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HL-1 Myocytes Exhibit PKC and K_{ATP} Channel-Dependent Delta Opioid Preconditioning[1,2]

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Background. **Opioid preconditioning protects the myocardium against ischemia/reperfusion (IR) injury. By enhancing cardiomyocyte viability, opioids can enhance cardiac function and recovery from IR injury during acute cardiac care. The myocyte model HL-1 is an immortalized, mouse atrial cell line that expresses functional delta-opioid receptors. The HL-1 myocyte may be useful for IR injury research exploring opioid cardioprotection.**

Materials and methods. **In study I, microplates of HL-1 were subjected to 10 min pre-treatment with either basal media, delta-opioid agonist DADLE(10uM), or DADLE(10uM) + delta-antagonist naltrindole (10uM). Study II treatment groups included PKC inhibitor chelerythrine (2uM), K_{ATP} channel closer glybenclamide (100uM), or mitochondrial K_{ATP} channel opener diazoxide (100uM) administered in various combinations followed by DADLE (10uM) or control. Microplates were subjected to normal oxygen/substrate conditions or ischemic (<1% 0_2) and substrate deficient (10 uM 2-Deoxyglucose versus 10 mM glucose) conditions, then reperfused with normal oxygen and glucose-containing media. Microplate supernatants were subjected to lactate dehydrogenase (LDH) assay.**

Results. **Compared to untreated control, the LDH assay showed significant reduction in opioid-only pretreated groups at all time points. These effects were attenuated with delta-opioid antagonist co-administration. Co-administration of non-selective K_{ATP} channel closer glybenclamide and DADLE abolished DADLE cytoprotection, while selective mitochondrial K_{ATP} opener diazoxide mimicked DADLE cytoprotection Co-administration of chelerythrine and DADLE significantly reduced chelerythrine cytotoxicity.**

Conclusion. **Delta-opioid preconditioning of HL-1 myocytes significantly decreased necrosis from in vitro simulated ischemia/reperfusion as measured by LDH release; this effect was reversed by delta-antagonist naltrindole. Cytoprotection was PKC and K_{ATP} channel-dependent. HL-1 myocytes exhibit opioid-induced cytoprotection from IR injury, and present a novel model of pharmacologic preconditioning.**

Key Words: **opioid; ischemia; reperfusion; necrosis; pre-conditioning.**

INTRODUCTION

Cardiac ischemia/reperfusion injury impairs cardiac function, and can lead to the death of myocytes by both apoptosis and necrosis. Global ischemia, as occurs in cardiac surgical procedures requiring cardiopulmonary bypass, typically induces myocyte necrosis versus apoptosis. In conditions of global ischemia, approximately 90% of myocyte death occurs by necrosis as measured within a few hours of reperfusion [1–3]. Still, apoptosis is postulated to contribute to downstream signals that enhance necrotic death in late reperfusion [4, 5]. The severity of ATP depletion during ischemia partly determines which death pathway a myocyte will under go [6]. Besides ATP depletion, other consequences of ischemia/reperfusion injury include calcium overload,

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osmotic swelling, contractile dysfunction, and free-radical induced protein and DNA modifications [7].

Ischemic or pharmacologic preconditioning decreases the scope and severity of ischemia/reperfusion injury. Preconditioning involves the attenuated response of a larger ischemic insult by prior exposure to period(s) of cell stress, such as brief cycles of ischemia or heat stress. Pharmacologic preconditioning with opioids has been shown to reduce ischemia/reperfusion damage by limiting infarct size and enhancing functional recovery in whole-heart models [8, 9]. In addition, isolated myocyte models indicate that opioids can enhance cell viability [10–12] and function [13]. Although the end effectors of acute opioid preconditioning remain elusive, early events following opioid-receptor stimulation are better understood. Opioids agonists act through G_i protein-coupled opioid receptors, leading to the translocation and activation of protein kinase C. Active PKC then initiates cardioprotection through multiple kinase pathways which phosphorylate undetermined effectors [8, 14–16]. Mitochondrial K_{ATP} channels opened by opioid-agonist stimulation also play a critical role in PKC-mediated cardioprotection [17–21]. The temporal relationship between PKC activation and K_{ATP} channel opening continues to be revealed. Once believed to be an effector of opioid preconditioning, recent studies also indicate a trigger role for K_{ATP} openers [21].

Both ventricular and atrial tissue/myocyte studies support opioid cardioprotection against ischemia/reperfusion injury. Isolated myocyte studies in opioid cardioprotection often use ventricular myocytes, which offer a higher myocyte yield from costly primary isolations. However, atrial tissue has been another important cell source for investigations of opioid cardioprotection. Bell and colleagues found similar mRNA relative abundance profiles for delta, kappa, and mu opioid receptors between human ventricular and atrial tissue [22]. Importantly, both atrial and ventricular tissue cytoprotection is reversible by specific opioid antagonists, PKC inhibitors, and K_{ATP} channel closers such as mitochondrial K_{ATP} antagonist 5-HD or non-specific K_{ATP} channel closer glybenclamide [18, 21–26].

