Oxidative stress-induced formation of a positive-feedback loop for the sustained activation of p38 MAPK leading to the loss of cell division in cardiomyocytes soon after birth

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Abstract

Shortly after birth, mammalian cardiomyocytes irreversibly exit from the cell cycle and become terminally differentiated. The cellular mechanisms responsible for the cessation of cell division and terminal differentiation of cardiomyocytes soon after birth have intrigued developmental biologists as well as cardiovascular physicians, but the genetic cues for the irreversible exit from the cell cycle soon after birth remain largely unknown. We examined whether and if so how oxidative stress to mammalian hearts during fetal-neonatal transition produces changes in the proliferative activity and terminal differentiation of cardiomyocytes. Scavenging of reactive oxygen species (ROS) during fetal-neonatal transition, especially after birth, resulted in an increase in the proliferative activity and a decrease in the ratio of binucleated cardiomyocytes. Exposure to ROS in cultured cardiomyocytes increased the activity of p38 MAPK and the expression of connexin43 (Cx43). Not only knockdown of Cx43 using siRNA but also the inhibition of p38 MAPK activity resulted in a significant decrease in the production of ROS in cardiomyocytes, suggesting that the signaling pathway ROS–p38 MAPK–Cx43 (especially, Cx43 at mitochondria, mtCx43) constituted a closed regulatory system with positive-feedback. In addition, continuous scavenging of ROS or suppression of p38 MAPK activity for 4 days after birth resulted in a significant decrease in the expression of mtCx43 and in the number of binucleated cardiomyocytes. This study demonstrated that the ROS-induced formation of a positive-feedback loop ROS–p38 MAPK–mtCx43 for the sustained activation of p38 MAPK soon after birth possibly contributes to the loss of cell division and binucleation in mammalian cardiomyocytes.

Key words:

perinatal oxidative stress; reactive oxygen species; connexin43; positive-feedback loop; binucleation; p38 MAPK

Introduction

Mammalian cardiac and skeletal muscle are both derived from mesoderm and share many morphologic, physiologic, and biochemical properties [49]. However, one difference is that during embryonic development, cell proliferation and differentiation processes occur in skeletal muscle but not cardiac muscle [5]. In the developing embryo, committed skeletal muscle myoblasts proliferate until a certain cell mass has been reached and then start to fuse together, leading to permanent withdrawal from the cell cycle; that is, terminal differentiation [49]. In contrast, committed cardiac myoblasts differentiate into contractile cardiac muscle cells without withdrawing from the cell cycle. Many types of cells lose the ability to divide once they have produced specialized cytoplasmic structures characteristic of their fully differentiated state [3]. Carlson explained that such a characteristic feature of cardiomyocytes is not surprising in view of the requirement for early and continuous functioning of the heart during embryonic development [3]. However, shortly after birth, mammalian cardiac myocytes irreversibly exit from the cell cycle and become terminally differentiated as skeletal muscle does at earlier stages [47]. These findings have led to the idea that unlike other cell types, cardiomyocytes must have additional mechanisms for terminal differentiation functioning during fetal-neonatal transition. The cellular mechanisms responsible for the cessation of cell division and terminal differentiation of cardiomyocytes soon after birth have intrigued developmental biologists as well as cardiovascular physicians, but the genetic cues for the irreversible exit from the cell cycle soon after birth remain largely unknown.

Mammalian birth is characterized by a sudden transition from maternal-mediated respiration to autonomous pulmonary respiration. The fetus transfers from an intrauterine hypoxic environment with a pO_2 of 20-25 mmHg to an extrauterine relatively hyperoxic environment with a pO_2 of 100 mmHg [32,34]. Drastic changes in circulatory and respiratory functions during fetal-neonatal transition may cause physiological oxidative stress that can result in the generation of reactive oxygen species (ROS) after birth [14,34,39]. In general, newborn animals and infants develop systems to protect against oxidative stress during fetal-neonatal transition, such as the production of a variety of anti-oxidant enzymes. In fact, previous studies have revealed a surge in the activity of pulmonary antioxidants [13,34], and proposed it to be a defense mechanism against the acute changes in oxygen concentrations at birth [13,15]. However, de Haan *et al*. investigated the expression of the copper/zinc superoxide dismutase (SOD1) and glutathione peroxidase (GPX1) genes in a variety of murine

embryonic, fetal, and neonatal organs, and found an increase in both in the lungs, stomach, and liver of late-gestational mouse fetuses [7]. In contrast, the heart fails to show an appreciable elevation of SOD1 and GPX1 expression in late gestation or at birth [7], suggesting that the oxidative stress status of the heart during fetal-neonatal transition is different from that of other organs such as the lungs and liver.

We hypothesized that oxidative stress during fetal-neonatal transition regulates the rapid transition from hyperplasia to hypertrophy and is crucial to the terminal differentiation of cardiomyocytes shortly after birth. Here we demonstrated the ROS-induced formation of a positive-feedback loop for the sustained activation of p38 MAPK; that is, a ROS-induced and p38 MAPK-mediated increase in the expression of mitochondrial Cx43 (mtCx43), possibly leading to the loss of cell division, and binucleation (karyokinesis without cytokinesis) of mammalian cardiomyocytes soon after birth.

Materials and Methods

The animal experiments conformed to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996), as well as the "guide for the care and use of laboratory animals", Hokkaido University School of Medicine.

Treatment of pregnant and newborn rats with NAC

The treatment of pregnant rats with *N*-acetyl-L-cycteine (NAC, Sigma) was done according to the methods of Izzotti *et al* [22]. In brief, half of the pregnant rats were left untreated (control), and half received NAC. The drug was added to drinking water at a concentration accounting for a calculated daily intake of 1 g/kg body weight. Treatment with NAC started when the females were caged with the males, and continued until parturition.

In some experiments, NAC was administered systemically as an intraperitoneal injection to newborns from NAC-treated dams at a dose of 300 mg/kg [45]. The injection was done once a day from day 0 to day 4.

Measurement of heart weight

At 4 days of age, neonatal rats were decapitated and their hearts removed, trimmed of extracardiac tissue, and weighed. Hearts were dried to a constant weight in filter paper to eliminate water content. To assess changes in the development of the heart dependent on NAC treatment, heart weight was normalized to the body weight (HW/BW).

Isolation and culture of cells

Cardiomyocytes were prepared from the ventricles of Wistar rats aged 0 days, removed after decapitation. The ventricles were rinsed in a 25 mM HEPES buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1% collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37°C for 10 min. The digested fragments were centrifuged at 1000 rpm for 4 min (LC-100, TOMY, Tokyo, Japan) and precipitated cell components were washed twice with MSS to terminate the effects of the collagenase. The cell components were suspended in MCDB 107 (Research Institute for the Functional Peptides, Yamagata, Japan) containing 5% heat-inactivated fetal calf serum (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90 μm porosity) to remove large aggregates of cells; the filtered suspension contained cardiomyocytes and cardiac fibroblasts. To separate cardiomyocytes from cardiac fibroblasts

based on the selective adhesion technique, the cell suspension was poured into petri dishes (ϕ 60 mm, CELL STAR®, Greiner Bio-one), and incubated for 60 min at 37°C, in 5% CO_2 and 95% air. By virtue of the procedure, most cardiac fibroblasts adhere to the dish. After the incubation, the suspension, mostly containing cardiomyocytes, was collected. The suspension was centrifuged at 700 rpm for 5 min to separate the remaining blood cell components in the supernatant. The precipitated cells were resuspended in MCDB 107 containing 5% heat-inactivated fetal calf serum, transferrin (10 μg/mL, Sigma, St. Louis, MO), and insulin (10 μg/mL, SIGMA). The cell suspension was passed through a fine wire mesh screen (25 μm porosity) to remove remaining small aggregates of myocytes, and finally the isolated myocytes remaining were cultured at a density of about 5.0×10^5 cells/ml in a petri dish (ϕ 35 mm, CELLSTAR®, Greiner Bio-one) coated beforehand with fibronectin (1.0 μg/cm^2, Sigma). Cultures incubated for 6 hours were used in this study.

The method of culturing cardiomyocytes is described in detail in our previous papers [24-26,31,46].

RNA interference

We used chemically modified Cx43 small interfering RNAs (Cx43 siRNAs) generated by Santa Cruz (sc-60008) (Santa Cruz Biotechnology, Inc., CA). For transfection, 5×10^5 cells per well in 2mL of antibiotic-free normal growth medium supplemented with FBS were grown in 6-well plates, and transfected at the time of plating with Cx43 siRNAs using Transfection Reagent (Santa Cruz, sc-29528). The protocol for transfection was described in detail in our previous paper [31].

Cell counting

Cells were washed three times with ice-cold 0.02 mol phosphate-buffered saline (PBS), pH 7.4, trypsinized, and enumerated with a hemocytometer [31].

Inhibition of p38 MAPK activity

To inhibit the activity of p38 MAPK, cells cultured for 6 hours were treated everyday with SB203580 (10 μM) (p38 MAPK inhibitor, Calbiochem). This concentration of SB203580 significantly inhibits the activity of p38 MAPK in cultured cardiomyocytes [31]. In some experiments, the activity of p38 MAPK was continuously suppressed by intraperitoneal injection into newborns with SB203580 (50 μg/50 μL) after birth. The injection was done

once a day from day 0 to day 4.

