2-aminoethoxydiphenyl borate provides an anti-oxidative effect and mediates cardioprotection during ischemia reperfusion in mice

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Abstract

Excessive levels of reactive oxygen species (ROS) and impaired Ca²⁺ homeostasis play central roles in the development of multiple cardiac pathologies, including cell death during ischemia-reperfusion (I/R) injury. In several organs, treatment with 2-aminoethoxydiphenyl borate (2-APB) was shown to have protective effects, generally believed to be due to Ca²⁺ channel inhibition. However, the mechanism of 2-APB-induced cardioprotection has not been fully investigated. Herein we investigated the protective effects of 2-APB treatment against cardiac pathogenesis and deciphered the underlying mechanisms. In neonatal rat cardiomyocytes, treatment with 2-APB was shown to prevent hydrogen peroxide (H₂O₂)-induced cell death by inhibiting the increase in intracellular Ca²⁺ levels. However, no 2-APB-sensitive channel blocker inhibited H₂O₂-induced cell death and a direct reaction between 2-APB and H₂O₂ was detected by ¹H-NMR, suggesting that 2-APB chemically scavenges extracellular ROS and provides cytoprotection. In a mouse I/R model, treatment with 2-APB led to a considerable reduction in the infarct size after I/R, which was accompanied by the reduction in ROS levels and neutrophil infiltration, indicating that the anti-oxidative properties of 2-APB plays an important role in the prevention of I/R injury in vivo as well. Taken together, present results indicate that 2-APB treatment induces cardioprotection and prevents ROS-induced cardiomyocyte death, at least partially, by the direct scavenging of extracellular ROS. Therefore, administration of 2-APB may represent a promising therapeutic strategy for the treatment of ROS-related cardiac pathology including I/R injury.
Introduction

Cardiovascular diseases (CVDs) are the leading cause of death and disability worldwide. One of the most dominant causes of death by CVD is myocardial infarction (MI), which is accompanied by massive cardiomyocyte death. While recent progress in clinical cardiology led to a successful reduction of MI size by reperfusion therapies, the size-limiting effects are substantially lessened by ischemia-reperfusion (I/R) injury, due to the cellular damage caused by the restoration of coronary blood flow [1]. Therefore, novel therapeutic approaches for preventing myocardial I/R are required.

Whereas the mechanisms underlying I/R injury and myocardial protection have been intensively investigated during the last three decades [2, 3], the clinical efficacy of few pharmacological therapies have been demonstrated [4]. Two major intracellular second messengers, Ca^{2+} and reactive oxygen species (ROS), are known to play dominant roles in cardiac I/R injury [5, 6]. In the first several minutes of myocardial reperfusion, a burst of oxidative stress is induced from a variety of sources [7] [8], and leads to apoptotic and non-apoptotic cell death [9]. Animal model research indicated that the overproduction of ROS may be responsible for I/R injury [10, 11], but the results of clinical studies with the administration of antioxidant therapy at the onset of myocardial reperfusion have been mixed [12]. Therefore, further mechanisms underlying cardiac I/R or novel tools for the regulation of ROS should be investigated.

2-aminoethoxydiphenyl borate (2-APB) is a membrane-permeant, lipophilic compound that was originally reported as a blocker of intracellular inositol 1,4,5-trisphosphate receptor (IP$_3$R) [13, 14]. Currently, increasing evidence indicates that 2-APB has inhibitory effects on multiple ion channels such as transient receptor potential (TRP) family of cation channels, potassium channels [15], and volume-regulated anion channels [16]. Multiple members of TRP channels were shown to be sensitive to 2-APB, including TRPC3, TRPC5, TRPC6, TRPM2, TRPM3, and TRPM7 [17–22]. Therefore, the major biological function of 2-APB is generally considered to be based on channel inhibitory effect.

Reduced I/R injury by 2-APB treatment has been reported in multiple organs with promising results [23–25]. Therefore, we hypothesized that 2-APB may exert a protective effect during cardiac I/R injury through a channel inhibitory effect and assessed the effects of 2-APB administration on ROS-induced cardiomyocyte death or cardiac I/R injury in mice. Unexpectedly, we found that these protective effects are based on direct ROS inhibition, not channel blocking effect, in cardiomyocytes. The obtained results indicate that ROS scavenging by 2-APB may be a promising therapeutic approach in cardiac I/R injury.

Materials and methods

Animal care

The care of all animals complied with the Osaka University animal care guidelines. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals, promulgated by the US National Institutes of Health, and were approved by Animal Care and Use Committee in Graduate School of Pharmaceutical Sciences, Osaka University (approved as Douyaku 26–4, the animal experimental protocol number 26–4 of Graduate School of Pharmaceutical Sciences). All animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition, updated by the US National Research Council Committee in 2011. A total of 108 male mice underwent surgical procedures in this study, and all experimental protocols were completed within 24 h following the surgery. All efforts were made to minimize suffering and distress of the animals. Humane endpoints were used for all mice involved in the study and once animals reached endpoint criteria including cachexic...
condition evaluated by body weight loss (more than 10%), loss of voluntary motion and failure to react to stimuli, they are euthanized by inhalation of isoflurane in a euthanasia chamber as soon as possible. Death of the animals was confirmed by monitoring the absence of breath after the removal of the carcass from the euthanasia chamber. No animals died before meeting criteria for euthanasia except those that died of causes related to surgical procedures, such as bleeding.