Our laboratory first characterized the presence of functional delta-opioid receptors on an immortalized mouse atrial cell line, HL-1 [27]. As such, HL-1 cell culture could serve as an alternative cell model for investigations in delta opioid cardioprotection. Immortalized cell culture provides several benefits over primary cell isolations. Primary isolation of ventricular myocytes involves 1) animal use and care, 2) costly and lengthy preparation, 3) variable and limited yield for high-throughput approaches, and a 4) heterogeneous cell population. This study evaluated the effect of opioid preconditioning on the viability of HL-1 myocytes subjected to in vitro ischemia/reperfusion injury. To establish a relationship between HL-1 and established myocyte models of opioid cardioprotection, we also evaluated the PKC and K_{ATP} channel-dependence of the preconditioning mechanism.

MATERIALS AND METHODS

Chemicals and Reagents

HL-1 cardiomyocytes were obtained from their originator, Dr. Willam C. Claycomb, Lousisiana State University Health Sciences Center. Claycomb Media™ was obtained from JRH Biosciences(Lenexa, KS), and fetal bovine serum from Life Technologies/GibcoBRL (Carlsbad, CA). DADLE was a generous gift from Dr. Tsung-Ping Su, NIH-NIDA. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), and cell-culture disposables from Fisher Scientific (Hanover Park, IL). Diazoxide, chelerythrine, and glybenclamide were purchased from Sigma-Aldrich. All agents were dissolved in DMSO, then diluted in a basic salt solution (BSS) containing (in g/L): 6.7 NaCl, 0.186 KCl, 1.85 $NaHCO_3$ .0197 $MgSO_47H_2O$, .120 NaH_2PO_4, .097 $CaCl_22H_20$, and 1.8 glucose, pH 7.4. The final concentration of DMSO was 0.01% across all treatments.

Cell Culture and Pretreatments

HL-1 cells were maintained in T-75 flasks for routine passaging, and grown in 48 and 96-well microplates for experimental procedures. Cell culture-ware was pre-coated with 0.00125% fibronectin in 0.02% gelatin. Claycomb Media™ for cell growth and maintenance was supplemented with 0.1 mM norepinephrine (Sigma-Aldrich), 2mM L-glutamine (Life Technologies), 100 U/ml/100ug/ml Penicillin/Streptomycin (Life Technologies), and 10% fetal bovine serum (JRH Biosciences). Norepinephrine supplementation of Claycomb Media™ allows HL-1s to display a beating phenotype and to maintain their differentiated state [28]. HL-1s were plated at a concentration of 2×10^4 cells/cm^2 into 48-well plates and 96-well plates, and incubated at 37°C in a 5% CO_2 water-jacketed incubator. Experiments were initiated at approximately 80% confluency. For treatment groups, all media was replaced with normoxic, glucose-containing BSS (recipe as detailed earlier). Based upon receptor binding studies and preliminary dose-response (LDH release) curves (data not shown), 10 uM DADLE provided the most cytoprotection from a 10 minute pre-treatment. Study I was designed to determine the cyotprotective ability of delta opioid DADLE pre-conditioning, and the ability of delta-opioid antagonist naltrindole to reverse this protection. Plates were treated for 10 min with either (normoxic, glucose-containing) BSS, BSS + DADLE (10uM), or BSS + DADLE (10uM) + naltrindole (10uM), followed by a 5 min washout with normoxic, glucose containing BSS before to the ischemia/reperfusion protocol.

Study II tested the PKC and K_{ATP} channel dependence of DADLE cytoprotection, Parallel treatments are illustrated in Fig. 1. Doses of treatment reagents chelerythrine, glybenclamide, and diazoxide were based upon preliminary studies conducted in our laboratory with HL-1 myocytes, and studies of others using human atrial trebeculae and a ventricular myocyte models [11, 21, 29]. Chelerythrine (CHE 2 uM), glybenclamide (GLYB100 uM), or diazoxide (DIAZ 100 uM) treatments diluted in normoxic, glucose containing BSS were administered alone or in combination for 10 min. Plates were washed twice with PBS, pH 7.4. The washes were immediately followed by 10 min of DADLE or control BSS in designated groups, followed by a 5 min PBS washout prior to the ischemia/reperfusion protocol.