Immunofluorescence staining

Cardiomyocytes were stained with antibodies against sarcomeric α-actinin and proliferating cell nuclear antigen (PCNA) (Sigma, St. Louis, MA). First, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then rinsed three times with 0.02 mol phosphate-buffered saline (PBS), pH 7.4. Fixed cells were permeabilized in 0.1% triton X-100 in PBS for 15 min, blocked with 1% normal goat serum in PBS for 30 min, rinsed three times with 0.02 M PBS, pH 7.4, and incubated with a primary antibody over a 24-hour period. After being washed with 0.02 mol PBS, they were incubated with a secondary antibody (Alexa fluor 532, 488, 405 (Molecular Probes), 1:200) for 1h at room temperature. After again being washed with 0.02 mol PBS, the specimens were mounted in PBS and sealed with nail polish. They were then observed under a confocal laser microscope (FLUOVIEW FV300, OLYMPUS) at the OPEN FACILITY, Hokkaido University Sousei Hall, Sapporo, Japan.

Western blotting

Lysed cells were sonicated and centrifuged at 20,000 × *g* for 20 min at 4°C, and protein concentrations were measured using the BCA (Bicinchoninic Acid) methods. Proteins (50 μg per lane) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred onto a difluoride membrane (Bio-Rad, Hercules, CA). Nonspecific binding sites were blocked with 5% non-fat milk or Blocking Buffer (Thermo Scientific, SuperBlock® Blocking Buffer in PBS) for 60 min, and the membrane was incubated overnight at 4°C with antibodies: Cx43, sarcomeric α-actinin (Sigma, St. Louis, MA), MAP kinases (phospho-p38, p38α; Santa Cruz), complex II (Santa Cruz), adenine nucleotide translocase (ANT) (Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz), copper/zinc superoxide dismutase (SOD1) (Santa Cruz), glutathione peroxidase (GPx-1) (Santa Cruz), catalase (Santa Cruz), and then goat anti-rabbit IgG Horseradish peroxidase (HRP)-conjugated antibody for Cx43, complex II, ANT, GAPDH, and MAP kinases or goat anti-mouse IgG HRP-conjugated antibody for sarcomeric α-actinin (Cell Signaling Technology). The immunoreactive bands were detected with an enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA). Quantification of the expression of Cx43, MAP kinases, sarcomeric α-actinin, and complex II was performed by densitometric analysis using Image J 1.42q for Windows (NIH,

USA).

Measurement of ROS

Intracellular ROS was measured using 2', 7'-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen, D399). Briefly, isolated cardiomyocytes were cultured for 4 days *in vitro* (4DIV) and then incubated for 24 h with the H_2O_2-sensitive fluorophore 2', 7'-dichlorofluorescein diacetate (H_2DCFDA, final concentration; 10 μM in MCDB107). The cells were washed three times with phosphate-buffered saline (PBS), pH7.4, and incubated in MCDB107 for 5min. Images of cardiomyocytes were recorded with a CCD camera (WV-BD400, Panasonic, Tokyo, Japan) through a phase-contrast microscope (IX70, OLYMPUS, Japan). Quantification of the production of ROS was performed by densitometric analysis using Image J 1.42q for Windows (NIH, USA).

Treatment of cultured cardiomyocytes with H_2O_2

To expose them to ROS, cardiomyocytes cultured for 6 hours were treated every second day with medium containing 0 (control) or 10 μM H_2O_2 (Wako).

Isolation of cardiac mitochondria

Cardiac mitochondria were isolated from rat hearts by Mitochondria Isolation Kit (#89874, Thremo Scientific, Waltham, Mass). The cells were washed three times with phosphate-buffered saline (PBS), pH7.4, and were scraped in a minimal volume (~1.0 mL/dish) of ice-cold PBS. Lysed cells on microcentrifuge tube were centrifuged at 850×*g* for 2 min at 4^{o}C. Pellets were added 400 μL of Mitochondria Isolation Reagent A, vortexed at medium speed for 5 seconds, and incubated tube on ice for exactly 2 minutes. And then, 5 μL of Mitochondria Isolation Reagent B was added, and vortexed at maximum speed for 5 seconds. The suspension was incubated on ice for 5 minutes, vortexing at maximum speed every minute, and added 400 μL of Mitochondria Isolation Reagent C. The suspension was centrifuged at 700×*g* for 10 min at 4^{o}C. The pellet was resuspended in a new reagent, and centrifuged at 12,000×*g* for 15 minutes at 4^{o}C. The supernatant was transferred to a new tube (The pellet contains the isolated mitochondria). 250 μL of Mitochondria Isolation Reagent C was added to the pellet, centrifuged at 12,000×*g* for 5 minutes, and discarded the supernatant. The pellet was used for experiments.

The mitochondria were isolated as described previously [1,20].

Statistical analysis

The data are expressed as the mean ± SD. Group comparisons were made using an analysis of variance (ANOVA) with Fisher's test. A *P*-value of less than 0.01 or 0.05 was considered significant.

Results

We first investigated the expression of antioxidants such as SOD1, GPX1, and catalase is developmentally regulated in fetal and neonatal rat's hearts. The expression of SOD1, GPX1, and catalase in the heart was significantly down-regulated in fetuses, but was gradually increased after birth in neonates (Suppl. Fig. 1), suggesting that the heart of newborns was actually exposed to oxidative stress during fetal-neonatal transition. To investigate the relationship between oxidative stress and the development of the heart, we then examined whether treatment of pregnant rats and isolated cardiomyocytes with a scavenger of ROS, *N*-acetyl-L-cycteine (NAC), changed the proliferative activity of cardiomyocytes. Cardiomyocytes isolated from the pups of NAC-treated dams were treated with NAC in culture (NAC+/c+). This treatment resulted in a significant increase in PCNA-positive cardiomyocytes as compared with control cultured cells (NAC–/c–), those from NAC-untreated dams with NAC in culture (NAC–/c+), and those from NAC-treated dams without NAC in culture (NAC+/c–) (Figs. 1A1-1B3 and 1C), suggesting that oxidative stress during fetal-neonatal transition, especially after birth, contributed to the exit from the cell cycle. To examine whether the scavenging of ROS induced reentry into the cell division cycle, we performed a cell count analysis. At 4 days *in vitro* (4DIV), the number of cardiomyocytes was significantly increased by continuous NAC treatment, suggesting that the scavenging of ROS resulted in an increase in activity for cell division (Fig. 1D). In addition, NAC treatment also decreased the proportion of binucleated cardiomyocytes (Fig. 1E, 1F). In general, the binucleation of cells reflects a phenomenon known as "karyokinesis without cytokinesis". These results suggested that oxidative stress during fetal-neonatal transition was responsible for a decrease in the proliferative activity and in the binucleation of cardiomyocytes.

Although the scavenging of ROS actually increased the proliferative activity of cardiomyocytes, the mechanisms involved in the increase remain largely unknown. Previous studies have demonstrated that ROS are crucially involved in the differentiation and hypertrophy of cardiomyocytes via the activation of p38 MAPK [10,40,48]. In addition, the activation of p38 MAPK increases the expression of Cx43 in many cell types including cardiomyocytes [23,38]. We have recently demonstrated that knockdown of Cx43 changes p38 MAPK activity and proliferative activity in cardiomyocytes [31]. We therefore analyzed whether the scavenging of ROS changed the activity of p38 MAPK and the expression of Cx43. The treatment of cardiomyocytes from NAC-treated dams with NAC (NAC+/c+) for 4 days significantly decreased the activity of p38 MAPK and the expression of Cx43 (Fig. 2A1-A3),

suggesting that ROS were responsible for the activation of p38 MAPK and the increased expression of Cx43 in cardiomyocytes. What then are the mechanisms involved in the ROS-induced decrease in the ability of cells to divide?

Cardiomyocytes were exposed to H_2O_2 (10 μM) for 4 days. Western blotting showed a significant increase in the activity of p38 MAPK (Fig. 2B1, 2B2) and expression of Cx43 (Fig. 2B1, 2B3), the latter antagonized by co-treatment with SB203580, an inhibitor of p38 MAPK, suggesting that ROS increased Cx43 expression via the activation of p38 MAPK in cardiomyocytes. Treatment with SB203580 under control condition also resulted in a decrease in *p*-p38 MAPK and Cx43, suggesting that p38 MAPK was consistently activated even under this culture condition. These results raise questions about the cause-and-effect relationship between the activation of p38 MAPK and the increased expression of Cx43, since we have recently demonstrated that the down-regulation of Cx43 expression using Cx43 siRNA decreases the activity of p38 MAPK, leading to increased proliferative activity in cardiomyocytes [31]. The cause-and-effect relationship seems to be reversed.

The present study demonstrated that ROS activated p38 MAPK and increased Cx43 expression (Figs 1 and 2), while knockdown of Cx43 decreased the activity of p38 MAPK [31]. We then investigated whether the knockdown of Cx43 decreased the production of ROS in cardiomyocytes. In this study, the production of ROS was assessed by an increase in the intensity of H_2DCF-DA fluorescence. Exposure of cardiomyocytes to H_2O_2 (10 μM) significantly increased the H_2DCF-DA fluorescence (Fig. 3A). In addition, the intensity of H_2DCF-DA fluorescence was significantly attenuated by treatment with NAC (data not shown), suggesting that H_2DCF-DA fluorescence reflected ROS generated in cardiomyocytes.

