2-APB protects against liver ischemia-reperfusion injury by reducing cellular and mitochondrial calcium uptake

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Nicoud IB, Knox CD, Jones CM, Anderson CD, Pierce JM, Belous AE, Earl TM, Chari RS. 2-APB protects against liver ischemia-reperfusion injury by reducing cellular and mitochondrial calcium uptake. Am J Physiol Gastrointest Liver Physiol 293: G623–G630, 2007. First published July 12, 2007; doi:10.1152/ajpgi.00521.2006.—Ischemia-reperfusion (I/R) injury is a commonly encountered clinical problem in liver surgery and transplantation. The pathogenesis of I/R injury is multifactorial, but mitochondrial Ca2+ overload plays a central role. We have previously defined a novel pathway for mitochondrial Ca2+ handling and now further characterize this pathway and investigate a novel Ca2+-channel inhibitor, 2-aminoethoxydiphenyl borate (2-APB), for preventing hepatic I/R injury. The effect of 2-APB on cellular and mitochondrial Ca2+ uptake was evaluated in vitro by using 45Ca2+. Subsequently, 2-APB (2 mg/kg) or vehicle was injected into the portal vein of anesthetized rats either before or following 1 h of inflow occlusion to 70% of the liver. After 3 h of reperfusion, liver injury was assessed enzymatically and histologically. Hep G2 cells transfected with green fluorescent protein-tagged cytochrome c were used to evaluate mitochondrial permeability. 2-APB dose-dependently blocked Ca2+ uptake in isolated liver mitochondria and reduced cellular Ca2+ accumulation in Hep G2 cells. In vivo I/R increased liver enzymes 10-fold, and 2-APB prevented this when administered pre- or postschemia. 2-APB significantly reduced cellular damage determined by hematoxylin and eosin and prevented this when administered pre- or postschemia. 2-APB significantly reduced cellular damage determined by hematoxylin and eosin and prevented this when administered pre- or postschemia.

The structure of the mCa2+ uniporter has not been elucidated, and in an attempt to better characterize the mechanism governing uniporter control, we recently described the novel presence of phospholipase C in mitochondria and demonstrated its involvement in regulation of mCa2+ uptake (31). Not surprisingly, selective inhibition of phospholipase C prevents mCa2+ uptake and minimizes I/R damage in the liver (4, 31, 32). During these previous studies, we discovered another novel cell-permeable inhibitor of mCa2+ uptake, 2-aminooxydiphenyl borate (2-APB). This compound was initially believed to be an inositol trisphosphate (IP3)-receptor antagonist (40), but further reports have concluded that 2-APB does not affect liver IP3 receptors, although it does appear to inhibit downstream SOC channels (19, 24). Because 2-APB is a novel inhibitor of mCa2+ uptake in isolated liver mitochondria (31) and blocking mCa2+ uptake is effective in mitigating liver I/R overload, loss of the mitochondrial membrane potential, and formation of the permeability transition pore (PTP), a terminal and likely irreversible event for the cell (11, 33, 44, 45, 60). PTP formation facilitates the release of cytochrome c, apoptosis-inducing factor, and other molecules that lead to mitochondria-associated caspase-dependent and caspase-independent cell death (26, 27, 37, 38, 55).

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injury (3), we examined the effects of 2-APB in the setting of liver I/R injury.

In the present study, we 1) establish the potency and efficacy of 2-APB for inhibiting \( Ca^{2+} \) uptake in isolated liver mitochondria, 2) identify that 2-APB exerts its protective effect by reducing cellular \( Ca^{2+} \) uptake and preventing m\( Ca^{2+} \) overload, 3) show that 2-APB is effective in preventing hepatic I/R damage when administered via the portal vein before ischemia in an in vivo model of liver I/R injury, 4) demonstrate that 2-APB is also able to attenuate I/R injury when administered following an ischemic event, and 5) provide evidence that the mechanism of protection for 2-APB rests in its ability to prevent mitochondrial permeability as assessed by cytochrome c release.