Reagents
Reagents used in this study included H$_2$O$_2$ (31 wt%; Santoku Chemical Industries Co, Japan), BAPTA-AM (Merck Millipore, Billerica, MA), xestospongin C (Wako Pure Chemical Industries, Japan), SKF-96365 (Focus Biomolecules, Plymouth Meeting, PA), mefenamic acid (Tokyo Chemical Industry Co, Japan), 2-APB, AA-861 and phenylephrine (Sigma-Aldrich, St. Louis, MO).

Neonatal rat cardiomyocyte (NRCM) isolation
Neonatal rats were purchased from Kiwa Laboratory Animals Co.Ltd. NRCMs were isolated and cultured as previously described [26]. In brief, hearts excised from neonatal rats were minced and digested with a solution containing 0.1% collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.1% trypsin (Thermo Fisher Scientific, Waltham, MA) to obtain a suspension of single cells. After incubating the cells in a culture dish for 60–90 min at 37˚C in 5% CO$_2$ atmosphere, floating cells were collected as NRCMs. Isolated NRCMs were cultured in Dulbecco’s Modified Eagles Medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing high glucose (4.5 g/L) with L-glutamine (4 mM) and sodium bicarbonate (3.7 g/L), supplemented with 10% fetal bovine serum (FBS) and bromodeoxyuridine (0.1 μg/mL; Sigma-Aldrich, St. Louis, MO).

Cell viability assays
The isolated NRCMs were cultured in a serum-free medium for 24 h and were pretreated with 2-APB, specific 1,4,5-Inositol trisphosphate receptor blocker (xestospongin C), TRP channel blocker (SKF-96365), TRPM7 blocker (AA-861) or TRPM3 blocker (mefenamic acid), 1 h before the addition of H$_2$O$_2$. An equal volume of vehicles (i.e., ethanol for xestospongin C and DMSO for SKF-96365, AA-861, mefenamic acid) was added as a control 1 h before the addition of H$_2$O$_2$. Four hours after the addition of H$_2$O$_2$, cell viability was measured using the Cell-Titer Blue assay kit (Promega, Madison, WI), according to the manufacturer’s protocol. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent product (resorufin). The cells in 96-well culture plate were incubated with 20 μL of CellTiter Blue reagent for 3 h at 37˚C in a humidified, 5% CO$_2$ atmosphere. The fluorescence was measured using a 96-well plate reader (SpectraMAX M5e, excitation at 560 nm and emission at 590 nm; Molecular Devices, Sunnyvale, CA).

Intracellular calcium measurements
To elucidate the underlying mechanism of Ca$^{2+}$ chelator mediated inhibition in H$_2$O$_2$-induced cardiomyocyte death, intracellular calcium levels were monitored as previously reported with modifications [27]. NRCMs were cultured on a flexiPERM disc (Sarstedt, Germany) using DMEM with 10% FBS. The cells were washed with Hank’s balanced salt solution (HBSS) containing 1.26 CaCl$_2$, 0.493 MgCl$_2$, 0.407 MgSO$_4$, 5.33 KCl, 0.441 KH$_2$PO$_4$, 4.17 NaHCO$_3$, 137.93 NaCl, 0.338 Na$_2$HPO$_4$ and 5.56 D-glucose (in mM) and incubated in 10 μM Fura
2-AM (Sarstedt, Germany) /HBSS working solution for 1 h. Subsequently, cells were washed with HBSS three times, which was followed with the incubation for 30 min, and the resulting fluorescence was detected by AQUA COSMOS (Hamamatsu Photonics, Japan) monitoring the excitation spectra at 340 or 380 nm with fixed emission at 505 nm. The fluorescence ratio of 340/380 nm excitation was calculated to assess intracellular Ca\(^{2+}\) concentration.

**Intracellular ROS measurements**

Intracellular ROS levels were determined using the fluorescent marker 2',7'-dichlorofluorescin diacetate (DCFH-DA, D6883; Sigma-Aldrich, St. Louis, MO), according to the manufacturer’s protocol. Briefly, NRCMs were cultured in 96-well culture plate and incubated with 5 μM DCFH-DA for 30 min at 37˚C, and they were washed twice with the phenol red-free DMEM (Sigma-Aldrich, St. Louis, MO). After the pretreatment with/without 2-APB for 1 h, the cells were stimulated with 100 μM H\(_2\)O\(_2\) or 2 mM phenylephrine (PE) and fluorescence intensity was measured using a 96-well plate reader (SpectraMAX M5e, excitation at 485 nm and emission at 590 nm; Molecular Devices, Sunnyvale, CA) to assess the increase in the intracellular ROS levels. Based on DCF fluorescent intensity levels, the ROS increase ratio was calculated as difference from values of those at 0 min and represented as %Fluorescence increase which is normalized to those at 60 min of vehicle-treated control.

