profound.exe), was used to identify proteins by peptide mass fingerprinting. The spectra were calibrated using the trypsin autodigestion ion peak signals (m/z 842.510 and 2211.1046) as internal standards.

Quantitative PCR (Real-time PCR)

Ctrl- and VPA-treated MSCs were harvested and the total RNA extracted using Trizol reagent according to the manufacturer’s instructions (Invitrogen). PrimeScript® Reverse Transcriptase (Takara, Japan) was used to reverse-transcribe 3–5 µg of total RNA with 0.25 µM oligo dT primer, 1 mM dNTPs, and the supplied buffer. First strand cDNAs were amplified using SYBR Premix Ex Taq™ II (Takara, Japan) with primers for human cyclin D1 (forward 5’-CATCTTACAC CGAAACCTCCC-3’; reverse 5’-TCTGGCATTGGAG GAGGAAACAG-3’), FoxO1 (forward 5’-CAGGATCCCTC CATCAACAC-3’; reverse 5’-TAAGTGAACCTGTCACTAA CCC-3’), SOD2 (forward 5’-GGACACTTACAGATTGCTGC TTGT-3’; reverse 5’-AGTAAAGCTGCTCCACACACAT-3’), KRIT1 (forward 5’-GAAAGAGGAAGGAGGAGGCAACGCA-3’; reverse 5’-ATGGGTGGGACAGTCACCG-3’), and ß-actin (forward 5’-TGAGAGGAAATCTCTGCAACACAC-3’; reverse 5’-GATGCGCAACTGTGGTGACC3-3’). Quantitative PCR cycling parameters were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C with an ABI 7500 real-time PCR system (Applied Biosystems, Inc., Foster City, CA). Primers were synthesized by GenoTech (Daejeon, Korea). All data are presented as the means ± standard deviation (SD) from three independent experiments. Statistical comparisons between groups were conducted with LSD post hoc test after one-way ANOVA.

Immunoblot analysis

Cells were extracted with 400 µl of cell lysis buffer containing protease and phosphatase inhibitors (iNtRON, Korea) for...
30 min at $-20^\circ$C, centrifuged at 12,000 $\times$ g for 15 min, and then subjected to immunoblotting with antibodies against KRIT1 (1:1000 SC-23997, Santa Cruz Biotechnology, Santa Cruz, CA), FoxO1 (1:1000, ab36970, Abcam, Cambridge, MA), SOD2 (1:1000, ab13533, Abcam), and $\beta$-actin (1:5000, A-5316, Sigma, St. Louis, MO) followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA). Immunoblots were quantified with ImageJ software.

**Fluorescence staining and microscopy**

1 $\times$ 10$^4$bBM-MSCs were seeded onto pre-coated coverslips in four-well plates and further incubated with 10% FBS containing growth media for a day. The cells were then treated with 200 $\mu$g/ml VPA for 12 hours. After washes with PBS, 0–1.5 mM H$_2$O$_2$ containing serum-free media were replaced with growth media and incubated for 30 min to induce oxidative injury. Cells were then washed with PBS and the intracellular ROS stained with 10 $\mu$M 2,$'$7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) following the incubation for 30 min. After washes with PBS, the cells were fixed with 4% paraformaldehyde for 10 min and mounted with 40,6-diamidino-2-phenylindole (DAPI) containing mounting solutions (Vectashield, Vector Laboratories, Burlingame, CA). Cells were visualized using a fluorescence microscope (Nikon, Japan).

**Gene silencing by KRIT1 siRNA**

MSCs were transfected with synthetic siRNAs in Lipofectamine RNAi max containing Opti-MEM I at a final concentration of 10 nM. At 6 hours post-transfection, the cell culture medium was replaced with growth medium. Next day, cells were transfection again with the siRNAs mixture for 6 hours and washed with growth medium. The cells were then incubated for additional 12 hours prior to use in experiments. Specific gene silencing was verified by RT-PCR analysis. The KRIT1 siRNAs used in this study were purchased from Santa Cruz (sc-43884, Santa Cruz Biotechnology).

**Cell viability assay**

To evaluate the protective effects of VPA-treated MSCs against H$_2$O$_2$-induced oxidative stress, the MTT assay (Sigma) was used according to the manufacturer’s instructions. Briefly, 1 $\times$ 10$^4$hBM-MSCs were seeded onto 96-well plates. The next day, the cells were incubated with/without 200 $\mu$g/ml of VPA for 12 hours. Cells were then treated with 0–1.5 mM H$_2$O$_2$ for 1 hour and subjected to the MTT assay. All data are presented as the mean standard deviation (SD) of the means from four independent experiments. Statistical comparisons between groups were made using an independent $t$-test.