**EXPERIMENTAL PROCEDURES**

**Reagents and animals.** Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO). All experiments were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by and carried out under the guidelines of the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (Nashville, TN). Male Sprague-Dawley rats (~300 g) were purchased from Harlan (Indianapolis, IN) and were housed in a climate-controlled institutional animal facility on a 12:12-h light/dark cycle where food and water were provided ad libitum.

**Mitochondrial isolation.** Rats were anesthetized with isoflurane and were killed by cervical dislocation. Livers were perfused and stored as previously reported (7). Briefly, an abdominal incision was made and the portal vein was cannulated. The liver was perfused with 30 ml of 4°C University of Wisconsin solution (UW). The liver was minced and homogenized in liver homogenization buffer (0.2 M mannitol, 50 mM sucrose, 10 mM KCl, and 1 mM Na\(_2\)EDTA, adjusted to pH 7.4 with KOH). The homogenate was filtered through gauze and was centrifuged for 10 min at 3,000 g. The supernatant was collected. The pellet was washed twice with 1,000 g. The supernatant was collected and was centrifuged for 10 min at 3,000 g; the resulting pellet was washed twice. This pellet was then resuspended in \( Ca^{2+} \)-free liver homogenization buffer without EDTA. Final mitochondrial protein concentration was 2 mg/ml. All steps were performed at 4°C, and we have previously shown that this technique yields pure mitochondria free of plasma membrane contamination (7,31). MitoTracker Red (Molecular Probes, Eugene, OR) fluorescence microscopy was used to ensure that mitochondria maintained their transmembrane potential which is indicative of viability.

**Measurement of \( mCa^{2+} \) uptake.** All \( mCa^{2+} \)-uptake studies were performed in incubation buffer (IB; 100 mM KCl, 20 mM HEPES, and 5 mM MgCl\(_2\), adjusted to pH 7.4 with KOH) by using a method similar to that previously described by Knox et al. (31). All components of the IB solution were filtered by using Chelex resin or were similar to that previously described by Knox et al. (31). All experiments without \( 45Ca^{2+} \) were incubated for 30 min. They were then filtered through a gauze and were centrifuged at 10,000 g at 4°C. MitoTracker Red (Molecular Probes, Eugene, OR) fluorescence microscopy was used to ensure that mitochondria maintained their transmembrane potential which is indicative of viability.

**Measurement of total cellular \( Ca^{2+} \) uptake.** The human Hep G2 hepatoblastoma cell line (catalog no. HB-8065; ATCC, Manassas, VA) was chosen because of its stability and predictable growth, retained characteristics of primary hepatocytes, and our prior experience with this cell line (3). Hep G2 cells are well described and have been used by many other authors in the study of ischemic injury as well as hepatic regeneration (1,14,15,29). \( Ca^{2+} \) uptake in Hep G2 cells was measured by using radioactive \( 45Ca^{2+} \). Cells were grown in MEM with Earle’s salts (ATCC) supplemented with 10% fetal bovine serum (Cascade Biologicals, Winchester, MA), 1% penicillin/streptomycin, and 1% nonessential amino acids (Gibco, Grand Island, NY), which contained 1.8 mM of calcium. Separate aliquots of complete MEM and UW were placed in a 37°C hypoxic incubator (95% \( N_2 \) -5% \( CO_2 \)) overnight to deplete the solutions of oxygen. To track \( Ca^{2+} \) accumulation in the cells, we added 1.8 \( \mu \)M of radioactive \( 45Ca^{2+} \) (1:1,000 proportion) in the medium. Cells were seeded in polystyrene-coated (50 \( \mu \)g/ml) 24-well plates in equal amounts with 1.08 \( \times \) 10\(^5\) cells per well. Cells were then subjected to the following conditions: 1) cells spent 9 h under normal conditions in either normal growth medium or UW (control) and 2) cells were subjected to 6 h of normothermic ischemia, which was achieved by adding of hypoxic solutions containing \( 45Ca^{2+} \) and displacement of the air by a gas mixture containing nitrogen and \( CO_2 \) (95:5% respectively), followed by 3 h of reperfusion, which was achieved by replacing hypoxic medium or UW with fresh oxygenated medium containing \( 45Ca^{2+} \). At the end, medium was removed, cells were briefly rinsed with 1X PBS without radioactive \( 45Ca^{2+} \), and cells were harvested by lysing with 10% SDS. This solution was transferred to scintillation vials containing scintillation fluid (Bio-Safe II, RPI, Mount Prospect, IL). To ensure complete harvesting of the cells, the wells were washed a second time with scintillation fluid, which was transferred to the scintillation vials. Radioactivity of the samples was measured by using a Beckman L6000IC beta counter (Beckman Coulter, Fullerton, CA). Parallel experiments were performed for the same conditions without \( 45Ca^{2+} \) for determination of cell number and protein concentration, which were used to normalize calcium content between groups.