Cx43 siRNA significantly decreased ROS production (Fig. 3C), but scrambled RNA did not (Fig. 3D), suggesting that the signal transduction pathway ROS−p38 MAPK−Cx43 is not unidirectional, but constitutes a closed regulatory loop; that is, a positive-feedback loop. Treatment with Cx43 siRNA for 6 h resulted in a significant decrease in the expression of mitochondrial (Fig. 6C) and total Cx43 [31]. As shown in Fig. 2, treatment of cultures with H_2O_2 increased the expression of Cx43 via the activation of p38 MAPK. If the ROS−p38 MAPK−Cx43 pathway is a closed positive-feedback loop, the suppression of p38 MAPK activity may decrease the production of ROS. Treatment of cultures with SB203580 (10 μM) significantly decreased ROS production in cardiomyocytes (Fig. 4B). The inhibition of p38 MAPK activity antagonized the ROS-enhanced expression of Cx43 (Fig. 2), supporting the notion that the ROS−p38 MAPK−Cx43 pathway constituted a positive-feedback loop. If the

ROS–p38 MAPK–Cx43 pathway constituted a closed system, the increase in p38 MAPK activity caused by exposure to ROS would persist even after removal of oxidative stress. Indeed, the H_2O_2-induced increase in the activity of p38 MAPK persisted for at least 2 days after the washout of H_2O_2 (Fig. 5). All these findings suggested that the ROS–p38 MAPK–Cx43 pathway constituted a closed positive-feedback loop. It should be noted that persistent exposure to oxidative stress might be required for the formation of such a positive-feedback loop, since treatment of cardiomyocytes with H_2O_2 for a short duration (1 day) did not result in the persistent activation of p38 MAPK; that is, the p38 MAPK activity returned to the control level at 1 day after the washout of H_2O_2 (Suppl. Fig. 2).

We next investigated the functional link between the expression of Cx43 and production of ROS in cardiomyocytes. We have previously revealed that treatment with heptanol, a blocker of gap junctions, do not affect the proliferative activity of cardiomyocytes [31], suggesting that gap junctions themselves are not involved in the functional link. Recently, Cx43 has been detected in the mitochondria of cardiomyocytes [1,18,36,37]. In fact, the mitochondrial Cx43 expression was also detected in cultured cardiomyocytes used in the present study (Fig. 6A). Mitochondria are considered the most relevant site for the formation of ROS in cardiomyocytes [30], and mitochondria-derived ROS are involved in myocardial failure [30] as well as cardioprotection [19,28]. In addition, Boengler *et al*. found Cx43 in subsarcolemmal mitochondria (SSM), but not in interfibrillar mitochondria (IFM) [2], and more ROS (H_2O_2) in the SSM than IFM under pathophysiological conditions [4]. These findings imply that mitochondrial Cx43 (mtCx43) seems crucial to the ROS–p38 MAPK–Cx43 pathway; that is, the ROS-induced increase in the expression of mtCx43 would further increase ROS production.

A Western blot analysis was performed to confirm the purity of mitochondrial extracts (Fig. 6A). In rat myocardial mitochondria, signals for markers of the plasmamembrane (Na^+/K^+-ATPase) and the cytosol (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were hardly detected, but inner mitochondrial membrane proteins (adenine nucleotide translocase, ANT and the respiratory chain complex II) were stained. Treatment of cardiomyocytes with H_2O_2 increased the expression of mtCx43, and the effect was reversed by co-treatment with SB203580, an inhibitor of p38 MAPK (Fig. 6B). Cx43 siRNA significantly reduced mtCx43 expression (Fig. 6C) in addition to the overall (mitochondrial, cytosolic, and sarcolemmal) expression of Cx43 [31].

mtCx43 is a nuclear encoded mitochondrial protein, and translocated into the inner mitochondrial membrane through a heat shock protein 90 (Hsp90)-dependent TOM (translocase

of the outer membrane) pathway [35]. Treatment of isolated rat hearts with geldanamycin, a blocker of Hsp90 [43], resulted in a rapid (15 min) reduction in mtCx43 content [35]. Therefore, we next treated cardiomyocytes with geldanamycin, and found that it reduced the expression of mtCx43 (Fig. 7A) and production of ROS (Fig. 7B). The treatment also suppressed the activity of p38 MAPK activity (Fig. 7C). In addition, geldanamycin reduced total Cx43 content, although the reduction was not significant. These results supported our notion that the ROS−p38 MAPK−Cx43 (mtCx43) pathway constituted a positive-feedback loop. We next investigated whether such a closed regulatory loop actually function *in vivo* after birth, and is it involved in the loss of cell division, leading to the binucleation of cardiomyocytes.

A previous study has demonstrated a rapid switch from myocyte hyperplasia to hypertrophy between postnatal days 3 and 4 in rat hearts [29]. Therefore, a Western blot analysis was conducted with heart tissue 4 days after birth (Fig. 8). Continuous scavenging of ROS for 4 days after birth in newborns from NAC-treated dams (NAC+/+) resulted in a significant decrease in the expression of mtCx43 and total Cx43 and in the activity of p38 MAPK (Figs. 8A-8E). In addition, NAC treatment significantly reduced the number of binucleated cardiomyocytes at 4 days after birth (Figs. 8G-8J). The ratio of heart weight to body weight (HW/BW) was significantly greater in the NAC+/+ animals than that in the control (NAC−/−) animals (Fig. 8F3). If the ROS-induced activation of p38 MAPK were critical to the loss of cell division after birth, the continuous suppression of the activity of p38 MAPK with SB203580 would reduce binucleated cardiomyocytes. We finally investigated this possibility (Fig. 9).

The continuous suppression of the activity of p38 MAPK with intraperitoneal injection into newborns with SB203580 for 4 days after birth resulted in a significant decrease in the expression of mtCx43 and total Cx43 and in the activity of p38 MAPK (Figs. 9A-9E). In addition, SB203580 treatment significantly reduced the number of binucleated cardiomyocytes at 4 days after birth (Figs. 9F1-F3). The ratio of heart weight to body weight (HW/BW) was significantly greater in the animals treatment with SB203580 than that in the control animals (Fig. 9G3).

Discussion

The present study has revealed that ROS generated during fetal-neonatal transition, especially soon after birth, resulted in the formation of a positive-feedback loop for the sustained activation of p38 MAPK, and that the ROS-induced and p38 MAPK-mediated increase in the expression of mitochondrial Cx43 (mtCx43) is critical to such a loop. This positive-feedback-induced sustained activation of p38 MAPK possibly contributed to the loss of the cell's ability to divide and to binucleation (karyokinesis without cytokinesis) of mammalian cardiomyocytes soon after birth.

DNA synthesis in mammalian cardiomyocytes is associated with cell proliferation (cytokinesis) during fetal development, and with binucleation (karyokinesis without cytokinesis) after birth [6]. A detailed analysis of the growth pattern of cardiomyocytes has suggested that a rapid "trigger" mechanism may control cardiac growth in the early postnatal period, and that a sequence of master switch genes may regulate this rapid transition [29]. Such a rapid switch from myocyte hyperplasia to hypertrophy possibly occurs between postnatal days 3 and 4 in rat hearts [29]. The oxidative stress-induced formation of a positive-feedback loop, the ROS-p38 MAPK-mtCx43 pathway, and the resultant sustained activation of p38 MAPK described in the present study may be one of the "triggers" responsible for a switch from myocyte hyperplasia to hypertrophy soon after birth.

The present study has demonstrated the ROS-enhanced expression of Cx43, especially mtCx43, to be a critical component of the positive-feedback pathway responsible for the loss of cell division in cardiomyocytes after birth. Many cell types exhibit intercellular communication via gap junctions. Recent studies have revealed that Cx43 is present in the mitochondria of cardiomyocytes and that ischemic preconditioning (IP) enhances this mitochondrial localization [1,37]. During IP, generation of ROS is impaired in Cx43-deficient ($Cx43^{+/-}$) mice, suggesting that it is regulated by mitochondrial Cx43 [37]. The ROS generated during reperfusion are known to trigger the protection of isolated hearts from ischemia/reperfusion injury [9], and the activation of p38 MAPK is crucially involved in the development of IP-induced ischemic tolerance of pig hearts [41]. In addition, Kulisz et al. have suggested that in embryonic chick cardiomyocytes, mitochondrial ROS such as H_2O_2 and superoxide are crucial to the activation of p38 MAPK [27]. These previous results seem to support the present finding that Cx43-knockdown also reduced mitochondrial expression of Cx43, resulting in the suppression of ROS levels, leading to decreased activity of p38 MAPK

(Figs. 3 and 6C).

Gap junctions are composed of connexin which has several isoforms, whose expression differs among cell types. Although connexin-40, -43, and -45 are all expressed in rat ventricular cardiac myocytes, connexin43 (Cx43) is the primary subtype, and plays a principal role in transmitting electrical signals among the cells [17]. Recent studies, however, have demonstrated that connexins are involved in the regulation of a variety of cellular functions other than intercellular communication. We recently revealed that the knockdown of Cx43 using siRNA produced a significant increase in the proliferation of cardiomyocytes via suppression of the activity of p38 MAPK [31], suggesting that Cx43 is involved in the regulation of cell growth in cardiomyocytes. The activity of p38 MAPK and the expression of Cx43 seem to be functionally coupled, since Schulz et al. have suggested that Cx43 is co-localized to a signaling complex with p38 MAPK [42]. It has been revealed that the long C-terminus of Cx43 has many residues phosphorylated by kinases such as PKC, PKA, MAPK, and cdc2/cyclinB kinase [16]. In addition, the C-terminus of Cx43 directly and/or indirectly via ZO-1 interacts with cytoskeletons such as β-tubulin, a major component of microtubules, and filamentous actin [16]. The present study has suggested that the oxidative stress-induced formation of a positive-feedback pathway, ROS-p38 MAPK-mtCx43, resulted in a marked increase in the sarcolemmal and cytosolic expression of Cx43 as well. The enhanced interaction between the C-terminus of Cx43 and cytoskeletons seems to be one of the factors inhibiting cytokinesis in cardiomyocytes after birth.

