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Signals regulating necrosis of cardiomyoblast by BTG2$^{/TIS21/PC3}$ via activation of GSK3β and opening of mitochondrial permeability transition pore in response to H_2O_2

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ABSTRACT

To investigate signal transduction pathway of cell death regulated by a tumor suppressor after oxidative stress, cardiomyoblasts were virally transfected with BTG2$^{/TIS21/PC3}$ (BTG2) and subsequently treated with H_2O_2. Heart muscle rarely expresses BTG2 unless oxidative stress occurs, however, ischemia induced BTG2 expression and necrosis, not apoptosis, of cardiomyoblasts. BTG2-expressioning cardiomyblasts showed impaired recoveries of survival kinases, Akt and Erk, thus sustaining GSK-3β activity in 30 min of H_2O_2 exposure, in contrast to their rapid recoveries in LacZ control. The phenomenon was accompanied by the failure of ATP regeneration and the sustained activation of AMPK in the BTG2 expresser. Furthermore, H_2O_2 treatment markedly induced BTG2 translocation from nuclei to mitochondria along with cell death by cyclophilin D activation and mPTP opening. Exogenous and endogenous effect of BTG2 was confirmed by chemical inhibitors and BTG2-KO-MEF, respectively. Here, we suggest tumor suppressor, BTG2, as one of the regulators of necrosis in myocardium *via* inhibiting Akt/Erk, but activating GSK3β and cyclophilin D, which resulted in mPTP opening in response to H_2O_2.

1. Introduction

Oxidative stress and cell death are the main cause of heart failure due to myocardial injury and hypertensive cardiac hypertrophy. Various endeavors have been undertaken to elucidate the pro- and anti-survival signals regulated by oxidative stress in cardiac pathology. Consequently, proto-oncogenes, Akt and Erk, have been identified as the pro-survival mediator, making cardiac cells resistant to ischemic cell death [1,2], and c-myc, c-fos and H-Ras render cardiac cells resistant to the pressure overload by inducing cardiac hypertrophy [3]. Whereas tumor suppressor RASFF1, as an anti-Ras/MAPK signal mediator, protects cardiomyocytes from pathologic hypertrophy induced by Ras/MAPK signal pathway [4]. On the other hand, inactivation of tumor suppressors such as PTEN and p53 enhance cell survival against ischemic reperfusion (I/R) injury [5] and doxorubicin-induced cardiac toxicity [6], suggesting them as an anti-survivor mediator. In addition to the oncogenes and tumor suppressor genes, oncogenic microRNA, miR21, also exhibits a cardioprotective effect against I/R injury by inhibiting PDCD4 [7]. The above findings suggest significant roles of oncogenes and tumor suppressors in not only cancer biology but also in cardiac pathophysiology.

BTG2$^{/TIS21/PC3}$ (BTG2), initially reported as a primary response gene [8], antiproliferative gene [9] and tumorsuppressor in several organs [10,11], exhibits diverse activities as a linker between cancer and aging phenotypes [12]. Recently, we found BTG2 as a binding partner of Ant2 protein in mitochondria [13], however, the mechanism and signal pathway in mitochondria are still largely unknown. Heart muscle contains the largest number of mitochondria per single cell in our body, therefore, we were strongly tempted to examine whether BTG2 regulates cardiac cell survival in response to oxidative stress. Since the activity of oncogenic Ras can be downregulated by BTG2 directly or indirectly [14,15], and the downstream genes of H-Ras, Akt and Erk protect heart muscle from oxidative stress-induced cell death and induce pathologic cardiac hypertrophy, we assumed that BTG2 might have an important role in heart muscle. Moreover, BTG2 is also a target of miR21 in addition to PTEN and PDCD4, and it protects neuronal cell death as opposed to induction of cell death by p53 [16]. Therefore, BTG2 may reveal an opposite activity against miR21 not only in cancer but also in heart [17,18].

To our best knowledge, there is no report on the role of BTG2 in heart after oxidative stress. Therefore, we employed adenoviral transduction of BTG2 gene and evaluated its effect on H9c2 cardiomyoblasts after oxidative stress along with signal

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transduction pathways. The results led us to suggest BTG2 as an inducer of cardiomyoblasts necrosis by activating cyclophilin D and mPTP opening through GSK3βactivation and failure of ATP regeneration.

2. Materials and methods

2.1. Cell culture and in vivo ischemic injury analyses

H9c2 cells were maintained in DMEM (Gibco BRL) with 10% fetal bovine serum and antibiotics in 5% CO_2 incubator at 37 °C. Cells with 80% confluence were used for experiments. Wild type and BTG2-knockout mouse embryo fibroblasts (wt-MEF, BTG2-KO-MEF) were prepared in our laboratory [19] and cultured in DMEM with 10% fetal bovine serum, and used for study under the passage No 5. *In vivo* expression of BTG2 was measured in organs obtained from 7 week-old Sprague–Dawley male rats after cervical dislocation. For ischemic injury, the anterior descending branch of left coronary artery was tied for 12 h, and the infarct and surrounding tissues were dissected.