**Rodent model of hepatic I/R.** Animals were anesthetized by using isoflurane (2%, 2 \( \mu \)min \( O_2 \)). Following midline laparotomy, an atrumatic vascular clamp (S&T Microclamp HD-S; Fine Science Tools, Foster City, CA) was placed across the portal vein, hepatic artery, and bile duct branches to the left and median liver lobes, leaving ~70% of the liver ischemic. This method is widely reported (12,20,23,43,49) and prevents mesenteric congestion by allowing portal venous shunting through the right lobe and caudate lobe.

Ten minutes before initiation of ischemia, 2-APB (2 mg/kg) or ruthenium red (50 \( \mu \)g/kg) was administered intravenously via the portal vein by using a half-inch 27-gauge needle. 2-APB was prepared for intravenous injection by reconstitution in DMSO and then dilution in normal saline to achieve a final concentration of 10% DMSO. Animals received 2 mg/kg 2-APB injected directly into the portal vein in a total of 0.5 ml normal saline, which resulted in animals receiving <5 \( \mu \)l of DMSO. Control animals received the equivalent volume of DMSO in saline. Ruthenium red was dissolved in saline and was administered at 50 \( \mu \)g/kg in 0.5 ml. Control animals were given the same volume of sterile water in saline (0.5 ml).

Ischemia continued for 60 min. Sham-operated animals underwent laparotomy, mild liver manipulation, and equivalent anesthesia. Surgical procedures were performed under aseptic conditions, and warm, sterile, moistened gauze was placed over the laparotomy to avoid dehydration. Heparin was not administered. Body temperature was maintained at 36.5–37.5°C throughout the procedures.

Reperfusion was initiated by removal of the vascular clamp, and the abdominal incision was closed in two layers with 4-0 polypropylene suture. Prewarmed saline (1 ml) was introduced intraperitone-
ally before closure. Buprenorphine hydrochloride (0.05 mg/kg, Buprenex Injectable; Reckitt and Colman, Richmond, VA) was given subcutaneously immediately following closure to provide postoperative analgesia. Animals were allowed to recover and reperfuse for 180 min, after which time they were anesthetized and blood and tissue were collected.

The degree of hepatocellular injury was assessed by evaluating serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels. Approximately 4 ml of whole blood was collected by dividing the pulmonary artery and removing blood from the thoracic cavity; blood was allowed to clot at room temperature for 30 min and was centrifuged at 1,500 g for 15 min. Serum was carefully extracted, and analysis was performed by Antech Veterinary Laboratories (Memphis, TN).

**Immunohistochemical staining of paraffin-embedded tissue.** Half of the left lobe was collected at the time of death, placed in an immunohistochemical cassette, and submerged in 4% paraformaldehyde in PBS for 18 h, after which the cassettes were transferred to 70% ethanol for storage until paraffin embedding. Specimen processing was performed by the Human Tissue Acquisition and Pathology Shared Resource Facility at Vanderbilt University Medical Center. Five-micrometer sections were cut and mounted, and hematoxylin and eosin (H&E) staining was performed on the first section. Serial sections were used to evaluate cell necrosis.

