Opioid-Induced Second Window of Cardioprotection

Potential Role of Mitochondrial K_{ATP} Channels

Ryan M. Fryer, Anna K. Hsu, Janis T. Eells, Hiroshi Nagase, Garrett J. Gross

Abstract—Opioids have been previously shown to confer short-term cardioprotection against a prolonged ischemic insult. Therefore, the present study was designed to determine whether opioids can induce a delayed or “second window” of cardioprotection and to assess the potential involvement of the mitochondrial K_{ATP} channel. All rats were subjected to 30 minutes of ischemia and 2 hours of reperfusion (I/R). Control animals, injected with saline 24 hours before I/R, elicited an infarct size/area at risk (IS/AAR) of 62.9±3.4. TAN-67, a δ1-opioid receptor agonist, was administered 10 or 30 mg/kg IP 12, 24, 48, or 72 hours before I/R. TAN-67 (10 mg/kg) 12- or 24-hour pretreatment did not significantly reduce IS/AAR (62.1±6.3 and 43.3±7.3, respectively). Similarly, 12-hour pretreatment with TAN-67 (30 mg/kg) did not reduce IS/AAR (60.0±5.6); however, 24-hour pretreatment significantly reduced IS/AAR (34.5±5.9). Forty-eight–hour pretreatment with TAN-67 maximally reduced IS/AAR (29.2±7.0), and opioid-induced cardioprotection was lost after 72-hour pretreatment (61.7±3.8). TAN-67–induced cardioprotection could be abolished by pretreatment with the selective δ1-opioid receptor antagonist 7-benzylidenenaltrexone, BNTX, administered either 30 minutes before TAN-67 given 48 hours before I/R or 10 minutes before I/R in rats previously treated for 48 hours with TAN-67 (59.6±3.1 and 58.7±3.5, respectively). The involvement of the K_{ATP} channel was investigated with 2 inhibitors: glibenclamide, a nonselective K_{ATP} channel inhibitor, and 5-hydroxydecanoic acid, selective for the mitochondrial K_{ATP} channel in rabbits. Glibenclamide, administered 30 minutes before I/R in 48-hour TAN-67–pretreated rats, completely abolished cardioprotection (60.4±3.2). Similarly, 5-hydroxydecanoic acid, administered 5 minutes before I/R in rats pretreated 48 hours previously with TAN-67, completely abolished cardioprotection (57.8±2.5). These results suggest that δ1-opioid receptor stimulation, 24 to 48 hours before an ischemic insult, produces a delayed cardioprotective effect that is possibly the result of mitochondrial K_{ATP} channel activation.

(Circ Res. 1999;84:846-851.)

Key Words: quinolines ■ cardioprotection ■ mitochondria ■ receptors, opioid

Opioids have been widely used as therapeutic agents for the treatment of pain; however, our laboratory has recently demonstrated that opioids also produce cardioprotection in the in vivo rat heart against a prolonged ischemic insult and subsequent reperfusion.1 We demonstrated that this cardioprotective effect is the result of δ1-opioid receptor activation and is elicited through a G_{i/o} protein-mediated mechanism and subsequent opening of the ATP-sensitive potassium channel (K_{ATP} channel).2 Similarly, Miki et al3 have recently demonstrated that in the rabbit heart, morphine mimics ischemic preconditioning through the activation of protein kinase C (PKC).