**Results**

**Profiling of protein expression in VPA-treated hBM-MSCs under H$_2$O$_2$-induced oxidative stress conditions**

hBM-MSCs (ctrl-MSCs) were incubated with 200 $\mu$g/ml of VPA for 12 hours (VPA-MSCs) (Cho et al., 2012), and then treated with/without 1.5 mM H$_2$O$_2$ for 30 min (H$_2$O$_2$-MSCs, VPA-H$_2$O$_2$-MSCs). To determine the differentially expressed proteins between the designed groups, we performed two-dimensional gel electrophoresis (2DE) analysis. Among the hundreds of spots in the 2DE gels (Figure 1A, left panel and Supplementary Figure 1), several spots showed differential intensities (Figure 1A, right panel). The proteins in the spots were identified using MALDI-TOF MS. Four proteins - EBP1, KRIT1, annexin1, and synphilin-1, were identified as differentially expressed proteins and are summarized (Figure 1B).

**Up-regulation of KRIT1 in VPA-treated BM-MSCs**

A previous study indicated that the up-regulation of KRIT1 limits the accumulation of intracellular reactive oxygen species (ROS) (Goitre et al., 2010). In this study, we focused on KRIT1 and ROS homeostasis. To confirm the differential expression of KRIT1, ctrl-MSCs and VPA-MSCs were incubated with H$_2$O$_2$. Total proteins from each group were analyzed by immunoblot analysis with anti-KRIT1 and anti-$\beta$-actin antibodies. KRIT1 was decreased in H$_2$O$_2$-MSCs compared to ctrl-MSCs (Figure 2A). H$_2$O$_2$-treated VPA-MSCs (VPA-H$_2$O$_2$-MSCs) and VPA-MSCs had a recovery in their expression compared with H$_2$O$_2$-treated MSCs (H$_2$O$_2$-MSCs; Figure 2A). KRIT1-specific bands were quantified using ImageJ software and normalized with $\beta$-actin-specific bands. Values represent as the mean ± standard deviation (SD) of the means from four independent experiments (Figure 2B). Consistent results were obtained from qPCR with KRIT1 specific primers (Figure 2C).

**VPA-induced KRIT1 modulates the expression of FoxO1, SOD2 and cyclin D1**

The modulation of KRIT1 is associated with the expression of FoxO1, SOD2, and cyclin D1 (Goitre et al., 2010). To further evaluate the effects of KRIT1 in VPA-MSCs, KRIT1-related targets, FoxO1, SOD2, and cyclin D1, were measured by qPCR. FoxO1 and SOD2 were significantly increased at the transcriptional level in VPA-MSCs, while cyclin D1 levels were significantly decreased compared with ctrl-MSCs (Figure 3A; one-way ANOVA). The decreases of FoxO1 and SOD2 transcripts in H$_2$O$_2$-MSCs were reversibly increased by VPA-H$_2$O$_2$-MSCs (Figure 3A). Consistent results were obtained from immunoblot analysis using specific antibodies (Figure 3B).

**VPA treatment in BM-MSCs decreases intracellular ROS**

Since VPA-treated MSCs increased the expression of KRIT1, Foxo1, and SOD2, while decreasing cyclin D1 expression, we estimated ROS levels in VPA- and H$_2$O$_2$-treated MSCs. Ctrl- and VPA-MSCs were exposed to different concentrations of H$_2$O$_2$ (0, 0.2, 0.75, and 1.5 mM) for 30 min. The ROS levels were visualized by DCFH-DA staining. The intracellular ROS increased in an H$_2$O$_2$-dose-dependent manner in ctrl-MSCs but were attenuated in VPA-MSCs (Figure 4A). ROS-positive cells from each group were counted. Values represent the means from seven independent experiments (Figure 4B).
To further examine the effects of VPA, we applied siRNA mediated knock-down of KRIT1 expression in BM-MSCs (Figure 4C). The intracellular ROS were increased even in KRIT1 knock-down VPA-MSCs (siKRIT1 VPA-MSCs; Figure 4D), while decreased in non-specific siRNA transfected VPA-MSCs (siGFP VPA-MSCs; Supplementary Figure S2). It indicates that the increases of intracellular ROS by H2O2-induced oxidative stress were significantly reduced by VPA.

Figure 1. Profiling of protein expression in VPA-treated hBM-MSCs using proteomic techniques. VPA-treated MSCs (VPA-MSCs) and non-treated MSCs (ctrl-MSCs) were incubated with H2O2 (VPA-H2O2-MSCs, H2O2-MSCs). (A) Total proteins were displayed on a two-dimensional electrophoresis gel (2D-gel) stained with Coomassie blue (left panel). Differentially expressed spots from each group are presented (right panel). (B) The spots presented in the right panel were analyzed using MALDI-TOF MS. Four differentially expressed proteins were identified and are summarized in this figure.