**\(^1\)H-NMR measurements and thin-layer chromatography**

To examine the direct interaction between 2-APB and H\(_2\)O\(_2\), \(^1\)H-NMR measurement and thin-layer chromatography were performed [28] [29]. Five milligrams of 2-APB (0.0222 mmol) were dissolved in methanol-d\(_4\) (0.6 mL) in an NMR tube at room temperature, and the mixture was subjected to \(^1\)H-NMR analysis to obtain an authentic spectrum (spectrum A). H\(_2\)O\(_2\) (31% w/v, 25 μL, 0.222 mmol) was then added to this mixture. After shaking for a few seconds, the mixture was subjected to the \(^1\)H-NMR analysis and the second spectrum was recorded in 5 min (spectrum B). After 60 min, the mixture was analyzed to obtain the third spectrum (spectrum C). All \(^1\)H-NMR spectra were recorded on a 300 MHz JEOL AL-300 spectrometer in the deuterated solvent, and the chemical shifts were reported with a residual peak of CD\(_2\)HOD as an internal standard. The reaction products were further examined by thin-layer chromatography (TLC) using TLC plates (TLC silica gel 60 F\(_{254}\); Merck Millipore, Billerica, MA).

**I/R model and 2-APB treatment in mice**

Murine I/R model was generated following the protocol which we have established previously [30]. Briefly, C57BL/6 male mice (7–9 weeks old; Japan SLC) were anesthetized using 1.5% isoflurane (Pfizer, New York City, NY) carried in 100% oxygen followed by the intubation, and they were ventilated with 100% oxygen containing 1.5% isoflurane. After left-side thoracotomy, 7–0 silk suture was tied around the left coronary artery with a slipknot, which can be released by pulling one end coming out from the body. Infarction was confirmed by the discoloration of the ventricle. The chest and the skin were closed with 5–0 silk sutures. Sham operation was performed as a control. The mice were revived during the 30-min ischemic period, and anesthetized shortly at the end of ischemia to release the slipknots, and the heart was allowed to reperfuse for 24 h. Using this experimental protocol, 27 out of 32 mice survived after the surgical procedure and the mortality was minimized to less than 20%. Twenty-four hours after the reperfusion, the mice were euthanized and the slipknot was retied. Evans blue (5%; Wako Pure Chemical Industries, Japan) was injected into the left ventricle (LV), and the hearts were removed. Isolated hearts were sectioned, and viable myocardium was stained with
2% triphenyl tetrazolium chloride (Nacalai Tesque, Japan), as described previously [30]. Myocardial area not at risk, area at risk (AAR), and infarcted area (IA) were quantified using the ImageJ Analysis software (National Institutes of Health). In the 2-APB group, 10 mg/kg of 2-APB were intravenously administered immediately before the reperfusion (indicated concentrations in 125 μL of saline / 25 g of body weight), whereas the control group received the same volume of saline containing vehicle over the same period. There was no difference in mortality between two groups.

**Quantitative RT-PCR**

To assess the expression levels of proinflammatory cytokines that are involved in the recruitment of the inflammatory cells that produce ROS, quantitative RT-PCR was performed according to the manufacturer’s instructions. Hearts were obtained from mice that underwent 30 min ischemia followed by reperfusion with the period of 30 min or 6 h. Using this protocol, 31 out of 40 mice survived after the surgical procedure. Total RNA was isolated from hearts after I/R using QIAzol reagent (QIAGEN, Germantown, MD). First-strand cDNA was synthesized from 1 μg of total RNA with oligo (dT) primers and then used to determine the expression of the genes of interest. The mRNA expression for il-6, il-1β and tnf-α in the LVs from the ligation point to the apex was quantified by real-time RT-PCR using the Applied Biosystems StepOne Real-Time PCR systems with the SYBR Green system (Applied Biosystems, Carlsbad, CA). As an internal control, the expression of Gapdh was estimated using the SYBR Green system. Primer sequences are displayed in S1 Table.

**Immunohistochemical analyses**

For the immunohistochemical analysis, hearts were obtained from mice that underwent 30 min ischemic procedure followed by 24 h reperfusion. Hearts were embedded in O.C.T. Compound (Sakura Finetech, Japan), frozen in liquid nitrogen, and cut into 5 μm-thick sections using Leica CM1950 (Leica Biosystems, Germany). Immunohistochemical analyses were performed using the indirect immune-peroxidase method. After the inhibition of endogenous peroxidase activity, the sections were incubated overnight followed by incubation with the primary antibody such as anti-Ly-6G for neutrophils (Gr-1) (1:100) (clone 1A8; BioLegend, San Diego, CA). An appropriate immunoglobulin-conjugated peroxidase-labeled polymer amino acid (anti-rat IgG; Vector Laboratories, Burlingame, CA) was used as a secondary antibody. After visualization with 3,3’-diaminobenzidine, the sections were mounted with Entellan™ New (Merck Millipore, Billerica, MA). Staining was examined using FSX100 Inverted Microscope (Olympus, Hickersville, NY). The number of Gr-1-positive cells in the AAR was counted in five random fields in three nonconsecutive sections per heart by a researcher who was blinded to the experimental conditions and displayed as a number per mm².