Ischemic preconditioning (IPC), a phenomenon in which brief episodes of ischemia and reperfusion before a prolonged ischemic event limit myocardial cellular damage, has been shown to elicit both an acute and delayed phase of cardioprotection or a second window of protection.4 Short-term protection as a result of IPC provides immediate protection to the myocardium against a prolonged ischemic insult for 30 to 90 minutes after stimulus depending on the species studied. Baxter et al and others5-6 have demonstrated a second window of protection as a result of a previous IPC stimulus and have shown that an adenosine A_{1} receptor agonist also produces delayed cardioprotection. These investigators demonstrated that IPC and a selective adenosine A_{1} agonist can limit cell death as a result of a prolonged ischemic event in the rabbit heart 24, 48, or 72 hours after brief ischemia or pharmacological intervention. However, this delayed cardioprotection was not observed 12 hours after IPC and usually disappeared 72 to 96 hours after IPC.7 Baxter et al8 have also demonstrated that delayed cardioprotection produced by IPC is mediated by both PKC and tyrosine kinase activation. It has been hypothesized that this delayed protection may result from the translocation of PKC to the nucleus; increased transcription and subsequent synthesis of cardioprotective proteins such as the small heat shock protein, hsp 27,9 other stress proteins;4 and endogenous antioxidant enzymes.4

Although no work has been done concerning delayed cardioprotection due to opioids, Ventura et al11 have shown that the nucleus of hamster ventricular myocardial cells...
contain opioid binding sites and that cardiomyopathic cells had increased basal levels of opioid gene transcription. Opioids have been shown to activate other pathways thought to be involved in the protective effect of IPC-induced delayed protection. Gutstein et al.\(^{12}\) have examined the involvement of opioids on the mitogen-activated protein kinase signaling cascade and have demonstrated that opioids may induce activation of both extracellular signal-related kinase and p38. As previously mentioned, opioids may activate PKC to confer cardioprotection in acute IPC.\(^{5}\) Similarly, PKC-signaling events have been demonstrated in delayed cardioprotection due to IPC.\(^{8}\) Evidence exists that implicates the mitochondrial K\(_{ATP}\) channel as the end effector of acute IPC\(^{13–15}\); however, controversy exists as to the importance of either the sarcolemmal and/or mitochondrial K\(_{ATP}\) channel to confer delayed cardioprotection due to IPC. Recently, Liu et al. have identified a mitochondrial selective K\(_{ATP}\) channel antagonist, 5-hydroxydecanoic acid, in isolated rabbit myocardial cells. Therefore, the present study sought to examine the possibility of opioid-induced delayed cardioprotection in a rat model of ischemia/reperfusion injury and examined the potential involvement of the K\(_{ATP}\) channel in this protection. We hypothesize that \(\delta_1\)-opioid receptor activation can initiate a delayed cardioprotective effect to a prolonged ischemic insult in the rat and that the mitochondrial K\(_{ATP}\) channel may be the end effector of this cardioprotection.

**Materials and Methods**

This study was performed in accordance with the guidelines of the Animal Care Committee of the Medical College of Wisconsin, which is accredited by the American Association for Laboratory Animal Care.

**General Surgical Preparation**

Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, Ind), 350 to 450 g, were used for all phases of this study. Rats were administered a drug or saline 1, 12, 24, 48, or 72 hours before the surgical protocol through intraperitoneal injection. Subsequently, rats were anesthetized via intraperitoneal administration of thiobutabarbital sodium (Inactin, Research Biochemical International; 100 mg/kg). A tracheotomy was performed, and the trachea was intubated with a cannula connected to a rodent ventilator (model CIV-101, Columbus Instruments, or model 683, Harvard Apparatus). Rats were ventilated with room air supplemented with \(\text{O}_2\) at 60 to 65 breaths per minute. Atelectasis was prevented by maintaining a positive end-expiratory pressure of 5 to 10 mm H\(_2\)O. Arterial pH, \(\text{PCO}_2\), and \(\text{PO}_2\) were monitored at control, 15 minutes of occlusion, and 60 and 120 minutes of reperfusion by a blood gas system (AVL 995 pH/blood gas analyzer, AVL Medical Instruments) and maintained within a normal physiological range (pH 7.35 to 7.45; \(\text{PCO}_2\) 25 to 40 mm Hg; and \(\text{PO}_2\) 80 to 110 mm Hg) by adjusting the respiratory rate and/or tidal volume. Body temperature was maintained at 38°C by the use of a heating pad, and bicarbonate was administered intravenously as needed to maintain arterial blood pH within normal physiological levels.