Terminal deoxynucleotidyl (TdT)-mediated dUTP nick-end labeling (TUNEL) was performed by using the ApopTag Plus Fluorescein Direct In Situ Apoptosis Detection kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Briefly, slides were deparaffinized and rehydrated, followed by pretreatment for 15 min in 20 µg/ml proteinase K. Slides were treated with equilibration buffer for 10 s, followed by aspiration and application of TdT enzyme for 1 h at 37°C. The reaction was terminated by submerging slides in stop/wash buffer, agitating for 15 s, and incubating for 10 min at room temperature. After the washing, antidigoxigenin conjugate (fluorescein) was applied and the slides were submerging slides in stop/wash buffer, agitating for 15 s, and incubating for 10 min at room temperature. After the washing, antidigoxigenin conjugate (fluorescein) was applied and the slides were incubated in a dark humidified chamber for 30 min. The slides were washed again in PBS and were mounted using fluorescent mounting medium (Dako, Carpinteria, CA) containing 1.0 µg/ml propidium iodide and a glass coverslip. The slides were allowed to dry overnight protected from light, and the coverslip edges were sealed the next day with enamel.

Slides were viewed on an Axioplan 2 microscope (Carl Zeiss, Hallbergmoos, Germany) equipped with an argon laser and 450- to 490-nm and 546- to 590-nm filters. Images were acquired by using both filter sets without changing the x-y orientation by using an AxioCam HRC (Zeiss) remotely operated by a computer running Axiovision version 3.1 software (Zeiss). Image overlays were performed by using Adobe Photoshop CS version 8.0 (Adobe Systems, Sunnyvale, CA). All cell death percentages were obtained by averaging the estimated percent positivity for five random fields per slide on at least three slides from separate animals per condition. Slides were reviewed in a blinded fashion.

**In vitro model of IR.** In the current experiments, Hep G2 cells were grown at 37°C, 5% CO2 in complete medium (as described in *Measurement of total cellular Ca2+ uptake* to 60% confluence on poly-D-lysine-coated glass coverslips. Cells were then transfected with a cytochrome c-green fluorescent protein (GFP) expression plasmid (gift from Dr. A. L. Nieminen) as described by Nieminen and co-workers (22) and were allowed to grow for 6 h to ensure proper gene expression. To initiate experiments, hypoxic UW solution containing 2-APB (final concentration, 100 µM) or vehicle DMSO was added to cells, after which the cells were submitted to 6 h of hypoxia at either 37°C or 4°C; UW solution was used. We have previously tested ruthenium red in similar experiments and have published those findings (3). The 4°C experiments were carried out to determine the relevance of these data to situations such as transplantation, when cold, rather than warm, ischemia is encountered. Hypoxia was achieved by placing the cells in an airtight incubator (Forma Scientifica, Marietta, OH), which was flushed with 5% CO2 and 95% N2 until the oxygen content in the container reached <0.1%, as verified by using a dissolved O2 meter (model 4000; VWR Scientific Products, Swavanaugh, GE). Additionally, the UW solution was preincubated in the hypoxic chamber in an open sterile container for 8 h before experiments were carried out, resulting in a final O2 concentration of <0.1% as measured with the dissolved O2 meter. After 6 h, the hypoxic UW solution containing the compounds was aspirated and was replaced with fresh, warmed, oxygenated medium containing no compounds, and the cells were returned to normoxic conditions. This was considered the beginning of reperfusion, which was continued for 3 h. A set of cells from each condition was fixed before reperfusion to observe the effects of ischemia without reperfusion. The remaining cells were fixed for fluorescence microscopy immediately following 180 min of reperfusion. MitoTracker Red (100 nM), which is taken up by respiring mitochondria, was added to the medium for 7 min before fixation to study the relationship of cytochrome c-GFP to the mitochondria. Both an untreated control and a negative control were studied. Additionally, the experiments were repeated in full by using 6 h of normoxic, rather than hypoxic, incubation as an additional control for comparison.