In the present study, the ROS-p38 MAPK-mtCx43 pathway was involved in the persistent activation of p38 MAPK (Fig. 5). A previous study has revealed that the sustained activation of p38 MAPK, distinct from temporary cytokine- or stress-activated responses, is required for growth arrest and terminal differentiation of rhabdomyosarcoma cells [33]. In addition, a recent study demonstrated ROS-induced activation of p38 MAPK to be essential for the differentiation of murine embryonic stem cells into cardiomyocytes [8]. Engel *et al*. recently indicated that binucleation of cardiomyocytes is caused by a localization defect of Anillin, one of the crucial molecules responsible for cytokinesis [44], and that p38 MAPK activity has a regulatory role in cytokinesis, since the inhibition of p38 MAPK activity rescues the mid-body formation and cytokinesis failure [11]. It is generally accepted that appropriate and inappropriate production of ROS, together with the ability of organisms to respond to oxidative stress, is intricately connected to aging and life span [12]. Iwasa *et al*. reported that the ROS-induced activation of p38 continued even after the initial oxidative stress was withdrawn,

which may explain the irreversible nature of cellular senescence [21]. The cellular mechanisms responsible for such conditions are not known [21]. The positive-feedback loop described in the present study may provide a universal cell signaling mechanism for the sustained activation of p38 MAPK crucial to the terminal differentiation as well as senescence of the cells.

Acknowledgments

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan (21650103 & 22300148) to KK.

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Figure legends

Figure 1

Scavenging of ROS increased the proliferative activity, and decreased the ratio of binucleated cardiomyocytes in culture. Isolated cardiomyocytes from dams either with or without NAC treatment were cultured for 4 days. The treatment of cardiomyocytes from newborn hearts of NAC-treated animals with NAC (NAC+/c+) resulted in a significant increase in the proportion of PCNA-positive cardiomyocytes as compared with control (NAC–/c–), NAC–/c+, and NAC+/c– cardiomyocytes (A-C). White arrows in A and B show PCNA-positive nuclei of α-actinin-positive cardiomyocytes. C: n=7, mean ± SD, *p<0.05 vs. Control (Cont.). Figure D shows an increase in the number of cardiomyocytes at 4 days in vitro (DIV) on treatment with NAC (n=8, mean ± SD, *p<0.05 vs. Control). In addition, NAC treatment decreased the proportion of binucleated cardiomyocytes (E-H). In Figures E1-E4, yellow circles indicate binucleated cardiomyocytes. In phase-contrast images (F1-F4), black arrows indicate binucleated cardiomyocytes. White arrows in Figures G1-G4 show α-actinin-positive cardiomyocytes (red) with multiple nuclei stained with Hoechst (blue). H: Data are expressed as the mean ± SD (n=7). **p<0.01 vs. Control. Western blot analysis revealed that both the activity of p38 MAPK and the expression of Cx43 were significantly decreased in NAC+/c+ cardiomyocytes as compared with control (NAC–/c–) cardiomyocytes (G-I). H and I: Data are expressed as the mean ± SD (n=6). *p<0.05 vs. Control.

Figure 2

ROS involved in the activation of p38 MAPK and in the increased expression of Cx43. Western blot analysis revealed that both the activity of p38 MAPK and the expression of Cx43 were significantly decreased in NAC+/c+ cardiomyocytes as compared with control (NAC–/c–) cardiomyocytes (A1-A3). A2 and A3: Data are expressed as the mean ± SD (n=6). **p<0.01, *p<0.05 vs. Control. Cardiomyocytes were exposed to H_2O_2 (10 μM) for 4 days (B1-B3). Western blot analysis showed that treatment with H_2O_2 resulted in a significant increase in the activity of p38 MAPK and expression of Cx43, and the H_2O_2-induced increase in Cx43 was antagonized by co-treatment with SB203580 (SB), an inhibitor of p38 MAPK. Treatment with SB203580 under control condition also resulted in a decrease in p-p38 MAPK and Cx43. Data are expressed as the mean ± SD (n=8). **p<0.01, *p<0.05 vs. Control; $^{\#}p$<0.05 vs. H_2O_2

treatment.

Figure 3

Cx43-knockdown-induced decrease in the production of ROS in cardiomyocytes. Figure A illustrates changes in H_2DCF-DA fluorescence with time when cardiomyocytes were exposed to H_2O_2 (10 μM). A4: Data are expressed as the mean ± SD (n=16 cardiomyocytes from 2 cultures). Figure B shows changes in H_2DCF-DA fluorescence in the control cultures. The fluorescence did not change significantly for 6 hours (B5). Data are expressed as the mean ± SD (n=37 cardiomyocytes from 2 cultures). Cx43 siRNA significantly decreased ROS production (C, n=62 cardiomyocytes from 3 cultures; $^{**}p<0.01$), but scrambled RNA did not (D, n=54 cardiomyocytes from 2 cultures).

Figure 4

Inhibition of p38 MAPK activity decreased the production of ROS in cardiomyocytes. The cultures were treated with SB203580 (10 μM), an inhibitor of p38 MAPK. SB203580 significantly decreased ROS production in cardiomyocytes (B5). Data are expressed as the mean±SD (n=63 cardiomyocytes from 2 cultures, $^{**}p<0.01$).

Figure 5

ROS-induced sustained increase in the activity of p38 MAPK. Western blot analysis demonstrated that the H_2O_2-induced increase in the activity of p38 MAPK persisted for more than 2 days after the washout of H_2O_2 (B). Data are expressed as the mean±SD (n=7, $^{*}p<0.05$ vs. Control). Abbreviation: WO, washout.

Figure 6

Exposure of cardiomyocytes to ROS increased the mitochondrial expression of Cx43. Western blot analysis was performed to confirm the purity of mitochondrial extracts (A). Treatment of cardiomyocytes with H_2O_2 (10 μM) increased the expression of mtCx43, and the effect was reversed by co-treatment with SB203580, an inhibitor of p38 MAPK (B). Data are expressed as the mean±SD (n=7, $^{*}p<0.05$ vs. Control). Cx43 siRNA significantly reduced mtCx43 expression (C). Data are expressed as the mean±SD (n=7, $^{*}p<0.05$ vs. Control). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANT, adenine nucleotide translocase; SB, SB203580 (10 μM).

Figure 7

Reduced expression of mitochondrial Cx43 decreased the production of ROS in cardiomyocytes. The treatment of cardiomyocytes with geldanamycin (5 μM), an inhibitor of Hsp90, for 15 min significantly reduced mtCx43 expression (A). The treatment also reduced total Cx43 levels, although not significantly. Data are expressed as the mean±SD (n=5, *p<0.05 vs. Control). In addition, treatment with geldanamycin (5 μM) suppressed the production of ROS (B). Data are expressed as the mean±SD (n=39 cardiomyocytes from 2 cultures, *p<0.05). The treatment also decreased the activity of p38 MAPK activity (C). Data are expressed as the mean±SD (n=5, *p<0.05 vs. Control).