Ischemia/Reperfusion Protocol

Ischemia was obtained by both substrate and oxygen deprivation. Ischemic BSS (pH 6.8) contained 10 mM 2-deoxyglucose substituted

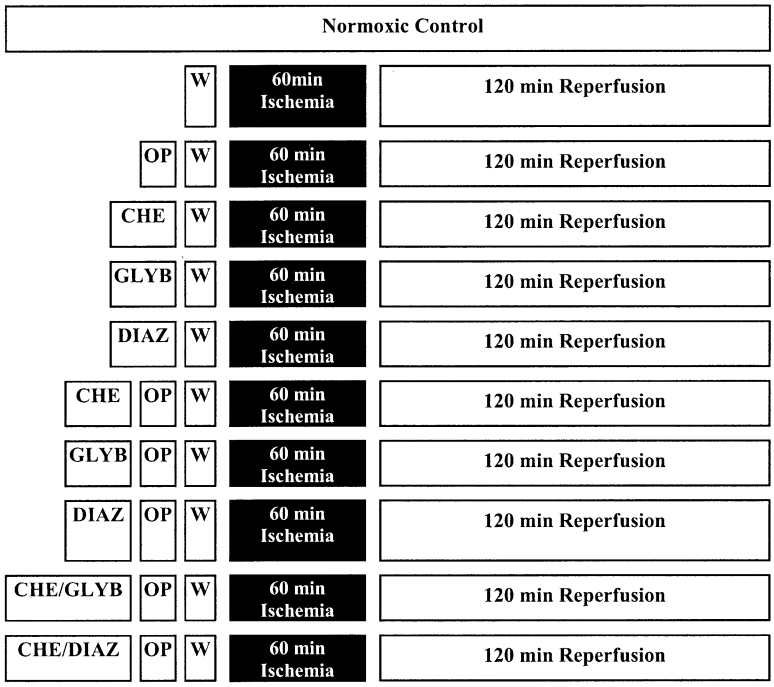

FIG. 1. Experimental Design to Assess the Impact of PKC inhibitor and K_{ATP} channel agonists/antagonists on DADLE cardioprotection of HL-1 cells. In a 96-well plate format, 10 uM Opioid DADLE(OP), 2uM (CHE), 100 uM Glybenclamide (GLYB), 100 uM Diazoxide (DIAZ) were administered in the above combinations for 10 min/washout/10 min of OP or control/washout. Plates then subjected to 60 min of simulated ischemia/180 min reperfusion.

for 10 mM glucose to limit glycolysis. Ischemic BSS was pre-equilibrated to limit dissolved oxygen by bubbling with 95%N_2/5%CO_2 for 30 min at 37°C. Next, simultaneous, parallel media changes with normoxic and ischemic BSS occurred before placement in a Billups-Rothenburg Modular Incubator Chamber (Billups-Rothenburg, Del Mar, CA). The chamber was flushed with 95%N_2/5%CO_2 at 20 L/min for 10 min, monitored by an in-line Flow-Meter. Oxygen levels were also monitored in-line using the Qubit Oxygen Sensor (Qubit Systems, Kingston, ON). The hypoxia chamber was then sealed and placed in the 37°C incubator, as was the normoxic control. After 1 h, both normoxic and ischemic plates were removed and exposed to room air. All plates then underwent a media change with fresh, normoxic BSS. Plates were subsequently returned to a 37°C incubator for 180 minute of reperfusion.

LDH Assay

From 48 (Study I) and 96-well (Study II) microplates, media was collected into microcentrifuge tubes and briefly spun at 200 × *g* to remove possible cell debris. Cytoplasmic LDH is released from cells exhibiting a loss of plasma membrane integrity, as typically occurs from primary and secondary necrosis. LDH activity was measured using the CytoTox 96® assay (Promega, Madison WI) by the reduction of lactate to pyruvate in the presence of NAD^+. The resultant NADH reduces INT, a tetrazolium salt, to form a red formazan product that is detectable at 490 nm. Results were read on a BioTek microplate reader (Bio-Tek, Winooski, VT) and quantified using Delta-Soft3 Software (BioMetallics, Princeton, NJ). Results were normalized to maximal LDH release following treatment with 0.8% Triton X-100 as directed by the manufacturer. Study I timecourse of LDH release was determined by sampling the media at 90, 120, and 180 minutes of reperfusion. Study II LDH release data was obtained only at 180 min of reperfusion. For graphical purposes, data from the LDH release was further transformed as % untreated control. Results were pooled from four separate experiments and expressed and mean ± SEM. Statistical analysis was performed using the paired *t* test, with $P < 0.05$ considered significant.

RESULTS AND DISCUSSION

LDH/Necrosis Measures

Study I time-course data from opioid-preconditioned HL-ls reveal a consistent and statistically significant decrease in necrosis at various points of reperfusion (Fig. 2). More importantly, this effect is reversed by delta-opioid antagonist naltrindole co-administration. This data supports previous findings in primary ventricular myocytes showing reduced necrosis from opioid preconditioning [10, 30].