Fluorescence microscopy was performed by using an AxioImag 2 with an AxioArc (Zeiss) fluorescent light source. Five random fields were acquired for each sample, and the number of TUNEL-positive cells was counted. A 450- to 490-nm filter was used to collect GFP fluorescence images, whereas a 546- to 590-nm filter was used to obtain MitoTracker images, all of which were recorded digitally by using an AxioCam HRC. The images were processed by using AxioVision version 3.1 software (Zeiss). Processed images were imported into Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA), and overlay screens were created to evaluate colocalization of cytochrome c-GFP and mitochondria.

**Statistical analysis.** Data analysis was carried out by using SPSS version 13.0 (SPSS, Chicago, IL). All values are presented as means or mean differences ± SE unless otherwise noted. The mean difference between groups was analyzed by using a one-way ANOVA with the least significant difference procedure for pair-wise comparisons, with P < 0.05 considered significant. Pair-wise comparisons were considered valid only when the overall model P value was ≤0.05, indicating significant difference between at least two groups.

**RESULTS**

2-APB dose-dependently inhibits Ca2+ uptake in isolated mitochondria. Our previous work suggests that 2-APB has a direct inhibitory effect on the mCa2+ unipporter (31). We expanded these studies to further characterize the effects of 2-APB on mitochondria. Isolated liver mitochondria demonstrated intense fluorescence (rhodamine filter) following staining with MitoTracker Red, indicating these were viable and maintained transmembrane potential throughout the isolation process (Fig. 1A). 2-APB dose-dependently inhibited mCa2+ uptake at dosages of 1–100 µg/ml (∼5–500 µM; Fig. 1B). The IC50 was ∼15.8 µg/ml (∼70 µM), which is consistent with previous data suggesting an IC50 of 50–75 µM (24, 31). Ruthenium red, the selective unipporter inhibitor, completely inhibited mCa2+ uptake, confirming that all mCa2+ uptake was through the unipporter. DMSO had no effect (data not shown, previously published in Ref. 31).

2-APB has no effect on cellular calcium uptake during ischemia. To address whether 2-APB could reduce cellular calcium accumulation in addition to mCa2+, we measured total cellular Ca2+ content by using radioactive 45Ca2+ in Hep G2 cells after 9 h normoxic storage in UW (control), 6 h hypoxia

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in UW (ischemia), or 6 h hypoxia with 3 h simulated reperfusion by using complete media and a normoxic chamber (I/R).

As illustrated in Fig. 2, 2-APB reduced cellular calcium accumulation in control and I/R conditions ($P = 0.034$ and $0.026$, respectively) but had no effect on cellular calcium accumulation during ischemia ($P = 0.088$).

2-APB decreases serum AST, ALT, and LDH following I/R. Direct inhibition of the mCa$^{2+}$/H uniporter by systemic ruthenium red pretreatment in a rat model of hepatic I/R injury is protective, so ruthenium red was used as a positive control in these studies. Our previous work indicates that peak aminotransferase levels occur following 60 min of ischemia and 180 min of reperfusion (3), and this time course was thus selected for these studies.

Sham-operated rats exhibited AST, ALT, and LDH levels of $155 \pm 21$, $71 \pm 24$, and $549 \pm 102$ U/l, respectively ($n = 3$; Fig. 2). In rats undergoing I/R alone, liver enzymes were significantly elevated compared with levels in sham-operated rats; AST, ALT, and LDH were $1,660 \pm 358$ ($P = 0.002$), $1,779 \pm 432$ ($P = 0.004$), and $2,907 \pm 786$ U/l ($P = 0.027$), respectively ($n = 9$). 2-APB (2 mg/kg) administered before ischemia via the portal vein (2-APB I/R) resulted in a significant decrease in serum markers, with an AST of $414 \pm 56$ ($P = 0.002$), ALT of $285 \pm 65$ ($P = 0.003$), and LDH of $813 \pm 87$ U/l ($P = 0.020$) ($n = 5$). Ruthenium red (50 µg/kg) also significantly decreased postreperfusion AST, ALT, and LDH to $442 \pm 129$ ($P = 0.004$), $282 \pm 120$ ($P = 0.005$), and $1,004 \pm 121$ U/l ($P = 0.046$), respectively ($n = 4$), compared with I/R alone. The values for these tissue-damage markers in both 2-APB I/R and ruthenium red I/R animals were not statistically different from those for sham-operated animals that underwent no ischemia at all (Fig. 3). Vehicle control (saline or DMSO) injection before ischemia had no effect on these values (data not shown).