2.2. Preparation and transduction of adenoviruses

Adenoviruses expressing BTG2 gene (Ad-BTG2-HA) and bacterial β-galactosidase (Ad-LacZ) were prepared in our laboratory [19], and 50 moi of Ad-BTG2 or Ad-LacZ were transduced to H9c2 cells for 48 h. LacZ and BTG2 expressers were treated with 800 μM H_2O_2 before subjected to various experiments. Maximum concentration of H_2O_2 and its treatment time were determined based on MTT assay.

2.3. Differential evaluation of apoptosis and necrosis

Cell viability was assessed by MTT test according to the published method [20]. To confirm apoptosis, nuclear condensation and fragmentation after Hoechst 33342 (Calbiochem) stain and caspase 3 cleavage were examined after etoposide (25 μM) or H_2O_2 treatment. To confirm necrosis, propidium (PI) staining and lactate dehydrogenase (LDH) release into culture media were measured by a kit (Takara). To confirm the effect of BTG2 expression on the regulation of necrosis, short interfering RNAs to $BTG2^{/Pc3}$ (siBTG2$^{/siPc3}$) were synthesized (Genolution Pharmaceuticals). H9c2 cells were transfected with 25–50 nM siRNA oligonucleotides for 4 h using Lipofectamine 2000 as described by the protocol (Invitrogen). Control siRNA (si-Ctrl) recognizes GFP mRNA, and siBTG2 detects four different sites of BTG2 mRNA. Sequences of the siRNAs directed against siCtrl and siBTG2 are as follows: siCtrl (5′-GUUCAGCGUGUCCGGCGAGTT-3′). siBTG2 #1 (5′-GCAUCAACCACAAGAUGGAUU-3′), siBTG2 #2 (5′-AGAACUACGUGAUGACUGUUU-3′), siBTG2 #3 (5′-GUAGAUGUGUGCAAUAUUUUU-3′), siBTG2 #4 (5′-GGCUAUAAGGCAGAUAUAAUU-3′).

2.4. Determination of ATP level and mPTP opening

ATP level was measured by determination kit (Perkin Elmer, Shelton, CT) according to the manufacturer's instruction after H_2O_2 treatment. mPTP opening was assessed using the calcein/cobalt method with minor modifications [21]; Calcein AM (1 μM) and $CoCl_2$ (1 mM) from Invitrogen were added to the LacZ and the BTG2 expressers, and then incubated at 37 °C for 30 min. The cells were washed with fresh medium and maintained with serum-free DMEM before exposure to H_2O_2. Degree of mPTP opening was measured by counting calcein AM fluorescence left in mitochondria using FACScan (BD Biosciences). To investigate the effect of cyclophilin D on the mPTP opening, the BTG2 or LacZ expressers were pretreated with cyclosporine A (CsA, Sigma–Aldrich) for 30 min before H_2O_2 treatment for 4 h. To evaluate the effect of GSK-3β on cyclophilin D activity, SB415286 (Sigma–Aldrich, 50 μM), inhibitor of GSK-3β, was applied.

2.5. Immunocytochemistry

BTG2 expressers were cultured on cover glass for 48 h with H_2O_2 and MitoTracker Red (100 nM, Invitrogen) treatment for 30 min, and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C for 15 min, followed by a standard method. BTG2 translocation to mitochondria after H_2O_2 treatment was evaluated in 500 cells from 3 independent experiments under a fluorescence microscope (Carl Zeiss, Axio Imager M1, Germany).

2.6. Reverse transcription-polymerase chain reaction

Total cellular RNAs (1.0 μg) were reverse-transcribed with oligo(dT) using Superscript II reverse transcriptase kit (Invitrogen). The RT product (1/50 v) was amplified by PCR kit (Takara); denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 40 s for GAPDH in 28 cycles and for BTG2 in 30 cycles by using the following primers: sense 5-ATGAGCCACGGGAAGAGAACCGAC-3 and anti-sense 5′-CTAGCTGGAGACAGTCATCACGTA-3′ for BTG2, and sense 5-CCATGGAGAAGGCTGGGG-3 and anti-sense 5′-CAAAGTTGTCATGGATGACC-3′ for GAPDH. Band intensities were determined by GEL-PRO® ANALYZER (Media Cybernetics, Inc., Bethesda, MD) based on the intensity of marker proteins.