Figure 2. Up-regulation of KRIT1 in VPA-treated BM-MSCs. (A) Differential expression of KRIT1 in VPA-MSCs under oxidative stress was confirmed by immunoblot analysis with a KRIT1-specific antibody. (B) KRIT1-specific bands were quantified and normalized with β-actin using ImageJ software. KRIT1 was significantly decreased in H2O2-MSCs compared to ctrl-MSCs, while VPA-H2O2-MSCs and VPA-MSCs had a recovery in their expression compared with H2O2-MSCs (one-way ANOVA, *p < 0.05, mean ± SD, n = 4). (C) The expression of KRIT1 was measured by qPCR (one-way ANOVA, *p < 0.05, mean ± SEM, n = 4).
pre-treatment in BM-MSCs and the VPA effects were abolished by KRIT1 gene knock-down.

Since VPA-treated hBM-MSCs suppress the intracellular ROS levels, we examined the viability assay. VPA-MSCs were incubated with 0, 0.2, 0.75, or 1.5 mM H2O2 for 30 min and evaluated by the MTT assay. Cell viabilities were significantly increased in VPA-treated MSCs compared with non-treated MSCs (Figure 4E; t-test, *p < 0.05, mean ± SD, n = 4).

**Discussion**

Valproic acid (VPA) protects cells against oxidative stress and improves their migratory ability by increasing the secretion of trophic factors in hBM-MSCs (Cho et al., 2012). In this study, we used proteomic techniques to investigate the molecular mechanism of VPA in hBM-MSCs. We identified the differentially expressed proteins under VPA and/or H2O2 treated conditions and identified several spots from 2D gel electrophoresis analysis (Figure 1A). Using MALDI-TOF MS analysis with four spots identified their proteins - EBP1, KRIT1, annexin1 and synphilin-1, (Figure 1B). KRIT1, which is involved in the homeostasis of intracellular ROS (Goitre et al., 2010), was the focus for further studies on the relationship between cell protection and ROS homeostasis.

The KRIT1 gene is responsible for cerebral cavernous malformations (CCM), a major cerebrovascular disease (Riant et al., 2010). Loss-of-function mutations in the KRIT1 gene are associated with increases of intracellular ROS through the down-regulation of SOD2 and FoxO1 expression (Goitre et al., 2010). Here, we show that VPA treatment of hBM-MSCs modulates KRIT1 (Figure 2), SOD2, FoxO1, and cyclin D1 (Figure 3). The finding that KRIT1 levels in hBM-MSCs are down-regulated under H2O2-induced oxidative stress conditions is consistent with a recent report based on a distinct cellular model (Goitre et al., 2014), suggesting that it may represent a general phenomenon.

In a previous study, VPA treatment affected stem cell growth and gene expression, improving ischemic myocardium protection in a mouse model of myocardial infarction (Burba et al., 2011). VPA also delayed spontaneous cell death in purified retinal ganglion cells (Biermann et al., 2011). The neuroprotective effects of VPA occurred via the inhibition of HDACs and the activation of gene expression, inducing the neuroprotective proteins ANG, BDNF, EGF, and GDNF (Chen et al., 2006; Cho et al., 2012; Yasuda et al., 2009). In the present study, we show that VPA modulates KRIT1, a modulator of ROS homeostasis. To further examine the relationship between these protective effects and ROS homeostasis, VPA-pretreated MSCs were exposed to H2O2 and the intracellular ROS level determined. The increased ROS levels induced by oxidative stress were reduced by VPA pretreatment in BM-MSCs compared with non-treated BM-MSCs (ctrl-MSC; Figure 4). These antioxidant effects were modulated through KRIT1 and subsequently through its target proteins, SOD2, FoxO1, and cyclin D1, suggesting that H2O2-induced oxidative stress produces intracellular ROS recovered through VPA-induced KRIT1 in hBM-MSCs.

The beneficial efficacy of stem cell therapy is severely limited due to death of engrafted stem cells, due to cell damage conditions such as oxidative stress and inflammation (Fan et al., 2012). We found that VPA-induced KRIT1 controls the steady-state levels of intracellular ROS,
preventing cellular oxidative damage. This suggests that VPA may be an excellent candidate for improving stem cell function and efficacy in stem cell therapy.

Previous studies suggested that loss-of-function mutations or down-regulation of KRIT1 may cause diseases such as CCM by impairing oxidative stress defences (Goitre et al., 2010, 2014). VPA could thus serve as a potential therapeutic drug for vascular malformation diseases. Here, we describe KRIT1 as a novel target of VPA. Based on the functional mechanism of VPA as a histone deacetylase inhibitor, VPA induces an increase in acetylated histones on chromatin, activating expression at several gene loci. Our proteomic analysis identified several differentially expressed proteins (data not shown), which may provide further clues for understanding the overall mechanisms of VPA and can be tested in future studies.

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Declaration of interest

The authors declare that they have no conflict of interest.

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Supplementary material available online

Supplementary Figures S1 and S2