**Dihydroethidium (DHE) fluorescence analysis**

DHE fluorescence analysis was performed to examine the generation of superoxide according to a previous report [30]. In brief, frozen sections (5 μm-thick) were prepared from hearts exposed to ischemia for 30 min and reperfused for 24 h, and stained with 10 μM DHE (Sigma-Aldrich, St. Louis, MO) in Krebs-HEPES buffer composed of (all in mM) 99.01 NaCl, 4.69 KCl, 1.87 CaCl2, 1.20 MgSO4, 1.03 K2HPO4, 25.0 NaHCO3, 20.0 Na-HEPES, and 11.1 glucose (pH 7.4) at 37˚C for 30 min in dark. The intensities of fluorescence were quantified using ImageJ Analysis software (National Institutes of Health) by a researcher who was blinded to the assay conditions.
Oxidative stress biomarker determination *in vivo*

To corroborate the involvement of ROS in 2-APB-induced cardioprotection *in vivo* further, oxidative stress levels were assessed in mice. The levels of diacron reactive oxygen metabolites (d-ROMs) and biological anti-oxidant potential (BAP) were measured using a free radical analyzer system (FREE Carrio Duo; Diacron International, Italy), as previously reported [31]. All analyses were performed using animal serum samples collected via inferior vena cava of mice exposed to ischemia for 30 min and reperfused for 30 min or 6 h. Using this experimental protocol, 19 out of 24 mice survived after the I/R surgical procedure. We chose those timepoints as an immediate period after reperfusion or a beginning phase of neutrophil infiltration. The d-ROMs test shows the amount of organic hydroperoxides, which corresponds to 0.8 mg/L of \( \text{H}_2\text{O}_2 \) per unit. The BAP test provides an estimate of the global anti-oxidant capacity of serum, by measuring the reduced ferric ion levels. BAP/d-ROM ratio was determined as a marker of relative anti-oxidant capacity.

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM). Comparisons between the two groups were performed with Student's t-test. One-way analysis of variance (ANOVA) followed by Tukey-Kramer test was used for multiple comparisons. Differences were considered statistically significant when the calculated (two-tailed) \( P \) value was <0.05.

**Results**

**Treatment with 2-APB suppresses ROS-induced cell death and intracellular \( \text{Ca}^{2+} \) increase in NRCMs**

First, we examined the effects of 2-APB treatment on ROS-induced cell death in NRCMs. Stimulation with \( \text{H}_2\text{O}_2 \) induced massive cell death 4 h following the administration, which was considerably decreased by pretreatment with 2-APB (Fig 1A). Treatment with 2-APB alone failed to induce cell death at a dose of 100 \( \mu \)M. Cell viability assay demonstrated that the pretreatment with 2-APB 1 h prior to \( \text{H}_2\text{O}_2 \) stimulation attenuated \( \text{H}_2\text{O}_2 \)-induced cell death in a dose-dependent manner and showed almost complete inhibition of cell death at 100 \( \mu \)M (cell death, 10.8±4.4% in pretreated vs 89.2±16.7% in non-pretreated samples; Fig 1A). Since previous reports suggested that \( \text{H}_2\text{O}_2 \)-induced cell death is associated with the impaired \( \text{Ca}^{2+} \) homeostasis in other cell types [32], we assessed intracellular \( \text{Ca}^{2+} \) concentrations during \( \text{H}_2\text{O}_2 \) treatment with or without 2-APB pretreatment in NRCMs. A considerable increase in intracellular \( \text{Ca}^{2+} \) concentration was observed within 10 min after \( \text{H}_2\text{O}_2 \) treatment, which was completely abolished by 2-APB pretreatment (Fig 1B). To elucidate the relationship between the 2-APB-mediated cell death inhibition and the prevention of an increase in the intracellular \( \text{Ca}^{2+} \) levels, we used BAPTA-AM, an intracellular \( \text{Ca}^{2+} \) chelator, to analyze whether the reduction of \( \text{Ca}^{2+} \) levels attenuates cardiomyocyte death following the \( \text{H}_2\text{O}_2 \) treatment. Pretreatment with a low dose of BAPTA-AM significantly attenuated \( \text{H}_2\text{O}_2 \)-induced cardiomyocyte death (9.1±3.9% vs 81.0±6.7%; Fig 1C). In addition, pretreatment with BAPTA-AM prevented the increase in \( \text{Ca}^{2+} \) concentration after \( \text{H}_2\text{O}_2 \) stimulation (Fig 1D). Finally, to determine whether the increase of \( \text{Ca}^{2+} \) levels is due to the extracellular \( \text{Ca}^{2+} \) entry, EDTA was used for the removal of the extracellular \( \text{Ca}^{2+} \), and intracellular \( \text{Ca}^{2+} \) levels were assessed during \( \text{H}_2\text{O}_2 \) treatment. The extracellular chelation of \( \text{Ca}^{2+} \) using EDTA-containing medium was shown to prevent an increase in \( \text{Ca}^{2+} \) levels after \( \text{H}_2\text{O}_2 \) stimulation (Fig 1F). Taken together, these results indicate that 2-APB treatment attenuates \( \text{H}_2\text{O}_2 \)-induced cell death through the inhibition of increase of intracellular \( \text{Ca}^{2+} \) levels.
Fig 1. Treatment with 2-APB attenuated H₂O₂-induced cell death and Ca²⁺ influx in cardiomyocytes. (A) Analysis of the viability of neonatal rat cardiomyocytes (NRCMs) with or without 100 μM 2-APB pretreatment for 1 h, followed by subsequent stimulation with 100 μM H₂O₂ for 4 h. Results are presented as mean ± SEM obtained in five to seven independent experiments. (B) Representative fura-2 ratios from NRCMs pretreated with or without 2-APB for 1 h, followed by stimulation with H₂O₂. (C) NRCMs were pretreated or not with 5 μM BAPTA-AM, which was followed by H₂O₂ treatment and the degree of cell survival is presented as mean values ± SEM obtained in six or seven independent experiments. (D) Representative fura-2 ratios from NRCMs pretreated or not with 10 μM BAPTA-AM for 1 h, followed by H₂O₂ treatment. (E) Representative fura-2 ratios obtained using NRCMs treated with 100 μM H₂O₂ in
2-APB-sensitive Ca\(^{2+}\) channels are not involved in H\(_2\)O\(_2\)-induced cardiomyocyte death