2.7. Immunoblot and immunoprecipitation analyses

Cell lysates (40 μg) prepared in RIPA buffer with phosphatase inhibitors were resolved on SDS–PAGE before transfer to PVDF membrane (Millipore Corp). Blots were hybridized with primary antibodies and visualized by ECL system (Amersham Biosciences). To examine interaction of BTG2 with either Akt or Erk1/2, cell lysates (1.0 mg) were subjected to our established method [11]. The antibodies were purchased: anti-caspase 3, anti-Akt, anti-p-Akt(S^{473}), anti-Erk1/2, anti-p-Erk1/2, and anti-p-GSK3β(S^{9}) were from Cell signaling; anti-α-tubulin and anti-HA from Santa Cruz Biotechnology.

2.8. Statistical analysis

Numerical data were presented as mean ± standard deviation of the independent determinations. Independent *t*-test or ANOVA was applied, and multiple comparisons were evaluated by Tukey HSD. $P < 0.05$ were considered as significant.

3. Results

To investigate the role of BTG2 in ischemic cardiomyoblasts, basal expression of BTG2 in heart was evaluated by RT-PCR and found to be least expressed among several organs without stimulation (Suppl. Fig. S1A). However, the expression of BTG2 clearly increased in the infarct (I/R) compared with the surrounding (NL) tissues (Suppl. Fig. S1B). The effect of oxidative damage on the BTG2 expression was evaluated in H9c2 cardiomyoblasts; Supplementary Fig. S1C shows that BTG2 expression was maximally induced in 60 min and maintained for 120 min after 800 μM H_2O_2 stimulation. By using 5 mM N-acetyl-L-cysteine pretreatment for 30 min, the induction of BTG2 expression in response to H_2O_2 was confirmed (Suppl. Fig. S1D). To evaluate whether the BTG2 expression

was protective or aggravating the oxidative stress, H9c2 cells with Ad-LacZ (LacZ expresser) or Ad-BTG2 (BTG2 expresser) were studied further after H_2O_2 treatment.

3.1. BTG2 enhances necrosis of H9c2 cells in response to H_2O_2

To evaluate the mode of cell death in H9c2 after oxidative injury, the LacZ and BTG2 expressers were treated with H_2O_2 for 4 h and evaluated by immunocytochemistry and immunoblot analyses; Fig. 1A shows that the treatment increased PI stain-positive cells only in the BTG2 expresser, whereas activation of caspase 3 was observed in the LacZ expresser, but not in the BTG2 expresser (Fig. 1B), suggesting induction of necrosis, not apoptosis, by BTG2 expression in response to H_2O_2. Moreover, LDH release into culture media was also significantly increased in the BTG2 expresser treated with 200 μM H_2O_2 as compared to LacZ (Fig. 1C). To further confirm the effect of BTG2 on necrosis, BTG2 knockdown was employed by transfection of siBTG2 (Supplementary Fig. S2A), and the changes of H_2O_2-induced PI positive cells and LDH release were examined. As expected, siBTG2 significantly regulated PI-positive cells (Suppl. Fig. S2B) and LDH release (Fig. 1D, $p = 0.000$) compared with those of the siControl and the BTG2 expressers. The data strongly suggest that endogenous and exogenous BTG2 induces necrosis in response to H_2O_2, as opposed to apoptosis induction in the LacZ expressers.

3.2. H_2O_2 induces BTG2 translocation to mitochondria and mPTP opening with cyclophilin D activation

To evaluate the location of BTG2 expression in response to H_2O_2, Ad-BTG2 transduced H9c2 cells were examined by immunocytochemistry (Fig. 2A); BTG2 was mostly expressed in the nuclei and partly in the cytoplasm, whereas it was translocated to mitochondria after being exposed to H_2O_2, strongly suggesting a potential role of BTG2 in mitochondria. When the degree of mPTP opening was evaluated by FACS, fluorescence intensity of calcein AM remaining in the mitochondria was significantly reduced in the BTG2 expresser than the LacZ in 60 min of H_2O_2 treatment (Fig. 2B), suggesting the regulation of mPTP opening by BTG2. Based on the reports that mPTP opening is regulated by cyclophilin D in necrosis [22,23], a possibility of cyclophilin D regulation by BTG2 was then evaluated by MTT assay with cyclosporine A (CsA), direct inhibitor of cyclophilin D, treatment; H_2O_2-induced cell death in BTG2 expresser was partially and completely recovered by 1.0 μM ($^{\#}p < 0.05$ *vs.* LacZ) and 4.0 μM CsA treatment ($^{+}p = 0.004$ *vs.* no CsA, $^{++}p = 0.001$ *vs.* 1.0 μM CsA), respectively. The data support the notion that BTG2 expression aggravates H_2O_2-induced necrosis via cyclophilin D regulation (Fig. 2C). Moreover, 4 μM CsA completely protected the LacZ expresser from H_2O_2 treatment ($^{*}p = 0.012$), strongly suggesting that BTG2 translocation to mitochondria in response to H_2O_2 enhances mPTP opening via cyclophilin D activation.