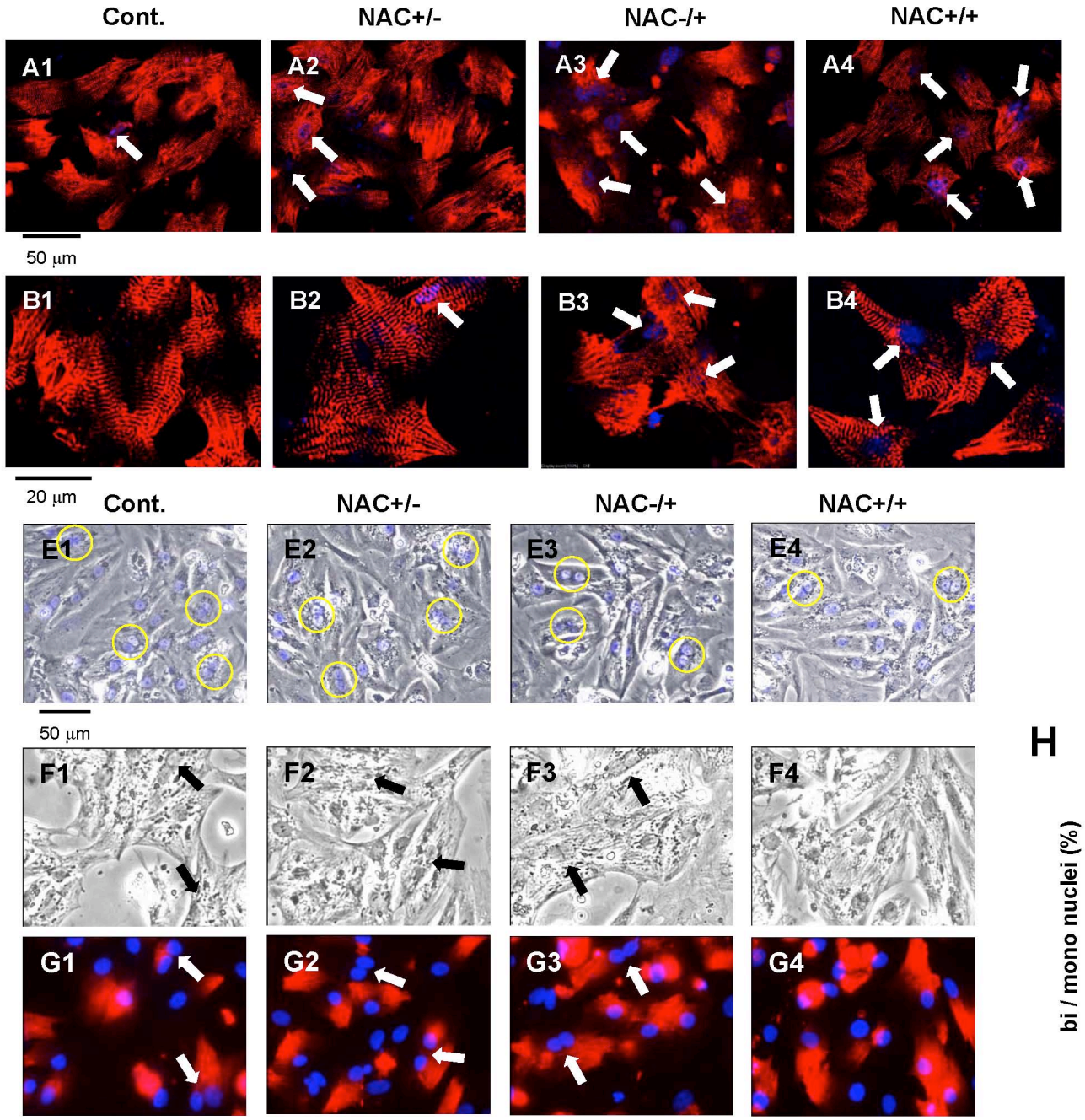
Figure 8

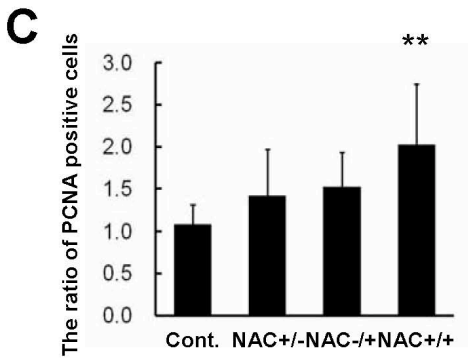
Continuous scavenging of ROS reduced the expression of mitochondrial Cx43, the activity of p38 MAPK, and binucleated cardiomyocytes in newborns at 4 days after birth. A Western blot analysis was done with heart tissue 4 days after birth (A-E). Continuous scavenging of ROS for 4 days in newborns from NAC-treated (NAC+/+) dams resulted in a significant decrease in mtCx43, total Cx43 levels, and in the activity of p38 MAPK. Data are expressed as the mean±SD (n=7, *p<0.05 or **p<0.01 vs. Control). In addition, NAC treatment significantly reduced the number of binucleated cardiomyocytes in NAC+/+ rats at 4 days after birth (G-J). Cells indicated by yellow circles in G1and G2 show binucleated cardiomyocytes. The α-actinin-positive binucleated cardiomyocytes are indicated by white arrows in I1. Data are expressed as the mean±SD (n=7, **p<0.01 vs. Control). The ratio of heart weight to body weight (HW/BW) in NAC+/+ animals was significantly greater than control animals (F3). Data are expressed as the mean±SD (n=12, *p<0.05, **p<0.01 vs. Control).

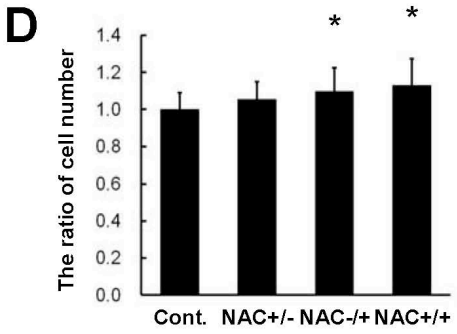
Figure 9

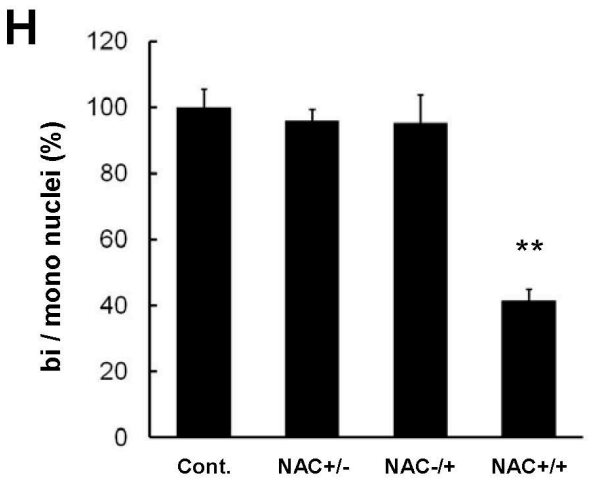
Continuous suppression of the activity of p38 MAPK reduced the expression of mitochondrial Cx43, the activity of p38 MAPK, and binucleated cardiomyocytes in newborns at 4 days after birth. A Western blot analysis was done with heart tissue 4 days after birth (A-E). Continuous suppression of the activity of p38 MAPK with intraperitoneal injection of SB203580 for 4 days in newborns resulted in a significant decrease in mtCx43, total Cx43 levels, and in the activity of p38 MAPK. Data are expressed as the mean±SD (n=6, *p<0.05 or **p<0.01 vs. Control). In addition, SB203580 treatment significantly reduced the number

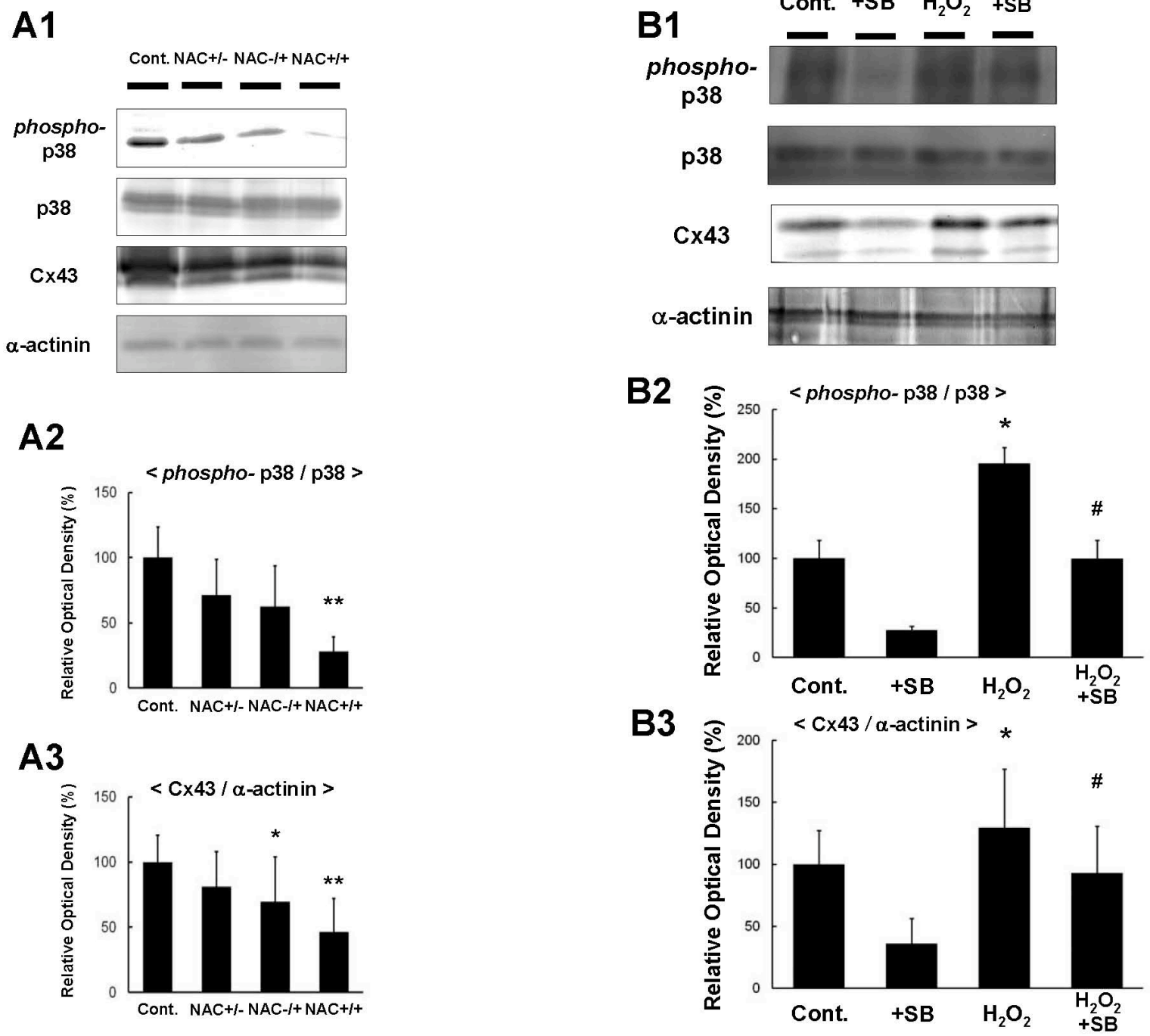
of binucleated cardiomyocytes at 4 days after birth (F). Cells indicated by yellow circles in F1 and F2 show the α-actinin-positive binucleated cardiomyocytes. Data are expressed as the mean±SD (n=8, $**p<0.01$ vs. Control). The ratio of heart weight to body weight (HW/BW) in SB203580-treated animals was significantly greater than control animals (G3). Data are expressed as the mean±SD (n=14, $*p<0.05$, $**p<0.01$ vs. Control).