We investigated whether IP\(_3\)R was involved in H\(_2\)O\(_2\)-induced cardiomyocyte death, since 2-APB was reported to be an IP\(_3\)R antagonist [33]. Pretreatment with xestospongin C, a specific IP\(_3\)R inhibitor, failed to suppress H\(_2\)O\(_2\)-induced cardiomyocyte death (Fig 2A). Consistently, NRCMs pretreated with xestospongin C displayed a similar increase in intracellular Ca\(^{2+}\) levels compared to those in the H\(_2\)O\(_2\)-treated control (Fig 2B), suggesting that IP\(_3\)R is not involved in 2-APB-mediated cytoprotection.

Multiple TRP channels were shown to be suppressed by 2-APB treatment [34, 35]. To date, multiple TRPCs and TRPM channels, such as TRPM2, TRPM3 and TRPM7 have been reported to be 2-APB sensitive [17, 20, 21]. We analyzed these channels using RT-PCR, and observed that TRPC1, 3, 5 and 6, as well as TRPM3 and 7 are expressed in NRCMs (Fig 2C). The inhibition of TRPCs by SKF-96365, TRPM3 by mefenamic acid, and TRPM7 by AA-861 failed to prevent H\(_2\)O\(_2\)-induced cell death (Fig 2D–2F), although the doses used were sufficient to inhibit the targeted channels based on the previously reported studies [36]. Pretreatment with SKF-96365 at a dose of 10 \(\mu\)M, which is twice higher compared to a previously reported one, was enough to inhibit capacitative calcium entry in NRCMs failed to prevent cell death, suggesting those TRPCs are not involved in H\(_2\)O\(_2\)-induced cardiomyocyte death (Fig 2D). Likewise, pretreatment with AA-861 which blocks TRPM7 activity at 10 \(\mu\)M in several previous reports [37, 38] failed to inhibit cell death with a similar dose (Fig 2E). Moreover, pretreatment with mefenamic acid, a specific inhibitor of TRPM3 whose EC\(_{50}\) is reported to be approximately 6 \(\mu\)M [39], also failed to attenuate cell death at 1, 10 and 100 \(\mu\)M (Fig 2F). Collectively, those results indicate that major 2-APB sensitive Ca\(^{2+}\) channels are not involved in the presented model of H\(_2\)O\(_2\)-induced cardiomyocyte death.

2-APB reacts with and scavenges extracellular ROS

To decipher the mechanism underlying 2-APB-mediated myocyte protection, we examined ROS inhibition by this compound. We assessed intracellular ROS levels in NRCMs and observed considerable increase in these levels after H\(_2\)O\(_2\) stimulation compared with those in the control, which are abolished after 2-APB pretreatment (Fig 3A). We demonstrated that 2-APB treatment led to a significant reduction in the H\(_2\)O\(_2\)-induced intracellular ROS increase 60 min after the treatment (DCF intensity, 87.0±7.0% vs. 45.8±7.6%; Fig 3B), suggesting that 2-APB directly inhibits ROS generation. In contrast to this, 2-APB treatment failed to inhibit a phenylephrine (PE)-induced increase in the intracellular ROS levels, which occurs through NADH/NADPH oxidase activation [40] (Fig 3C and 3D). Furthermore, pre-treatment with N-acetylcysteine (NAC), reported to inhibit G-protein coupled receptor-induced ROS level increase [41], prevented ROS level increase after PE administration (S1 and S2 Figs. These results indicate that 2-APB treatment does not inhibit an increase in intracellular ROS levels, but that it directly reacts with extracellular ROS, resulting in the inhibition of the ROS toxic effects in NRCMs. The direct reaction between 2-APB and H\(_2\)O\(_2\) was examined by \(^1\)H-NMR. The \(^1\)H-NMR spectra of 2-APB (spectrum B and C) recorded at 5 and 60 min, respectively, after the addition of 10 molar equivalents of H\(_2\)O\(_2\) to 2-APB, were shown to change significantly from the authentic spectrum (spectrum A). This observation suggests that 2-APB chemically reacts with H\(_2\)O\(_2\) (Fig 3E). TLC analysis showed that a vermilion spot identical to that of...
Fig 2. Specific inhibition of 2-APB-sensitive Ca\(^{2+}\) channels failed to prevent H\(_2\)O\(_2\)-induced cardiomyocyte death. (A) Neonatal rat cardiomyocytes (NRCMs) were pretreated or not with xestospongin C, a specific IP\(_3\)R inhibitor, for 1 hour, which was followed by H\(_2\)O\(_2\) treatment. Cell viability levels were determined. Results are shown as mean values ± SEM from three independent experiments. (B) Representative fura-2 ratio obtained using NRCMs pretreated or not with xestospongin C, followed by H\(_2\)O\(_2\) treatment. (C) The expression levels of 2-APB-sensitive TRP channels in NRCMs were analyzed by RT-PCR. A representative image with a 40-cycle amplification is shown. (D-F) NRCMs were pretreated or not with SKF-96365 (D, a TRPC inhibitor), AA-861 (E, a TRPM7 inhibitor) and mefenamic acid (F, a TRPM3 inhibitor), followed by H\(_2\)O\(_2\) treatment. Cell viability rate is presented. Results are presented as mean values ± SEM obtained in four independent experiments.