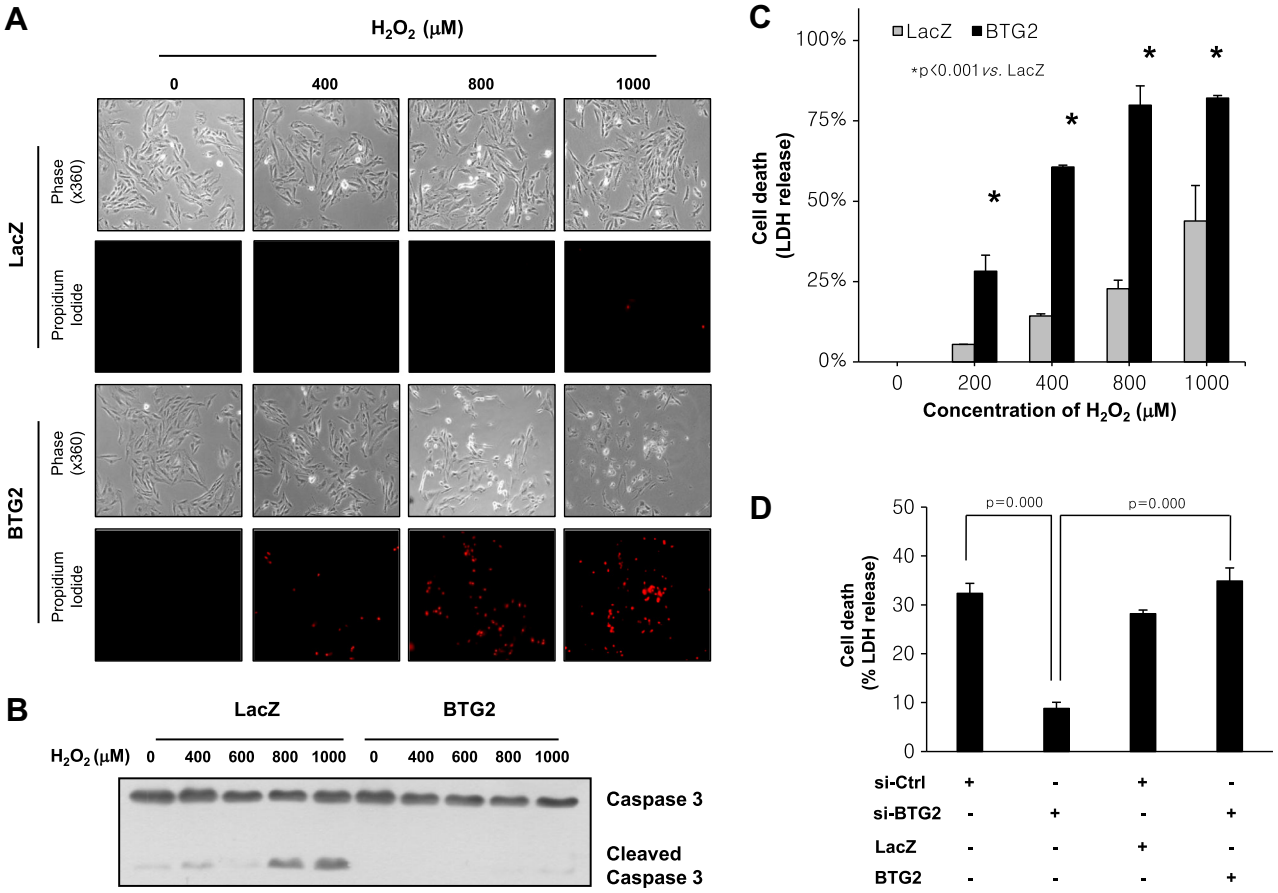

Fig. 1. Induction of necrosis in H9c2 cells with BTG2 expression after H_2O_2 treatment. H9c2cells with Ad-LacZ or Ad-BTG2 transduction were treated with H_2O_2 and then subjected to evaluations. (A) Microscopic observation and PI staining. Treatment with H_2O_2 for 4 h revealed PI-positive cells in the BTG2 expresser, but not LacZ, along with H_2O_2 concentration. (B) Immunoblot analysis showing caspase 3 cleavage by H_2O_2 in the LacZ expresser, but not BTG2. (C) Lactate dehydrogenase (LDH) released in culture media was significantly increased in the BTG2 expresser than the LacZ. (D) Inhibition of LDH release by short interfering BTG2 RNAs. H9c2 cells were transfected with siBTG2 for 48 h and treated with H_2O_2 (800 μM) for 60 min before LDH assay.