**Postischemic 2-APB administration reduces serum liver enzymes.** Currently, there are no widely used techniques for preventing liver I/R damage that are effective when treatment is administered following ischemia, during reperfusion. 2-APB (2 mg/kg) or ruthenium red (50 µg/kg) was injected into the portal vein immediately following 60 min of hepatic ischemia, and animals were allowed to reperfuse for 180 min. Serum

![Fig. 1. A: isolated liver mitochondria stained with MitoTracker Red demonstrating viability and maintained transmembrane potential throughout the isolation process. B: 2-aminoethoxydiphenyl borate (2-APB) concentration-dependently inhibits mitochondrial calcium (mCa$^{2+}$) uptake in isolated liver mitochondria, with an IC$_{50}$ of 15.8 µg/ml (dashed line).](http://ajpgi.physiology.org/)

![Fig. 2. Total cellular calcium uptake based on β-emission of Ca$^{2+}$, represented as counts per minute per cell. Cells stored in University of Wisconsin medium (UW) alone (solid bars) were compared with those stored in UW plus 100 M 2-APB (open bars). 2-APB reduced cellular Ca$^{2+}$ in cells stored in normoxic medium for 9 h and in those subjected to 6 h hypoxia with 3 h simulated reperfusion ($*P < 0.05$). Calcium measurements made immediately after 6 h ischemia demonstrated that 2-APB had no effect on cellular Ca$^{2+}$ during ischemia.](http://ajpgi.physiology.org/)

![Fig. 3. Serum aspartate aminotransferase (AST; solid bar), alanine aminotransferase (ALT; open bar), and lactate dehydrogenase (LDH; shaded bar, represented as LDH/3) were assessed following 3 h reperfusion. Preischemic treatment with 2-APB (2-APB I/R) or ruthenium red (RR I/R) significantly reduced enzymes compared with ischemia-reperfusion (I/R) alone ($*P < 0.05$); these levels were not statistically different compared with sham ($*P < 0.05$). Postischemic treatment with 2-APB (2-APB rpx), but not ruthenium red (RR rpx), significantly prevented I/R-induced elevations in AST and ALT.](http://ajpgi.physiology.org/)
analysis and histological assays were performed as described in EXPERIMENTAL PROCEDURES. Compared with baseline I/R values established above, transaminase levels following 180 min of reperfusion were lower with 2-APB treatment; AST was 933 ± 101 U/l (P = 0.05) and ALT was 834 ± 174 U/l (P = 0.04). Ruthenium red trended toward a significant effect (Fig. 3).

2-APB decreases histological cell death. In rats undergoing I/R with no pharmacological treatment, cellular damage was observed in an average of 18% of all cells as characterized by nuclear pyknosis, nuclear fragmentation, loss of distinct cell borders, and decreased eosin staining (proteolysis) (Fig. 4B). Animals that received 2-APB or ruthenium red before ischemia displayed no focal areas of damage and only 1.1% and 1.5% pyknotic nuclei, respectively (Fig. 4C). In animals that received 2-APB following ischemia, small foci of cellular damage were seen, but not nearly to the degree of that seen in untreated animals, with an average of only 0.3% damage observed (Fig. 4D). Table 1 indicates the average percent cellular damage observed by H&E and indicates P values for pair-wise comparisons between all conditions and I/R alone. Significant reductions (P ≤ 0.05) were observed after preischemic treatment with either 2-APB or ruthenium red, but only 2-APB had a significant effect when administered after ischemia (Table 1).