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Fig 3. Hydrogen peroxide directly reacts with 2-APB. (A) Neonatal rat cardiomyocytes (NRCMs) were loaded with 5 μM DCF, and treated with 2-APB and H₂O₂. Average results of %Fluorescence increase were obtained from three independent experiments. (B) Intracellular ROS levels at 60 min after H₂O₂ stimulation were estimated from DCF fluorescence levels in NRCMs pretreated or not with 2-APB. Results of %Fluorescence increase are presented as mean values ± SEM obtained in three independent experiments. *P<0.05 vs. untreated control, †P<0.05 vs. H₂O₂ treated samples, obtained using one-way ANOVA. (C) NRCMs were loaded with 5 μM DCF and treated with 2-APB for 1 h, followed by stimulation with 2 mM PE. Average results of %Fluorescence increase were obtained from three independent experiments. (D)
Intracellular ROS levels at 60 min after PE stimulation, estimated using DCF fluorescence intensity levels in NRCMs pretreated or not with 2-APB. Results of %Fluorescence increase are presented as mean values ± SEM obtained in three independent experiments. **P<0.01 vs. untreated control, using one-way ANOVA. (E) Representative 1H-NMR spectra obtained from 2-APB (spectrum A) or 2-APB in direct reaction with H₂O₂ for 5 min (spectrum B) or 60 min (spectrum C) are shown. (F) Representative thin-layer chromatography (TLC) images are presented. Reaction product indicates samples obtained from the reaction between 2-APB with H₂O₂ used for NMR measurement. Comparison between the reaction products and authentic H₂O₂ (left), 2-APB (middle), or phenol (right) is presented.

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an authentic phenol appeared on a TLC plate by the treatment of 2-APB with H₂O₂, supporting that the oxidation of 2-APB with H₂O₂ generates phenol (Fig 3F). Taken together, these results indicate that 2-APB has a potential to scavenge extracellular ROS and functions as an anti-oxidative compound.

Administration of 2-APB attenuates myocardial damage after I/R in mice

To examine the effects of 2-APB on cardiac injury in vivo, C57BL/6 mice were randomized to either 2-APB- or vehicle-treated group, and subjected to left coronary artery occlusion for 30 min followed by 24 h reperfusion. Vehicle or 2-APB was administered via intravenous bolus injection immediately before the reperfusion. We demonstrated that 2-APB treatment failed to affect the area at risk (AAR) (Fig 4A and 4B). However, the ratio between IA and AAR was shown to decrease significantly after a single injection of 2-APB (Fig 4A and 4C). In contrast, a single dose NAC treatment failed to attenuate cardiac I/R injury (S3 and S4 Figs), suggesting that 2-APB has unique anti-oxidant properties.

2-APB treatment attenuates ROS activity and inflammatory responses during I/R injury

To investigate the functional role of ROS scavenging properties of 2-APB in the reduction of I/R injury in mice, we analyzed ROS levels in the I/R hearts using DHE staining. DHE-stained I/R hearts showed distinct signals 24 h after the reperfusion, indicating ROS production (Fig 5A). Notably, treatment with 10 mg/kg 2-APB led to a decrease in signal intensity in DHE-stained I/R heart (Fig 5A), which was shown to be significantly lower when compared to the control (fold intensity, 1.32±0.11 vs. 2.11±0.32, respectively; Fig 5B), suggesting that ROS production was suppressed by 2-APB treatment. To examine the involvement of ROS in 2-APB induced cardioprotection, serum d-ROM and BAP levels were determined 30 min and 6 h after the reperfusion. While no significant differences in both parameters were obtained at 30 min after the I/R, d-ROM levels were shown to be significantly decreased in 2-APB treatment group, compared with those in the vehicle-treated group 6 h after the I/R (105±4 vs. 91±4 U. CARR in d-ROMs and 26.2±1.3 vs. 31.8±1.5 in μM/U.CARR in BAP/d-ROMs; Fig 5C). Moreover, the BAP/d-ROM ratio was shown to be significantly increased in mice treated with 2-APB, in comparison to that in the vehicle-treated group (Fig 5D), suggesting an increase in the antioxidant potential in the 2-APB-treated mice.

Since the source of oxidative stress is mainly from neutrophils and not from cardiomyocyte mitochondria in the subacute phase after the I/R, we further evaluated whether 2-APB affects the inflammatory response after myocardial I/R. Levels of il-6 and il-1β expression were shown to be significantly decreased in 2-APB-treated mice compared with those in the control-I/R mice at 6 h after reperfusion (Fig 5E). The expression of tnf-α tended to decrease in the 2-APB-treated mice as well. Consistent with this, subsequent infiltration of inflammatory cells into the ischemic myocardium was attenuated (Fig 5F) and the number of infiltrated Ly-6G-positive granulocytes was decreased significantly in the 2-APB-treated mice at 24 h after the
reperfusion (Fig 5G). Collectively, these results indicate that 2-APB treatment induces reduction of ROS and attenuates inflammatory responses in subacute phase.