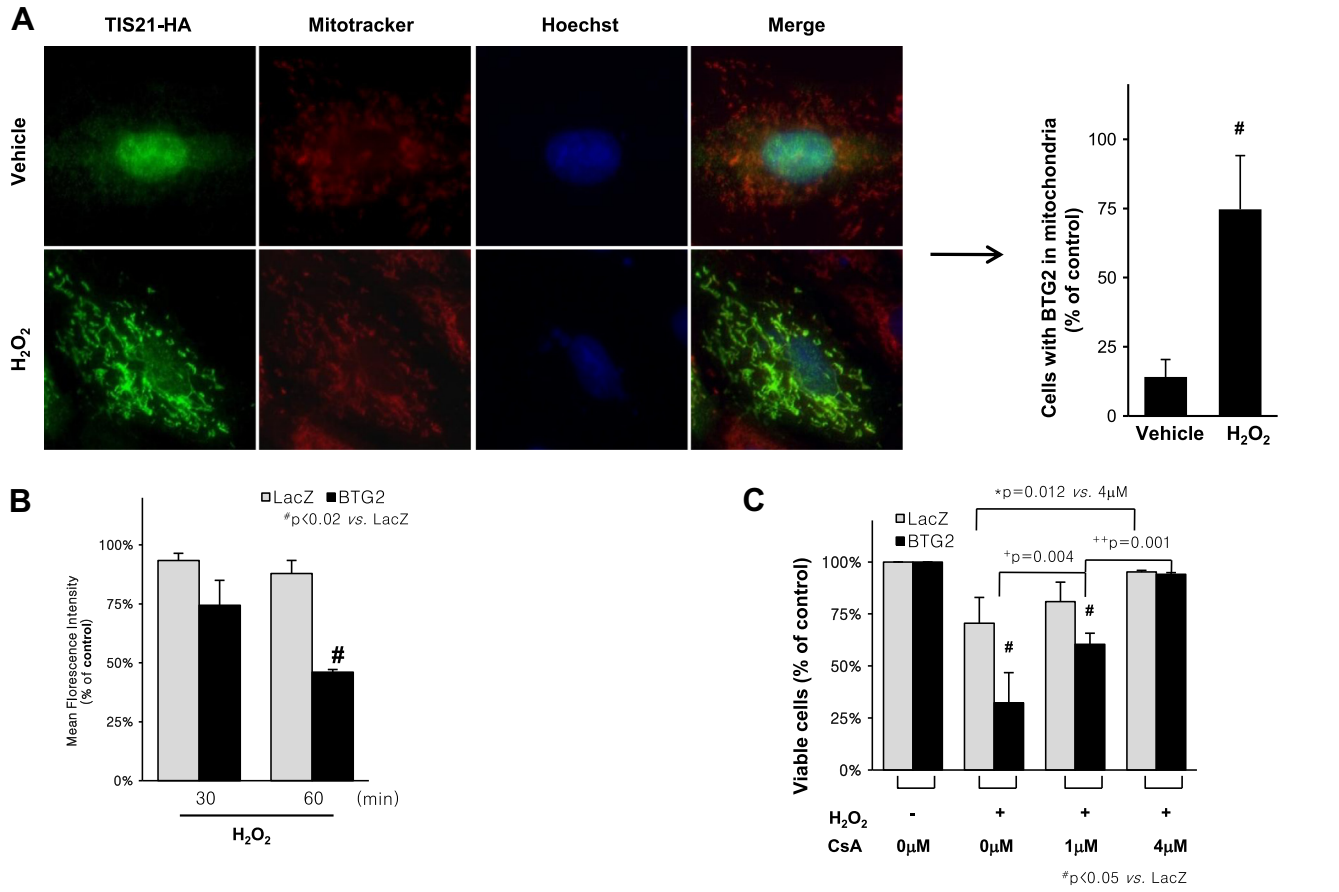

Fig. 2. BTG2 translocation to mitochondria and mPTP opening by cyclophilin D in response to H_2O_2. (A) Double immunocytochemistry of BTG2 expressers using anti-HA antibody and MitoTracker Red to stain BTG2 and mitochondria, respectively. H_2O_2 (800 μM) induced BTG2 translocation from nuclei to mitochondria. Cells with BTG2 in mitochondria were significantly increased by H_2O_2 treatment (75% *vs.* 10% control). (B) mPTP opening in the BTG2 expresser. The BTG2 or LacZ expressers were treated with calcein AM together with $CoCl_2$ and then treated with H_2O_2 for 60 min before measuring calcein AM fluorescence left in mitochondria by FACS. Loss of fluorescence in the BTG2 expresser indicates opening of mPTP in 60 min. (C) MTT assay. Cells were treated with an inhibitor of cyclophilin D, CsA, before incubating with H_2O_2 for 30 min. H_2O_2-reduced viability of the BTG2 expresser was recovered by CsA treatment. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