The right carotid artery was cannulated to measure blood pressure and heart rate via a Gould PE50 or Gould PE23 pressure transducer connected to a Grass (model 7) polygraph. The right jugular vein was cannulated for saline, bicarbonate, and drug infusion. A left thoracotomy was performed at the fifth intercostal space followed by a pericardiectomy and adjustment of the left atrial appendage to reveal the location of the left coronary artery. A ligature (6-0 prolene) was passed below the coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. The coronary artery was occluded by pulling the ends of the suture taut and clamping the snare onto the epicardial surface with a hemostat. Coronary artery occlusion was verified by epicardial cyanosis and a subsequent decrease in blood pressure. Reperfusion of the heart was initiated via unclamping the hemostat and loosening the snare and was confirmed by visualizing an epicardial hyperemic response. Heart rate and blood pressure were allowed to stabilize before the experimental protocols were initiated.

**Study Groups and Experimental Protocols**

Rats were randomly divided into 16 groups (Figure 1). Control rats were administered saline 24 hours before 30 minutes of regional ischemia and 2 hours of reperfusion (I/R). To reconfirm the effect of opioid-induced acute cardioprotection, TAN-67 was administered 1 hour before a prolonged ischemic insult. To determine whether opioid stimulation elicits delayed cardioprotection against an acute ischemic insult, TAN-67, a selective \(\delta_1\)-opioid receptor agonist,\(^{7}\) was administered at a level of 10 or 30 mg/kg either 12 or 24 hours before I/R (TAN 10 mg/kg at 12 hours, TAN 10 mg/kg at 24 hours, TAN

---

**Figure 1.** Protocol bar that depicts the experiments used to study the effects of opioid treatment in the in vivo rat. All groups underwent a 30-minute coronary artery occlusion and a 2-hour reperfusion period followed by TTC stain and infarct size analysis. Control rats were administered 0.9% saline (0.9 mL) 24 hours before 30 minutes of regional ischemia and 2 hours of I/R. TAN-67 (10 or 30 mg/kg) was administered 1, 12, 24, 48, or 72 hours before a prolonged ischemic insult. Gilbenclamide (1 mg/kg) was administered during either the control or 48-hour TAN-67 pretreatment protocol 5 minutes before I/R. TAN-67 (5-HD 10 mg/kg IV) was administered during either the control or 48-hour TAN-67 pretreatment protocol 10 minutes before I/R. BNTX (3 mg/kg IV) was administered 48 hours before a control protocol or 30 minutes before TAN-67 pretreatment 48 hours before I/R.
30 mg/kg at 12 hours, and TAN 30 mg/kg at 24 hours). TAN-67 was also administered at a level of 30 mg/kg either 48 or 72 hours before I/R (TAN 30 mg/kg at 48 hours and TAN 30 mg/kg at 72 hours). We examined the effects of δ-opioid receptor inhibition in the absence or presence of TAN-67 with the selective δ-opioid receptor antagonist, 7-benzylidenenaloxone (BNTX). BNTX 3 mg/kg was administered intravenously to either the control rats or those treated with TAN 30 mg/kg at 48 hour 10 minutes before I/R. Similarly, to examine the effect of BNTX given before TAN-67, we administered BNTX 6 mg/kg IP 48 hours before I/R and 30 minutes before saline injection. Tissue was dissected from the AAR under the illumination of a dissecting microscope (Cambridge Instruments). Infarct size (IS), AAR, and LV were determined by gravimetric analysis. IS was expressed as a percentage of the AAR (IS/AAR). AAR was expressed as a percentage of the LV (AAR/LV), and LV was subsequently cut into 6 thin, cross-sectional pieces. This allowed for the delineation of the normal area, stained blue, versus the AAR, which subsequently remained pink. The AAR was excised from the nonischemic area, and the tissues were placed in separate vials and incubated for 15 minutes with 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) stain in 100 mmol/L phosphate buffer (pH 7.4) at 37°C. TTC is an indicator of viable and nonviable tissue. TTC is reduced by dehydrogenase enzymes present in viable myocardium and results in a formazan precipitate, which induces a deep red color, whereas the infarcted area remains gray. Tissues were stored in vials of 10% formaldehyde overnight, and the infarcted myocardium was dissected from the AAR under the illumination of a dissecting microscope (Cambridge Instruments). Infarct size (IS), AAR, and left ventricular weight (LV) were determined by gravimetric analysis. AAR was expressed as a percentage of the LV (AAR/LV), and IS was expressed as a percentage of the AAR (IS/AAR).