**Discussion**

In the present study, we demonstrated that treatment with 2-APB prevents ROS-induced cardiomyocyte death by inhibiting Ca\(^{2+}\) overload caused by an extracellular Ca\(^{2+}\) influx and attenuates cardiac I/R injury in mice. Furthermore, we demonstrated that the protective effects in cardiomyocytes are based on a direct extracellular ROS scavenging by 2-APB, rather than the channel inhibitory effects or intracellular ROS chelating. To the best of our knowledge, this study is the first to demonstrate that the antioxidative effects of 2-APB induce cardioprotection.
Fig 5. Treatment with 2-APB attenuates reactive oxygen species (ROS) production and inflammatory responses after cardiac ischemia/reperfusion (I/R) in mice. (A) Representative fluorescence images of dihydroethidium (DHE)-stained heart sections obtained at 24 h of reperfusion from the 2-APB- or vehicle-treated mice. Scale bar: 50 μm. (B) Fluorescence intensity obtained from eight images, expressed as the mean fluorescence intensity normalized to the levels obtained from mice of the vehicle sham group. Results are presented as mean values ± SEM obtained from 4–5 mice. * P<0.05 vs. I/R 2-APB-treated sample. (C and D) Effect of 2-APB on diacron reactive oxygen metabolites (d-ROMs) (C) and biological antioxidant potential (BAP)/
d-ROMs (D) in mice that underwent 30 min of ischemia followed by 30 min or 6 h of reperfusion. Results are presented as mean values ± SEM obtained from 4–6 mice. 105 ± 4 vs. 91 ± 4 in U.CARR (6 h reperfusion in d-ROMs) and 26.2 ± 1.3 vs. 31.8 ± 1.5 in μM/U.CARR (6 h reperfusion in BAP/d-ROMs). *P < 0.05 vs. I/R vehicle-treated 6 hours reperfusion group, obtained using Student’s t-test. (E) IF, ILβ, and TNF-a expression levels in mouse hearts. Results are presented as mean values ± SEM obtained from 7–8 mice. *P < 0.05 vs. I/R vehicle-treated 6 h reperfusion group, obtained using Student’s t-test. (F) Representative images of heart sections obtained from different animal groups, stained with anti-Ly-6G antibody (Gr-1) for the detection of neutrophils. Scale bar: 50 μm. (G) Quantification of Gr-1-positive cells in ischemic myocardium presented as the number of positive cells/mm². Results are presented as mean values ± SEM obtained from 6–7 mice; 454 ± 52 cells/mm² vs. 140 ± 22 cells/mm² for Ly-6G-positive granulocytes. **P < 0.01 vs. I/R vehicle-treated group, obtained using Student’s t-test.

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We demonstrated that H₂O₂ treatment leads to an increase intracellular Ca²⁺ levels in NRCMs consistent with previous reports [42]. The increase in Ca²⁺ was prevented by the removal of extracellular Ca²⁺, suggesting that H₂O₂ treatment induces Ca²⁺ influx through unknown plasma membrane ion channels. Many studies have focused on the mechanisms underlying H₂O₂-induced cardiomyocyte death, involving both apoptosis and necrosis [3, 43–46], and while apoptotic mechanisms have been determined [47, 48], mechanisms underlying H₂O₂-induced necrosis remain unknown. Our results demonstrate that H₂O₂-induced Ca²⁺ influx plays a major role in cardiomyocytes, and it may represent myocyte necrosis inducer.

The results of several studies showed that 2-APB protects tissues against stress [23–25, 49–51]. In liver, pretreatment with 2-APB was shown to attenuate drug-induced cellular damage and the I/R injury in mice [23, 49, 50]. Similarly, in kidneys, 2-APB was shown to exert cytoprotection after I/R by TRPM2 inhibition [24, 51]. In the heart, perfusion with 2-APB improved cardiac function on reperfusion in a rabbit ex vivo I/R model [52]. Here, we showed that 2-APB treatment attenuates H₂O₂-induced cardiomyocyte death by inhibiting intracellular Ca²⁺ influx. Previously, all beneficial effects of 2-APB were considered to stem from channel inhibition, however, we showed that blocking of those channels did not prevent H₂O₂-induced cell death. These results indicate that major 2-APB-sensitive Ca²⁺ channels are not involved in the H₂O₂-induced cardiomyocyte death.

We used ¹H-NMR and TLC analysis to demonstrate that 2-APB chemically reacts with H₂O₂ and generates phenol, thereby exhibiting an antioxidant effect. We suggest the following process underlying the observed effects: 2-APB is oxidized by H₂O₂, generating a boronic ester that is hydrolyzed to produce phenol. This reaction was shown to occur within 5 min following the addition of H₂O₂ to 2-APB, indicating that 2-APB is highly reactive towards H₂O₂ and may represent a powerful antioxidant. To the best of our knowledge, this is the first evidence demonstrating a direct antioxidative effect of 2-APB. More than 800 studies using 2-APB have been published, but this compound has been, generally, used as a channel blocking reagent. Since our results indicate that 2-APB has strong antioxidative properties, we believe that previously reported results must be interpreted with caution, especially if H₂O₂ was used simultaneously.