3.3. ATP regeneration fails in the BTG2 expresser

To monitor changes of energy level in response to H_2O_2 exposure, the changes of ATP levels were monitored; H_2O_2 treatment decreased ATP for 30 min both in the LacZ and the BTG2 expressers (left panel, Fig. 3A), however, the BTG2 expresser, but not LacZ, failed to regenerate ATP at 60 min of the treatment (right panel, Fig. 3A). The significant failure of energy replenishment in 30 min was also revealed by immunoblot analyses with p-AMPK-Thr^{172} antibody (Fig. 3B); AMPK activation was reduced in the LacZ in 45 min, but persistent in the BTG2 expressers until 60 min. When the relative levels of ATP recovery and p-AMPK/AMPK in the LacZ and BTG2 expressers are presented as bar and line graphs, respectively, AMPK activation in the BTG2 expresser was severe and earlier than the failure of ATP regeneration (Fig. 3C), indicating that BTG2 significantly deteriorates ATP production in cardiomyoblasts exposed to H_2O_2.

3.4. BTG2 inhibits recovery of survival kinases, but activates GSK3β, after H_2O_2 stimulation

To investigate whether the BTG2-enhanced mPTP opening was regulated by survival kinases [24], phosphorylation of Akt and Erk was examined by immunoblot analysis. Fig. 4A shows transient inactivation of Akt and Erk in the LacZ expresser in 30 min of H_2O_2 treatment, whereas it was persistent until 60 min in the BTG2 expresser. Based on the report that GSK3β is critical in cardioprotection [25], we examined the activity of GSK3β by immunoblot analysis, and found its transient activation in the LacZ expresser from 15 to 30 min, bur prolonged in the BTG2 expressers (Fig. 4B). When the effect of GSK3β on cell death was evaluated after treatment of BTG2 expressers with H_2O_2 with or without SB-415286, MTT assay revealed that the co-treatment with the latter significantly recovered cell viability (Fig. 4C, $p = 0.002$ *vs.* H_2O_2 alone), confirming the effect of GSK-3β on the BTG2-enhanced necrosis. To confirm the *in vivo* effect of endogenous BTG2 on the sensitivity to H_2O_2, BTG2-KO-MEF were subjected to MTT assay; the absence of BTG2 gene significantly increased resistance to H_2O_2 compared to the wt-MEF (Fig. 4D). Data indicate that BTG2 regulates GSK-3β activation after exposure to H_2O_2, resulting in the regulation of mPTP opening by cyclophilin D activation.

4. Discussion

It has been known that mPTP, a regulated nonspecific protein channel spanning the inner and outer mitochondrial membranes, is opened by cytosolic accumulation of Ca^{2+} or oxidative damage-generating ROS, which mediates induction of necrosis [26,27]. Necrosis model of culture cells by H_2O_2 treatment is regulated by cyclophin D activity, the key component of mPTP. Therefore, CsA potently and specifically prevents mitochondrial permeability