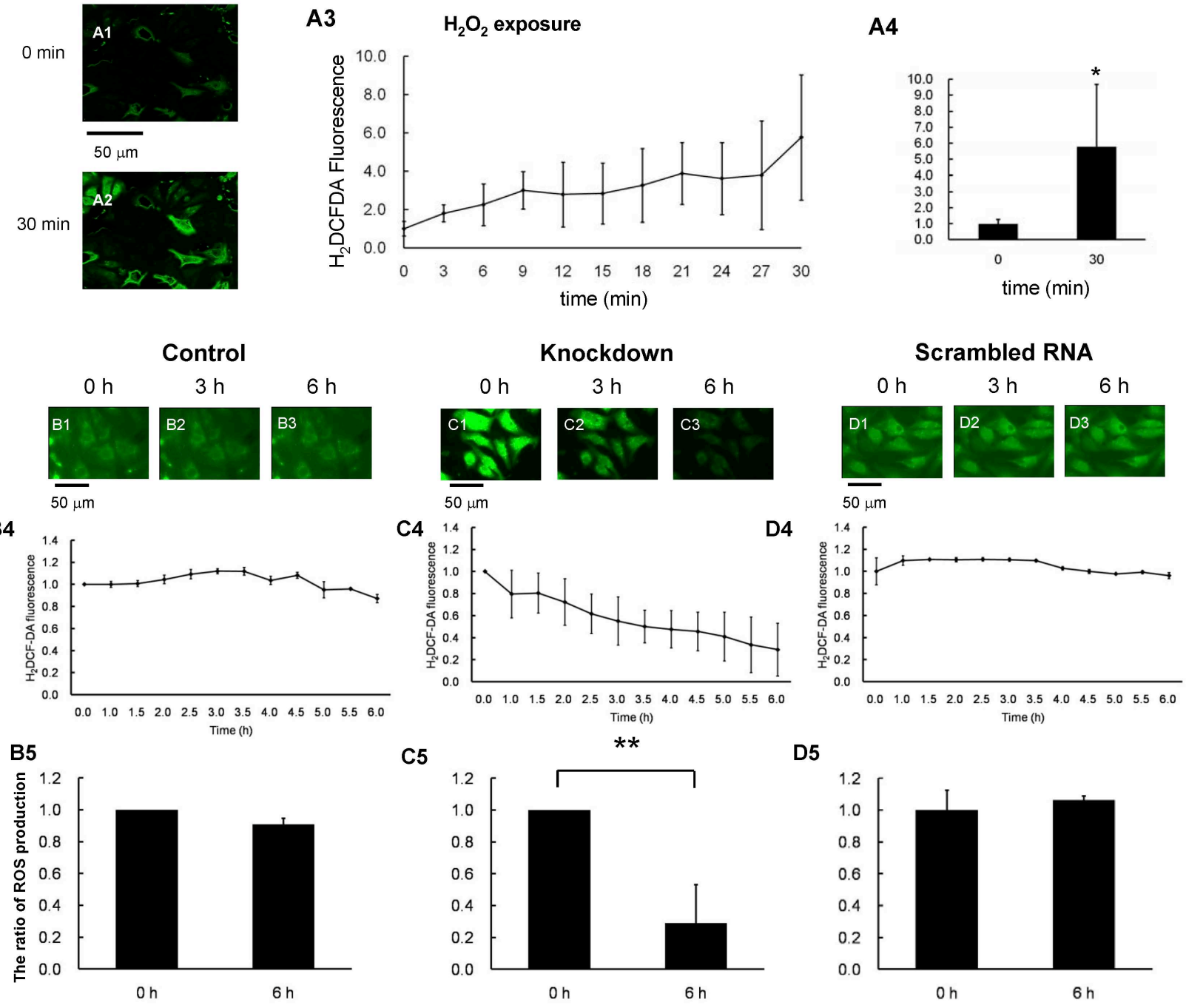

Cont.
NAC+/-
NAC-/+
NAC+/+
A1
A2
A3
A4
50 μm
B1
B2
B3
B4
20 μm
Cont.
NAC+/-
NAC-/+
NAC+/+
E1
E2
E3
E4
50 μm
F1
F2
F3
F4
G1
G2
G3
G4
25 μm


C
The ratio of PCNA positive cells
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Cont.
NAC+/-
NAC-/+
NAC+/+


D
The ratio of cell number
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Cont.
NAC+/-
NAC-/+
NAC+/+


H
bi / mono nuclei (%)
120
100
80
60
40
20
0
**
Cont.
NAC+/-
NAC-/+
NAC+/+


A1
Cont. NAC+/- NAC-/+ NAC+/+
phospho-p38
p38
Cx43
α-actinin
A2
< phospho- p38 / p38 >
Relative Optical Density (%)
150
100
50
0
**
Cont. NAC+/- NAC-/+ NAC+/+
A3
< Cx43 / α-actinin >
Relative Optical Density (%)
150
100
50
0
*
**
Cont. NAC+/- NAC-/+ NAC+/+
B1
Cont. +SB H2O2 H2O2 +SB
phospho-p38
p38
Cx43
α-actinin
B2
< phospho- p38 / p38 >
Relative Optical Density (%)
250
200
150
100
50
0
*
#
Cont. +SB H2O2 H2O2 +SB
B3
< Cx43 / α-actinin >
Relative Optical Density (%)
200
150
100
50
0
*
#
Cont. +SB H2O2 H2O2 +SB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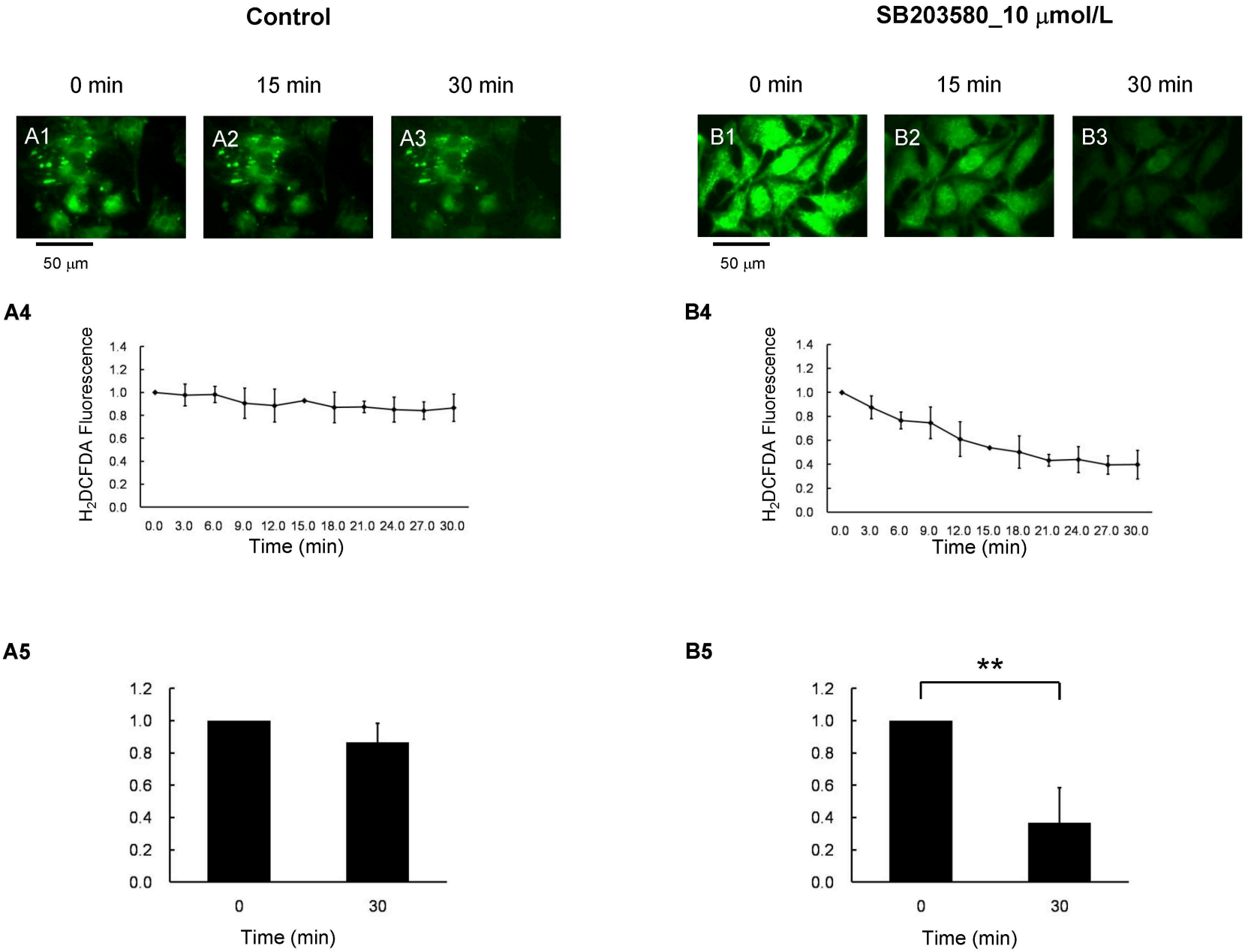
0 min
50 μm
A2
30 min
A3
H2O2 exposure
H2DCFDA Fluorescence
time (min)
A4
*
time (min)
Control
Knockdown
Scrambled RNA
0 h
3 h
6 h
B1
B2
B3
C1
C2
C3
D1
D2
D3
50 μm
B4
C4
D4
H2DCF-DA fluorescence
Time (h)
B5
C5
D5
**
The ratio of ROS production
0 h
6 h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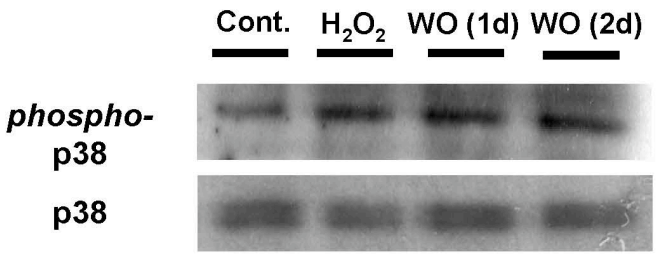
SB203580_10 μmol/L
0 min
15 min
30 min
A1
A2
A3
B1
B2
B3
50 μm
50 μm
A4
B4
H2DCFDA Fluorescence
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0 3.0 6.0 9.0 12.0 15.0 18.0 21.0 24.0 27.0 30.0
Time (min)
A5
B5
**
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
30
Time (min)