Fluorescent TUNEL staining for nicked DNA fragments (indicating cell damage) demonstrated 18.75% positivity in rats undergoing I/R with no treatment, compared with 1.5% or less in those receiving 2-APB or ruthenium red, regardless of whether the compounds were administered before or following ischemia (Fig. 4). Again, statistically significant reductions were seen after preischemic administration of either compound, but only 2-APB was statistically effective when administered after ischemia (Table 1).

2-APB prevents I/R-induced cytochrome c release in Hep G2 cells. In our in vitro model of I/R using Hep G2 cells, MitoTracker Red fluorescence localizes mitochondria, whereas GFP fluorescence identifies cytochrome c. If cytochrome c is located within the mitochondria, as it is during normal physiology, then the green and red images are very similar and the overlay of these images creates an orange-yellow appearance. If cytochrome c has been released from mitochondria, indicating initiation of cell-death pathways, then discrete areas of both green and red fluorescence remain visible when the images are overlaid. Ruthenium red was not included in these results because we have previously published similar data (3).

In control cells that underwent no hypoxia and were kept in normoxic UW solution for 6 h, there was no observable cytochrome c release from the mitochondria as indicated by tight colocalization of green and red fluorescence (Fig. 5, normoxia). Following 6 h of warm hypoxia, cytochrome c and mitochondria no longer colocalized in cells that received vehicle DMSO alone (Fig. 5, hypoxia + DMSO), whereas cells that were treated with 2-APB looked identical to normoxic control cells (Fig. 5, hypoxia + 2-APB). The results did not change when cells were allowed 3 h of “reperfusion” with warm oxygenated medium; 2-APB pretreatment prevented cytochrome c release, whereas DMSO did not (data not shown). The reperfusion media did not contain 2-APB or DMSO.

DISCUSSION

Multiple clinical scenarios, including trauma, shock, hemorrhage, thermal injury, resection, and transplantation, expose the liver to I/R injury. The most important finding in this manuscript was the identification of 2-APB as a novel com-
Table 1. Quantitated percent cell damage

<table>
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<th>Condition</th>
<th>% Cells Damaged</th>
<th>P</th>
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<tr>
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</table>

Data are average of 5 random fields per slide; n = 3 slides per condition. P values are indicated for pairwise comparisons to ischemia-reperfusion (I/R) alone. 2-APB, 2-aminoethoxydiphenyl borate; RR, ruthenium red; rpx, postischemic treatment with indicated substance; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling.

We have previously reported that ruthenium red prevents mCa2+ uptake in isolated mitochondria (6) and that systemic administration of ruthenium red 30 min before I/R prevents hepatocellular damage, as evidenced by decreased elevation of transaminases in a rat model of warm hepatic I/R (3). Ruthenium red is a hexavalent cationic glycoprotein stain that potently inhibits the mCa2+ uniporter and is considered to be a "gold standard" uniporter inhibitor (6, 17, 18, 47). Unfortunately, ruthenium red appears neurotoxic in vitro (58), making the clinical utility of this compound uncertain. However, our success with ruthenium red in preclinical experiments with respect to hepatic protection has prompted a search for alternative compounds affecting mCa2+ handling.

In terms of the progression of mitochondria-associated cell death, our data highlight the timing of mCa2+ release during the ischemic period; it was only following ischemia that total cellular calcium was affected. Other authors have provided similar evidence, demonstrating that that cytosolic free Ca2+ begins to increase rapidly following ischemia and that ischemia alone is sufficient to initiate hepatocyte death (28). Our data therefore suggest that during ischemia, 2-APB exerts a protective effect by inhibiting mCa2+ uptake, whereas subsequent reperfusion injury may be prevented by a combination of 2-APB’s effects on cellular and mitochondrial Ca2+ uptake.