While ROS plays central roles in the development of I/R injuries, the efficacy of antioxidant therapy remains controversial [53]. A single injection of NAC during ischemia failed to attenuate cardiac I/R injury [54], similar to the results obtained using our cardiac I/R model. ROS generation can affect I/R injury through two processes. First, during the acute phase of I/R injury, ROS efflux from mitochondria occurs immediately after the reperfusion in cardiomyocytes and induces cell death by triggering MPTP opening [2]. Second, ROS generated by endothelial or infiltrated inflammatory cells during the I/R extends the injury by activation of inflammatory responses [6]. Following the stimulation with proinflammatory mediators, neutrophils are recruited to the site of injury, where they produce superoxide anions through the
NADPH oxidase pathway, releasing ROS to damage cells [55]. The obtained results suggest that the latter mechanism represents the target of 2-APB, resulting in ROS scavenging and cardioprotection, since 2-APB failed to inhibit intracellular ROS increase caused by PE stimulation, where ROS is produced in cardiomyocytes through membrane NAPDH activation [40, 56]. These results indicate that 2-APB treatment induces not intracellular but mainly extracellular ROS inhibition, generated from inflammatory cells during I/R injury in mice. ROS levels were shown to be significantly lower in the later phase of the I/R injury development in 2-APB treated group. Since the mitochondrial ROS efflux occurs immediately after reperfusion, those results suggest that the 2-APB target is not mitochondrial ROS efflux. The results also indicate that 2-APB treatment modulates ROS levels from the infiltrated immune cells which could be the source of ROS in the later phase. The unique properties of this compound may be more suitable for cardioprotective use than previously reported antioxidants.

This study has several limitations. First, we administered 2-APB during ischemia, immediately before the reperfusion. We selected this, since this protocol simulates the clinical use of drugs during primary coronary interventions, as it is the earliest available pharmacological intervention point, however, it may explain why no differences were observed in ROS levels after 2-APB administration immediately after the reperfusion. Furthermore, we cannot exclude the possibility that 2-APB directly inhibits inflammatory cell channels, attenuating the inflammation [57, 58]. As previously reported, 2-APB treatment may affect neutrophil activity or inhibit inflammation [59, 60].

There are several reports regarding clinical trials with antioxidants for cardiac ischemia-reperfusion injury in acute coronary syndrome [61]. However, in terms of Jadad scale [62], only a few articles are of good quality. Among them, there were two clinical trials of NAC, those which were conducted using well-designed protocols [63, 64]. However, they showed controversial results. One of the major differences between 2-APB and NAC is the absence of chelation of ROS produced inside cells. This might be an advantage of 2-APB, since ROS signaling plays an important role in cellular functions, as well.

In summary, we demonstrated that 2-APB possesses antioxidative properties and the treatment with 2-APB attenuates cardiac I/R injury in mice. Furthermore, we demonstrated that the extracellular ROS mediates Ca\textsuperscript{2+} influx, inducing cardiomyocyte death, which can be prevented by 2-APB treatment. While the mechanisms underlying cardiac I/R injury have been intensely investigated, no pharmacological interventions that can alleviate the I/R injury are in clinical use. Our results suggest that the treatment with 2-APB is a promising novel therapeutic approach to the treatment of the cardiac I/R injury, at least partially by reducing ROS levels during subacute phase. Finally, the identification of channels responsible for H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} influxes is needed to develop novel therapeutics for improved cardioprotection.

**Supporting information**

S1 Fig. *N*-acetylcysteine treatment inhibited intracellular ROS increases after phenylephrine stimulation. NRCMs were loaded with 5 μM DCF and treated with *N*-acetylcysteine (NAC) for 1 hour, followed by stimulation with 2 mM phenylephrine (PE). Intracellular ROS levels are calculated as ROS increase ratio from DCF fluorescent intensity increase ratios normalized to those at 0 minute. The results of average value obtained from 3 independent experiments are depicted.

(TIF)

S2 Fig. *N*-acetylcysteine treatment significantly inhibited intracellular ROS increases in a dose-dependent manner. Intracellular ROS levels at the time point of 60 minutes after PE stimulation were estimated from the measurement of DCF fluorescence in NRCMs pretreated
with or without NAC (100 or 500 μM) for 1 hour. Values are shown as mean ± SEM of 3 independent experiments. **P<0.01 vs. non-treatment, ††P<0.01 vs. 2 mM PE treatment, by one-way ANOVA followed by Tukey-Kramer test.

(TIF)

S3 Fig. Single administration of NAC failed to affect areas at risk in cardiac I/R injury mouse model. C57BL/6 mice were exposed to 30 minutes ischemia, followed by 24 hours reperfusion. NAC (100 mg/kg) or vehicle was administered intravenously immediate before reperfusion. Areas at risk (AAR) were estimated by exclusion of area stained with Evans blue. The myocardial infarct areas were detected by staining with 2% triphenyl tetrazolium chloride (TTC). The ratio of AAR normalized to LV was quantitatively estimated.

(TIF)

S4 Fig. Single administration of NAC failed to attenuate cardiac I/R injury in mice. Size of infarct area (IA) normalized to AAR was quantitatively assessed. Values are shown as mean ± SEM (49.9±14.1% vs 41.3±9.2% in IA/AAR, n = 3 mice for vehicle; n = 5 mice for 100 mg/kg of NAC).

(TIF)

S1 Table. PCR primers used in the present study. The sequences of the primers used in the present study are shown.

(PDF)

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