**Exclusion Criteria**
A total of 106 rats successfully completed the above protocols. Rats were excluded from data analysis if they exhibited severe hypotension (<30 mm Hg systolic blood pressure) or if we were unable to maintain adequate blood gas values within a normal physiological range because of metabolic acidosis or alkalosis. Exclusion of animals from the present study were evenly distributed among the protocol groups.

**Statistical Analysis of Data**
All values are expressed as mean±SEM. One-way ANOVA with Bonferroni’s test was used to determine whether any significant differences existed among groups for hemodynamics, IS, and AAR. Significant differences were determined at P<0.05.

**Drugs**
Thiobutabarbital sodium (Inactin) and 5-HD were purchased from Research Biochemical International. TTC and glibenclamide were synthesized and furnished by Dr Hiroshi Nagase of Toray Industries (Kanagawa, Japan). Inactin was dissolved in distilled water. TAN-67 and 5-HD were dissolved in 0.9% saline. BNTX was dissolved in 0.9% saline in a 1:1:1:2 cocktail mixture. Glibenclamide was dissolved in polyethylene glycol 400 and dH2O. Glibenclamide was dissolved in polyethylene glycol 400, 0.1N NaOH, 95% EtOH, and 0.9% saline in a 1:1:1.2 cocktail mixture. This vehicle has been previously shown to have no effect on infarct size in this model. All drugs were administered in ~0.9 mL of vehicle. The maximally effective timing and dose of glibenclamide14 and 5-HD (R.M.F., G.J.G., unpublished observation, 1998) were previously determined in our laboratory.

**Results**

**Hemodynamics**
The Table summarizes heart rate, mean arterial blood pressure, and rate-pressure product in all groups, determined at baseline, 15 minutes of coronary artery occlusion, and 120 minutes of reperfusion. Blood pressure in the inhibitor protocols were maintained at baseline values after inhibitor treatment. No significant differences existed in the hemodynamics of all groups versus the control group; however, the rate-pressure product was significantly increased in the glibenclamide control protocol versus control at 2 hours of reperfusion (45±4 versus 30±3, respectively).

**Infarct Size and Area at Risk**
IS/AAR for all groups is shown in Figures 2 through 4. AAR/LV was not significantly different in any group versus the control (control, 50.7±2.4, rest of data not shown). Figures 2, 3, and 4 show IS/AAR for each group. The average IS/AAR in control rats was 62.9±3.4. TAN-67 administration 1 hour before ischemia significantly reduced IS/AAR (23.6±5.3). However, administration of TAN-67 at a level of 10 or 30 mg/kg, 12 hours before I/R, was not cardioprotective (62.1±6.3 or 60.0±5.6, respectively). Administration of TAN-67, 10 and 30 mg/kg, 24 hours before I/R reduced IS/AAR versus IS/AAR in control animals in a dose-dependent manner (43.3±7.3 and 34.5±5.9, respectively), which was significant at 30 mg/kg versus control. Injection of TAN-67 (30 mg/kg) 48 hours before I/R maximally reduced IS/AAR (29.2±7.0); however, cardioprotection was lost at 72 hours (61.7±3.8). In the absence of TAN-67 treatment, when BNTX (3 and 6 mg/kg) was administered either 48 hours or 10 minutes before I/R, it did not significantly affect IS/AAR versus control. However, when BNTX (3 or 6 mg/kg) was administered in the presence of TAN-67 either 48 hours or 10 minutes before I/R, TAN-67–induced cardioprotection was abolished (59.6±3.1 and 58.7±3.5). Inhibition of the KATP channel with glibenclamide or 5-HD in the absence of opioid pretreatment did not significantly affect IS/AAR (53.0±5.6 and 62.6±4.1, respectively). However, inhibition of the KATP channel 30 or 5 minutes before I/R with glibenclamide or 5-HD, respectively, after 48 hours of opioid pretreatment, completely abolished opioid-induced cardioprotection (60.4±3.2 and 57.8±2.5, respectively).