In our in vitro studies, 2-APB had no effect on total cellular Ca2+ uptake during ischemia but was able to significantly reduce cellular calcium accumulation following ischemia with reperfusion. Administering 2-APB before ischemia significantly reduced liver enzymes, cellular damage, and cytochrome c release.
chrome c release; liver enzymes were not statistically different from those for rats that underwent sham operation. In our previous report on the effect of ruthenium red in I/R, we used systemic administration and relatively long circulatory times before the I/R insult (3); in this manuscript, we found that delivery of these compounds directly into the portal vein near the hilum 10 min before ischemia was equally, if not more, effective in protecting the liver from I/R injury. Additionally, portal vein injection minimizes initial exposure of other organs to 2-APB and allows a significant portion of the compound to be delivered to the liver before being systemically absorbed, metabolized, or excreted. Based on a mean circulating blood volume of 64 ml/kg, the 2 mg/kg dose of 2-APB administered in these experiments resulted in a high initial concentration within liver portal veins, with a final circulating concentration between 2.7 μg/ml (assuming 2-APB distributes equally throughout total body water) and 32 μg/ml (assuming 2-APB remains completely within the vasculature). The IC50 for mCa2+ uptake inhibition in isolated mitochondria was 15.8 μg/ml. Considering the lipophilicity of 2-APB, the serum concentration of 2-APB could have fallen below 2.7 μg/ml because of sequestration by adipose tissue.

Few previous studies have examined the role of 2-APB during I/R, but a number of relevant reports deserve consideration given our new findings. Gysenbergh et al. (21) tested the effects of IP3 and 2-APB on mean infarct size after 30 min of coronary artery occlusion followed by 2 h of reperfusion in rabbits. 2-APB decreased the infarct size from 80 ± 5 to 55 ± 5% of the area at risk. Because administration of IP3 caused a similar effect, they concluded that the effects of 2-APB were due to actions against the IP3 receptor, but our data suggest that this is likely not the case, especially because 2-APB does not affect liver IP3 receptors (19, 24). Chinopoulos et al. published a thorough and well-designed study examining the effects of 2-APB on brain mitochondria. In their studies, 2-APB blocked PTP formation and cytochrome c release in the presence of high concentrations of extramitochondrial Ca2+, which corroborates our current findings (8). Their data were interpreted as suggesting that 2-APB prevented mCa2+ release, rather than uptake. However, they used much higher concentrations of Ca2+, different concentrations of electrolytes, and mitochondria isolated from rat neurons, which may account for the potential differences in 2-APB’s mitochondrial mechanism between their findings and ours. However, their overall results were consistent with those in this manuscript, and they also concluded that 2-APB acts at the mitochondrial level.

The known effect of 2-APB on SOC channels is a point of interest relevant to the discussion of our observed mechanism of action. Prior reports have suggested that 2-APB inhibits SOC entry in the liver (19, 24), and a recent study showed that I/R significantly increases SOC currents in hepatocytes and demonstrated that 2-APB was protective and produced a concentration-dependent decrease in SOC current with an IC50 of 65 ± 11 μM (24). The data in our current study suggest that 2-APB works by blocking mCa2+ uptake during ischemia. When considering our current and previous data together with the rest of the literature, and taking into account the potential contribution of SOC channels to I/R injury, we speculated that 2-APB may have a dual protective role, inhibiting both SOC channels and the mCa2+ uniporter. At the dose used, 2-APB did not prevent SOC-mediated cellular calcium uptake during ischemia; however, it did significantly reduce cellular calcium accumulation after ischemia with simulated reperfusion. Our collective data support a dual-protective role for 2-APB in controlling cellular and mitochondrial calcium in I/R injury.

In summary, our findings contribute to the growing volume of literature implicating mCa2+ uptake through the mCa2+ uniporter as a key player in pathophysiology of I/R damage. The use of 2-APB, a novel mCa2+ uniporter inhibitor, prevents mCa2+ overload, cytochrome c release, and cell death during I/R and provides further evidence that that postischemic pharmacological intervention in I/R injury is possible. Further studies will be required to test the efficacy of mCa2+ uptake inhibitors in alternative clinical scenarios, including in vivo cold I/R and orthotopic liver transplantation.

REFERENCES


2-APB PREVENTS WARM HEPATIC I/R INJURY