A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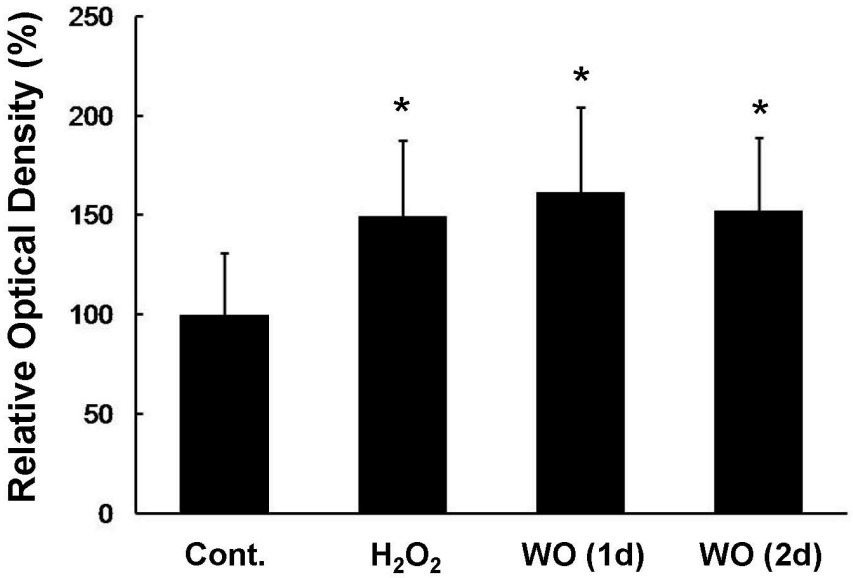
WO (2d)
phospho-p38
p38