**Discussion**
These experiments demonstrate for the first time that stimulation of the δ-opioid receptor induces delayed cardioprotection to the ischemic myocardium 24 or 48 hours after treatment. We also demonstrate that this cardioprotection is mediated via activation of the KATP channel and suggest that the mitochondrial KATP channel may mediate this cardioprotection.

IS/AAR was not affected by 12-hour pretreatment with TAN-67 at a level of 10 or 30 mg/kg or 24-hour TAN-67 (10 mg/kg) pretreatment. However, 24- or 48-hour pretreatment with TAN-67 (30 mg/kg) significantly reduced infarct size.
compared with control animals at 24 and 48 hours. This reduction was maximal with 48-hour pretreatment, and cardioprotection was lost with a 72-hour delay. Therefore, these data suggest that delayed protection to the ischemic myocardium can be mediated via δ-opioid receptor stimulation. These data are consistent with the observations of Yellow and Baxter, who showed in the rabbit model that pharmacological stimulation of the adenosine A1 receptor could induce delayed cardioprotection beginning 24 hours after a single pretreatment with the selective adenosine A1 agonist CCPA. However, opioid-induced cardioprotection in our study faded at 72 hours, whereas CCPA stimulation of the adenosine A1 receptor began to fade at 96 hours pretreatment.13

Our laboratory was the first to demonstrate that δ1 receptors were involved in mediating the cardioprotective effects of ischemic preconditioning to reduce myocyte cell death after a prolonged ischemic insult and subsequent reperfusion.14 Similarly, we have previously demonstrated that TAN-67 selectively acts through δ1-receptor...
activation to confer acute cardioprotection in the in vivo rat because this effect was completely abolished by the δ1-opioid receptor antagonist, BNTX. Similarly, the results of the present study demonstrate that BNTX pretreatment can abolish the TAN-67–induced second window of cardioprotection and are the first to demonstrate that δ1-opioid receptors can mediate delayed cardioprotection.

BNTX abolished cardioprotection induced by 48-hour TAN-67 pretreatment when administered either 30 minutes before TAN-67 pretreatment or 48 hours after TAN-67 treatment but 10 minutes before I/R. These data suggest that opioid-receptor stimulation may elicit signal transduction mechanisms necessary for delayed cardioprotection such as protein synthesis. However, because BNTX administered 10 minutes before I/R could abolish TAN-67 induced cardioprotection, these data suggest that reoccupation of δ1-opioid receptors may also be important in producing delayed cardioprotection during the lethal ischemic period.

Opioid receptor activation is thought to confer cardioprotection to an acute ischemic insult via coupling with a Gs protein. Evidence suggests that cardioprotection may subsequently signal through PKC, which may activate the KATP channel. Data from our laboratory are indicative of similarities between both acute and delayed cardioprotection due to opioid-receptor stimulation. With the use of both the nonselective KATP channel inhibitor glibenclamide and the putative mitochondrial KATP channel inhibitor 5-HD, we demonstrate for the first time that opioid receptor stimulation confers delayed cardioprotection from ischemia and reperfusion injury via activation of the KATP channel and suggest that the mitochondrial KATP channel may play a role in this cardioprotection. However, 5-HD has been shown to be specific only in the rabbit model, and the possibility exists that this agent may not exhibit the same selectivity in the rat model.