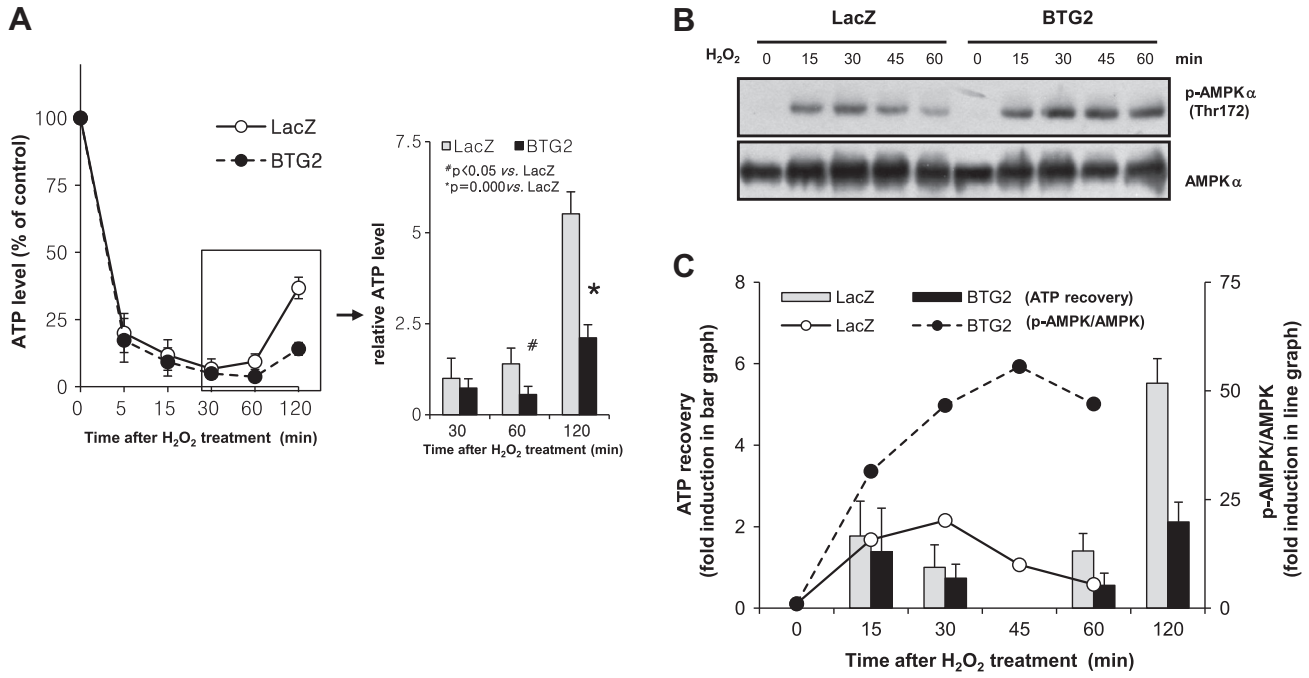

Fig. 3. Failure of ATP regeneration in the BTG2 expresser. (A) Changes of ATP levels after 800 μM H_2O_2 treatment. H_2O_2 rapidly reduced ATP level for 30 min both in the LacZ and BTG2 expressers, however, the LacZ control started to recover ATP within 60 min, but not the BTG2 expressers (right panel). (B) Immunoblot analysis. H_2O_2 treatment clearly induced AMPK phosphorylation of the LacZ and BTG2 expressers in 15 min, however, the inactivation was observed in the LacZ, not BTG2, expressers in 45 min of the treatment. (C) Comparison of p-AMPK levels (line graphs) to ATP generation (bar graphs). Solid line indicates the LacZ and the dotted line for BTG2 expresser, that is labeled in the right ordinate. Left ordinate indicates ATP level in the LacZ (gray bar) and the BTG2 (black bar) expressers. Data represent typical immunoblot analysis after repeated experiments.