B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*
*
*
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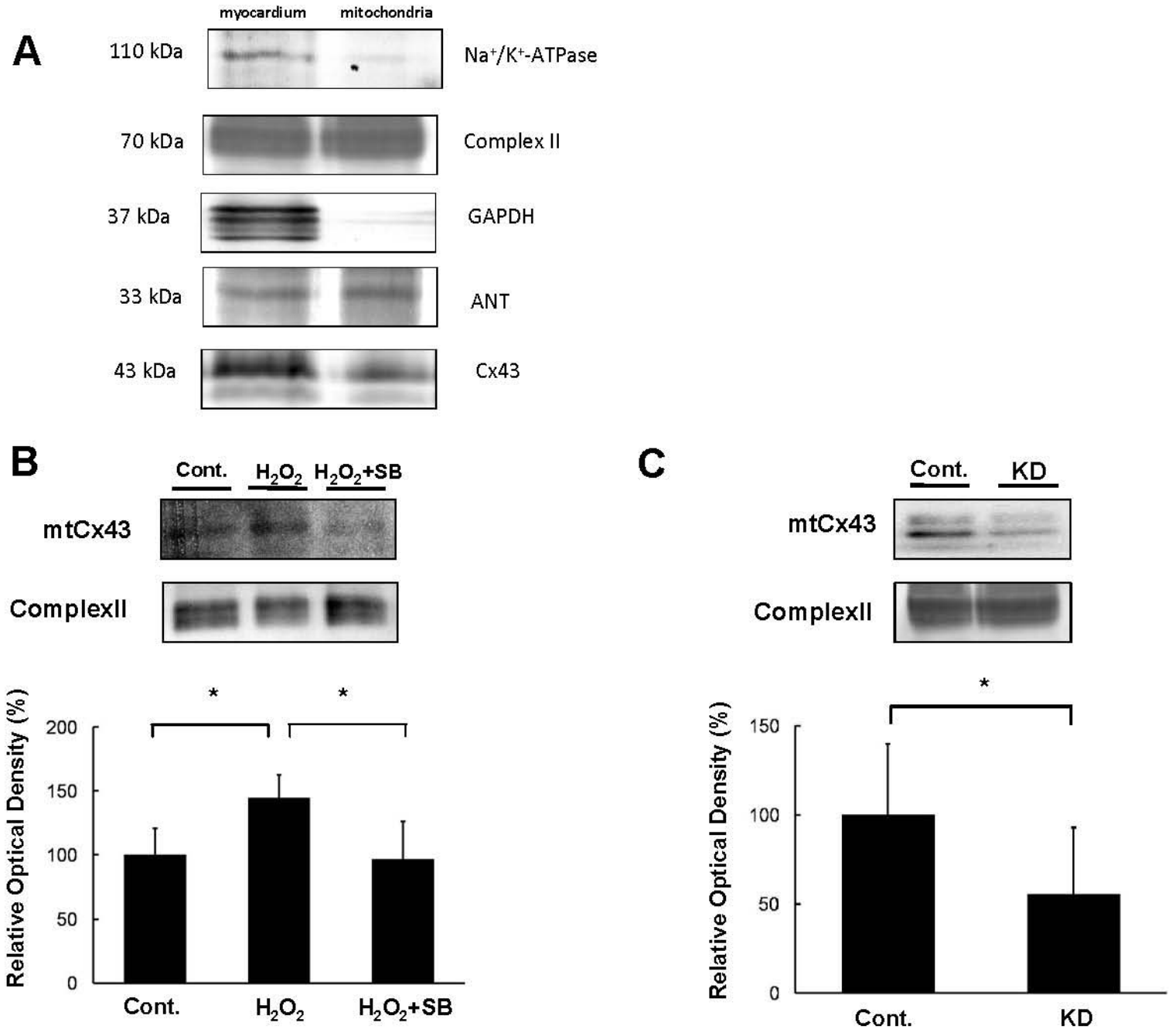
WO (1d)
WO (2d)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mitochondria
110 kDa
Na+/K+-ATPase
70 kDa
Complex II
37 kDa
GAPDH
33 kDa
ANT
43 kDa
Cx43
B
Cont.
H2O2
H2O2+SB
mtCx43
ComplexII
Relative Optical Density (%)
200
150
100
50
0
*
*
Cont.
H2O2
H2O2+SB
C
Cont.
KD
mtCx43
ComplexII
Relative Optical Density (%)
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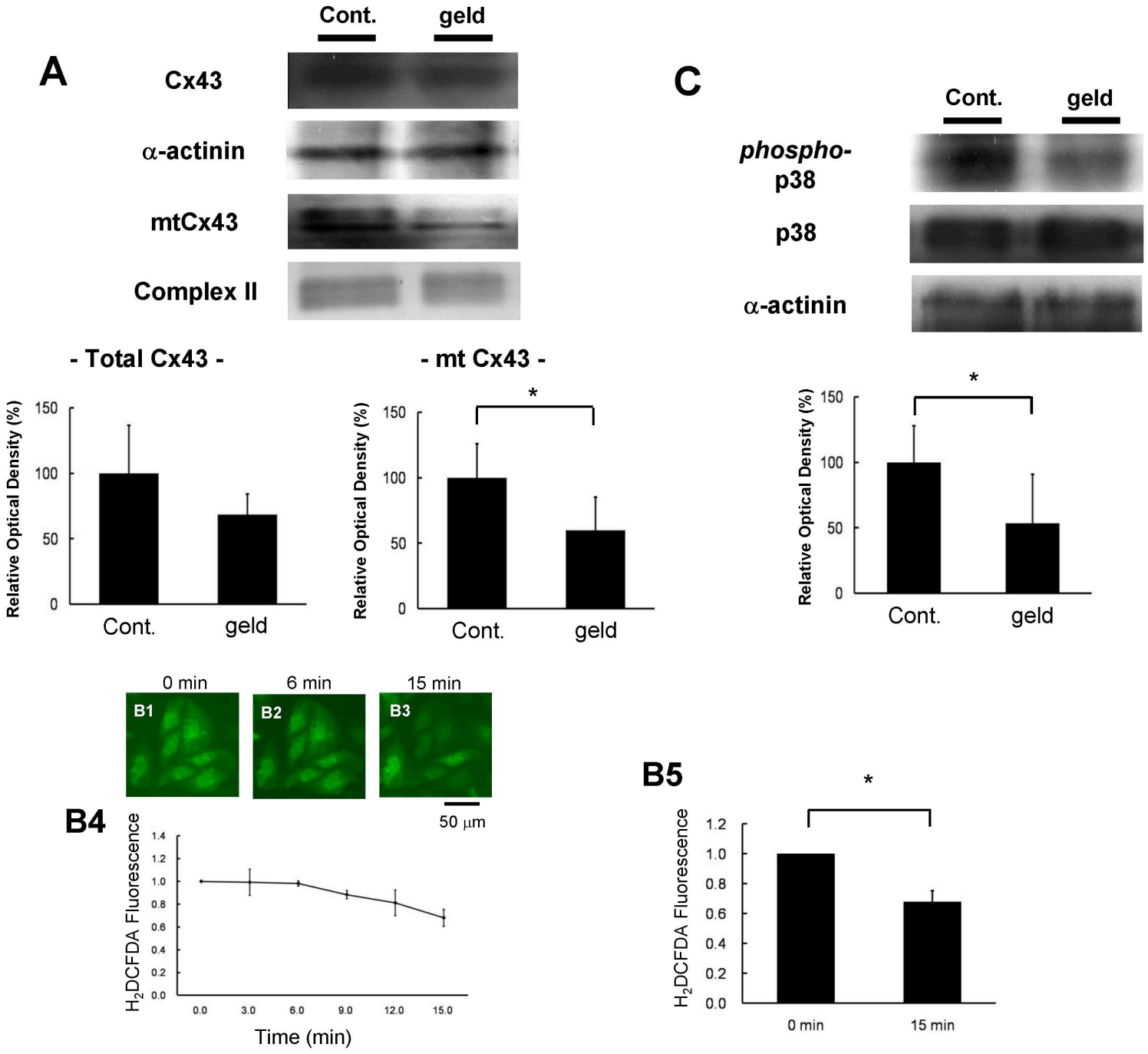
50
0
*
Cont.
KD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Cont.
geld
Cx43
α-actinin
mtCx43
Complex II
- Total Cx43 -
Relative Optical Density (%)
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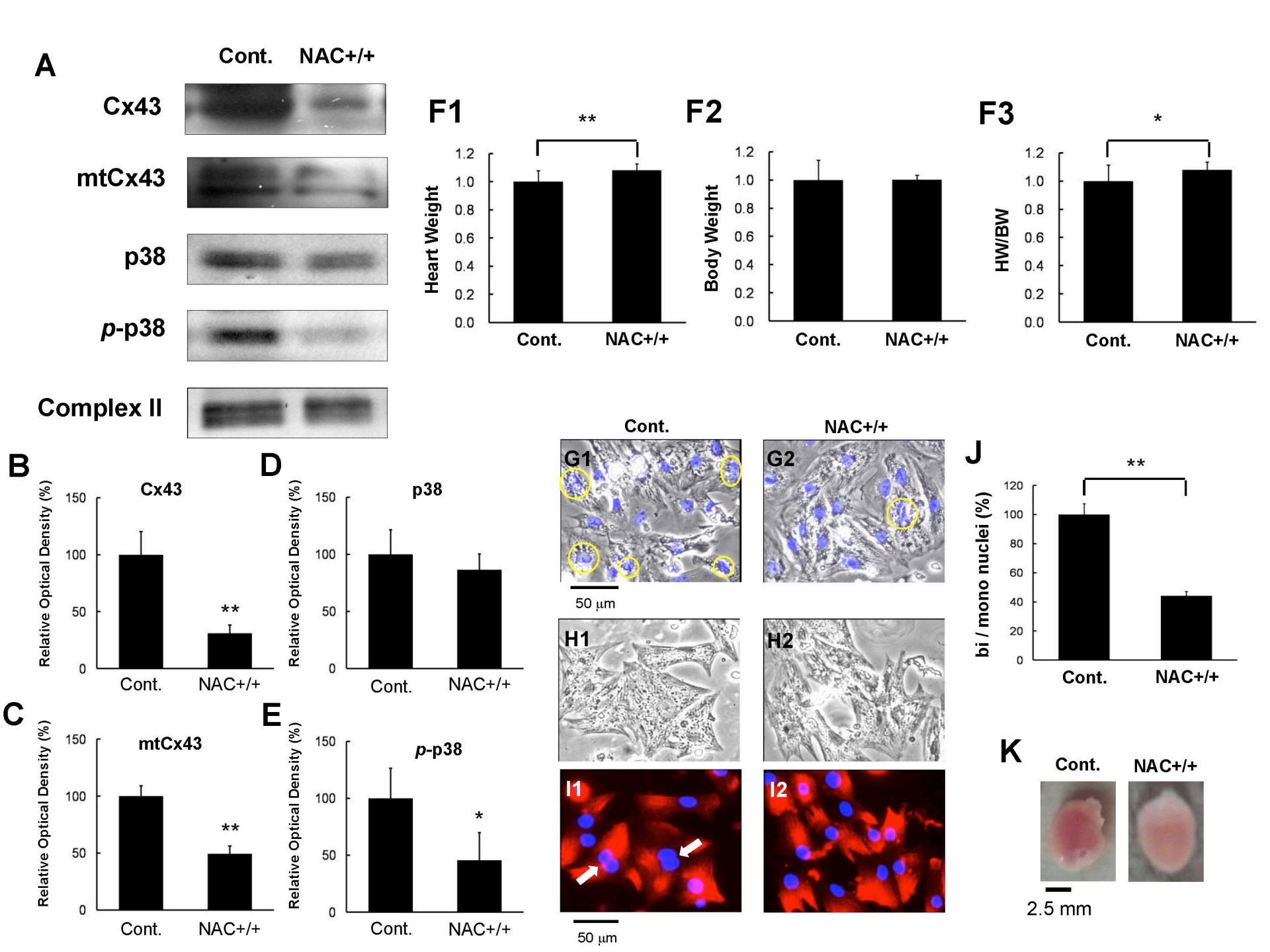
100
50
0
Cont.
geld
- mt Cx43 -
*
C
phospho-p38
p38
α-actinin
B1
B2
B3
0 min
6 min
15 min
50 μm
B4
H2DCFDA Fluorescence
Time (min)
B5
0 min
15 min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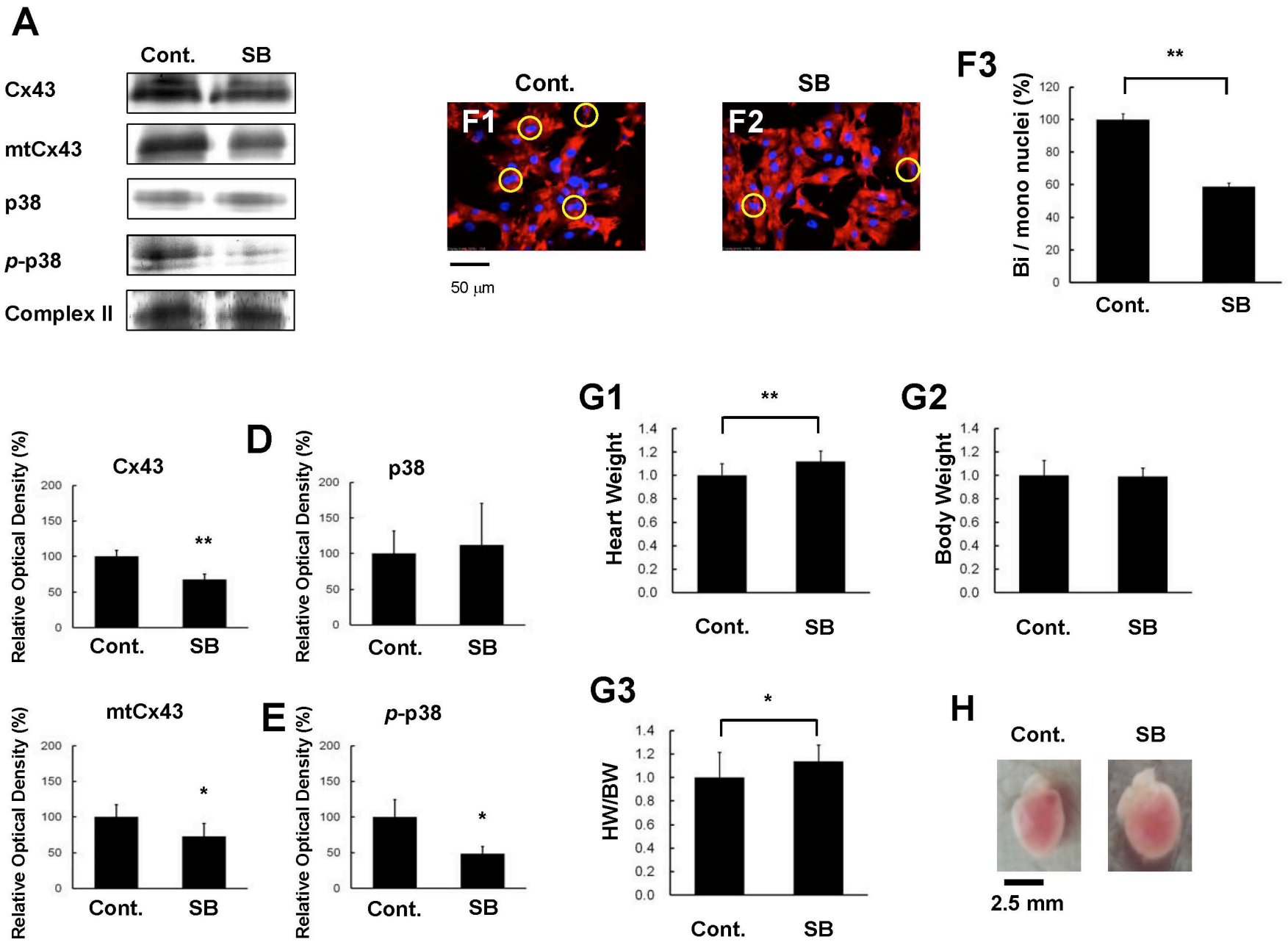
Cont.
NAC+/+
Cx43
mtCx43
p38
p-p38
Complex II
F1
**
Heart Weight
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
NAC+/+
F2
Body Weight
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
NAC+/+
F3
*
HW/BW
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
NAC+/+
B
Cx43
Relative Optical Density (%)
150
100
50
0
**
Cont.
NAC+/+
D
p38
Relative Optical Density (%)
150
100
50
0
Cont.
NAC+/+
C
mtCx43
Relative Optical Density (%)
150
100
50
0
**
Cont.
NAC+/+
E
p-p38
Relative Optical Density (%)
150
100
50
0
*
Cont.
NAC+/+
Cont.
NAC+/+
G1
G2
50 μm
H1
H2
I1
I2
50 μm
J
**
bi / mono nuclei (%)
120
100
80
60
40
20
0
Cont.
NAC+/+
K
Cont.
NAC+/+
2.5 mm


A
Cont.
SB
Cx43
mtCx43
p38
p-p38
Complex II
Cont.
SB
F1
F2
50 μm
F3
**
Bi / mono nuclei (%)
120
100
80
60
40
20
0
Cont.
SB
B
Cx43
Relative Optical Density (%)
200
150
100
50
0
**
Cont.
SB
D
p38
Relative Optical Density (%)
200
150
100
50
0
Cont.
SB
G1
**
Heart Weight
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
SB
G2
Body Weight
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
SB
C
mtCx43
Relative Optical Density (%)
200
150
100
50
0
*
Cont.
SB
E
p-p38
Relative Optical Density (%)
200
150
100
50
0
*
Cont.
SB
G3
*
HW/BW
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont.
SB
H
Cont.
SB
2.5 mm