Acute protection afforded to the ischemic myocardium via opioid receptor stimulation also appears to be due to KATP channel activation. Similarly, preliminary evidence by Bell et al demonstrates a role for the mitochondrial KATP channel in both preconditioning and opioid-induced cardioprotection in human cardiac tissue. As demonstrated by Baxter et al, acute cardioprotection due to ischemic preconditioning may disappear ~2 hours after stimulus. This may be due to a decrease in activated protein kinase second messengers or possible inactivation of the KATP channel. Extensive evidence exists to suggest the involvement of both PKC and the KATP channel in ischemic preconditioning. In this regard, Light et al have demonstrated that PKC can directly activate the KATP channel. The present study also demonstrates cardioprotection from an ischemic insult 1 hour after opioid treatment; however, cardioprotection was lost at 12 hours. The subsequent reappearance of cardioprotection 24 to 72 hours after ischemic preconditioning may be due to PKC translocation into the nucleus and subsequent induced transcription and synthesis of effector proteins. Similarly, the subsequent reappearance of cardioprotection 24 to 48 hours after opioid receptor stimulation may result from PKC signaling, because PKC has been demonstrated to be involved in opioid-induced acute cardioprotection in rabbits.

Considerable debate exists as to the specific KATP channel involved in cardioprotection. There are arguments both pro and con for the involvement of the sarcolemmal KATP channel. Opening of the KATP channel under conditions of ischemic stress and depletion of ATP from cells may effectively hyperpolarize the membrane and shorten phase 3 of the action potential. These actions may lead to decreased sodium and calcium entry into the cell. Subsequent decreases in calcium-induced calcium release and therefore decreased excitation-contraction coupling may conserve necessary ATP within the cell used to salvage vital mechanisms important for cellular homeostasis.

In contrast, the physiological role of the mitochondrial KATP channel is still unknown. KATP channels are present on both the sarcolemmal membrane and the inner mitochondrial membrane, and some studies have implicated the mitochondrial KATP channel in cardioprotection, possibly mediated by PKC. Activation of the mitochondrial KATP Channel has been shown to increase the influx of potassium into mitochondria, which results in both matrix expansion and mitochondrial depolarization. Recent studies in isolated cardiac mitochondria, in response to KATP channel activators, have demonstrated mitochondrial depolarization, increased rate of mitochondrial respiration, and decreased rate of ATP synthesis. As a compensatory response to this decreased ATP synthesis, it has been suggested that matrix expansion due to mitochondrial KATP channel opening stimulates electron transport and fatty acid oxidation. We speculate that increased respiratory chain activity and the possible increase in reactive oxygen species may confer delayed cardioprotection because it has been previously demonstrated that ROS can induce delayed cardioprotection in the ischemic heart. Alternatively, mitochondrial depolarization secondary to KATP channel activation may decrease the driving force for mitochondrial calcium uptake, which results in a reduction in mitochondrial calcium overload. Obviously, future studies are necessary to determine the mechanisms by which mitochondrial KATP channels elicit cardioprotection and to determine the selectivity of 5-HD for the mitochondrial KATP channel in other species.

In summary, these data demonstrate the involvement of the KATP channel to confer delayed cardioprotection against irreversible ischemia/reperfusion injury due to δ1-opioid receptor stimulation. The potential therapeutic benefits of
opioids as cardioprotective agents justifies further probing of the in vivo and in vitro mechanisms of opioid-induced protection.

Acknowledgment
This work was supported by the National Heart, Lung, and Blood Institute grant HL-08311.

References
Opioid-Induced Second Window of Cardioprotection: Potential Role of Mitochondrial KATP Channels
Ryan M. Fryer, Anna K. Hsu, Janis T. Eells, Hiroshi Nagase and Garrett J. Gross

Circ Res. 1999;84:846-851
doi: 10.1161/01.RES.84.7.846

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/7/846