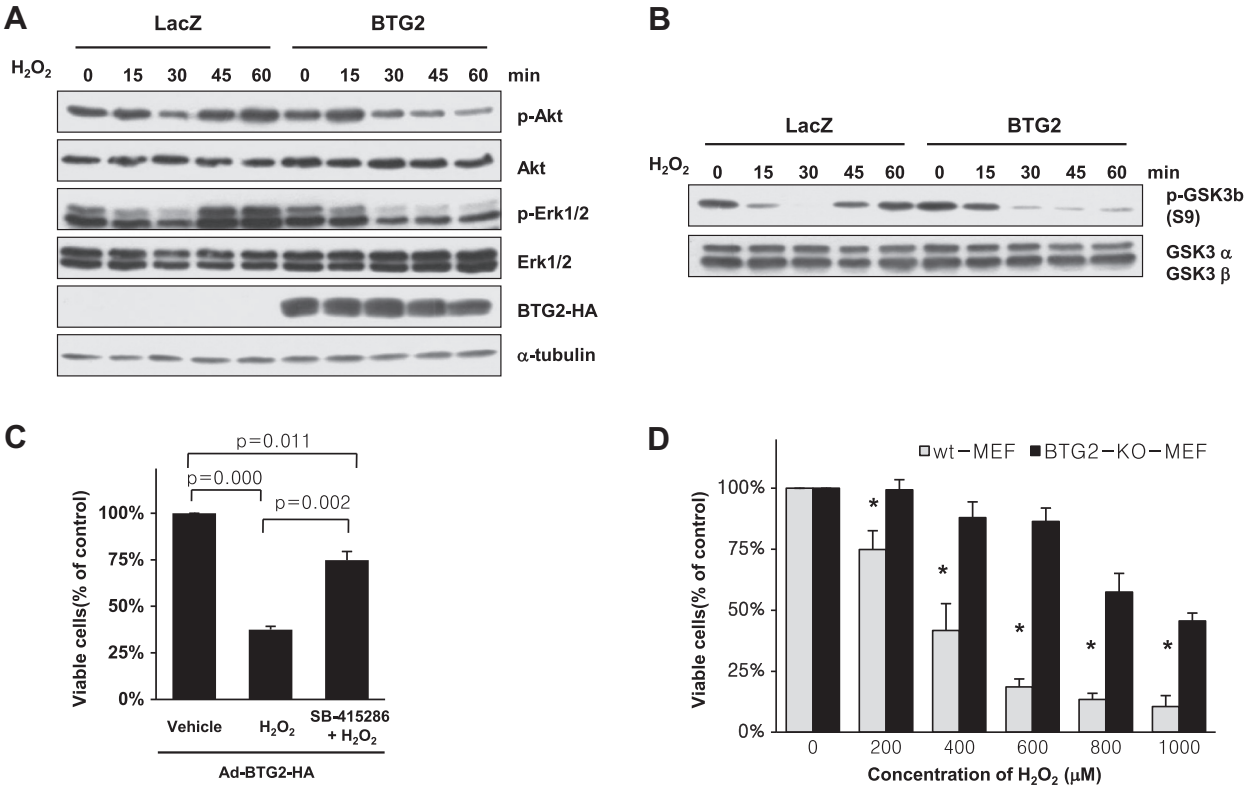

Fig. 4. Necrosis of BTG2 expressers by activating GSK3β. (A) Activities of Akt and Erk were transiently reduced at 30 min of H_2O_2 treatment and recovered in 45 min in the LacZ expresser, whereas it was inactive in the BTG2 expresser until 60 min. (B) GSK3β activation in the BTG2 expresser for 60 min after H_2O_2 treatment, but it was transient in the LacZ control, indicating sustained activation of GSK3β in the BTG2 expressers. (C) MTT assay; H_2O_2-induced cell death was partially ameliorated by pretreatment of BTG2 expresser with SB415286, suggesting the role of GSK3β in necrosis. (D) MTT assay showing the role of endogenous BTG2. Wild type-MEF was much more sensitive to H_2O_2 treatment than the BTG2-KO-MEF (*$p < 0.003$), indicating the cell death-enhancing effect of BTG2 in H9c2 cells.

transition by physical interaction with cyclophilin D, inhibits its activity, and then displaces it from mPTP [28].

Here, we presented results on the role of BTG2 in mitochondria of cardiomyoblasts; induction of necrosis in response to H_2O_2, via regulating mPTP opening and cyclophilin D activation. The results obtained by employing chemical inhibitors and BTG2-KO-MEF (Figs. 2 and 4) were well accordant with our previous report on the enhanced cell death by BTG2 expression after doxorubicin treatment [29]. The physiological function of mPTP core complex remains poorly understood. Nevertheless, the cell cannot generate ATP by oxidative phosphorylation, as long as mPTP is open.

A plausible explanation of the role of BTG2 might be found in the significant failure of ATP regeneration at 60 min after H_2O_2 stimulation along with severe activation of AMPK in the BTG2 expressers compared with those of the LacZ expressers (Fig. 3). Presently, we do not know how BTG2 is accumulated in mitochondria in response to H_2O_2, however, BTG2 is already present in mitochondrial matrix, as well as inner and outer membranes of the several human cancer cells such as Huh7, A549, HeLa and H9c2 cells [30], thus having an opportunity to regulate various mitochondrial proteins such as cyclophylin D, Erk and Akt in addition to GSK3β. Indeed, H_2O_2 partly increased BTG2 interaction with survival kinases (Suppl. Fig. S3A), and inhibited rapid recovery of the enzymes (Suppl. Fig. S3B), implying the importance of on-time regulation of GSK3β activity.

It has recently been reported that mitochondrial pools of GSK3β and hexokinase I and II protect cardiomyocytes from oxidative damage at the end of signal pathways which are directly responsible for the regulations of mPTP and mitochondrial depolarization [2]. Physiologically, BTG2 expression in heart muscle is almost absent under normal condition, whereas the expression is upregulated by ischemic damage and H_2O_2 stimulation (Suppl. Fig. S1), implying protective setting of myocardium from oxidative damage under normal respiration. Here, we suggest tumor suppressor, BTG2, as one of the regulators of necrosis in myocardium *via* inhibiting Akt/Erk, but activating GSK3β and cyclophilin D, ultimately resulting in mPTP opening in response to H_2O_2 (Suppl. Fig. S4).

On the other hand, senescent cells are ATP depleted compared with the young cells and resistant to apoptosis, whereas the cells are vulnerable to necrosis and inflammatory response [31]. Since the expression of BTG2 is higher in senescent cells than young cells [32], our data also imply that the role of a tumor suppressor in the process of cell death may be context dependent between the young and old cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.114.

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