Studies using whole heart and primary atrial/ventricular models indicate that opioid-preconditioning manifests in PKC activation, and that PKC inhibition limits or abolishes cardioprotection. In addition, the preconditioning pathway of PKC phosphorylation-mediated K_{ATP} channel opening is well documented in several species. To assess the utility of HL-ls as a model of opioid preconditioning, we evaluated the impact of PKC inhibitor chelerythrine (CHE), non-selective K_{ATP} channel closer glybenclamide (GLYB), and mitochondrial K_{ATP} channel opener diazoxide (DIAZ) (Fig. 1).

Study II findings shown in Figs. 3–5 indicate that

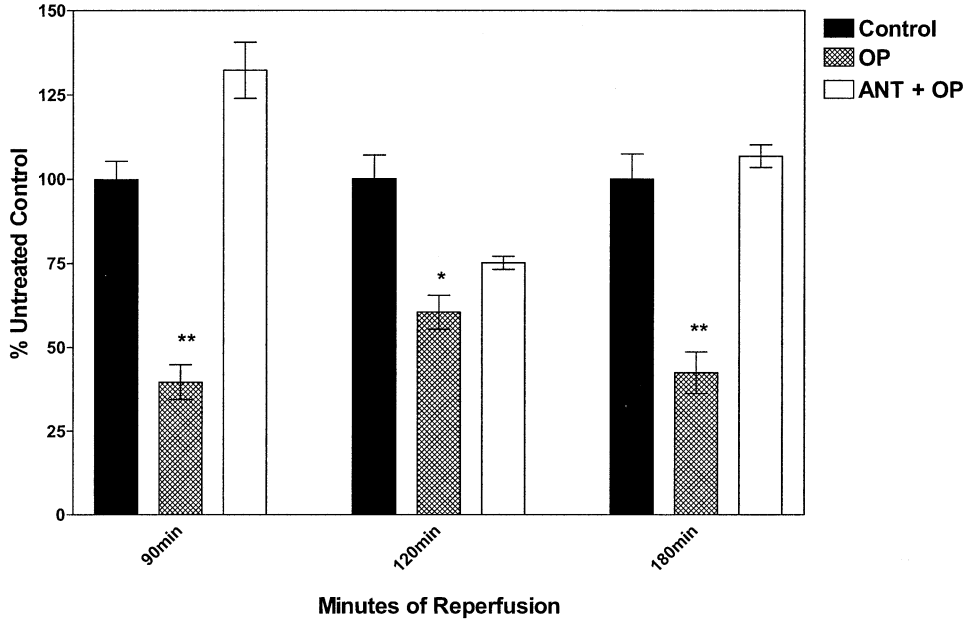

FIG. 2. Timecourse of LDH Necrosis Measures during Reperfusion of delta-opioid agonist/antagonist treated HL-1 Myocytes. Cells were pretreated with control media, 10uM DADLE(OP), or 10 uM DADLE + 10 uM antagonist naltrindole (ANT) for 10 min, followed by a 5 min washout, then 60 min of simulated ischemia/180 min reperfusion. Media sampled at 90, 120, and 180 min of reperfusion. $N = 9$ in each group, presented as % of untreated control. Paired t-test versus untreated control raw O.D.$^{**}P < 0.0001$, $^{*}P < 0.01$.

DADLE(OP) cardioprotection in HL-1 involves PKC activity. As shown in Fig. 3, OP-only pre-treatment significantly decreased necrosis by 44% versus ischemic control ($P < 0.05$). In contrast, pre-incubation with PKC inhibitor CHE significantly increased necrosis over untreated ischemic control (70% increase). However, CHE/OP co-administration decreased death by approximately 40% as compared to CHE-only treatment. Therefore, the known PKC agonist activity of opioid pretreatment appeared to counter the PKC antagonism of CHE, reflected in enhanced cytoprotection.

K_{ATP} channel closer results also reflect findings in

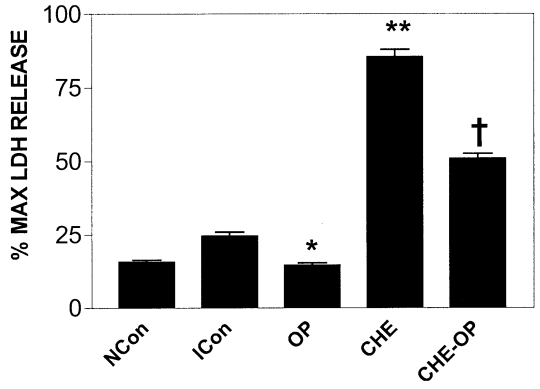

FIG. 3. Chelerythrine Inhibition of delta-opioid HL-1 myocyte cardioprotection. 10 uM DADLE(OP) or 2 uM (CHE) were administered for 10 min followed by washout before 60 min simulated ischemia followed by 180 min reperfusion. Additional CHE/OP group consisted of 10 min CHE/washout/10 min OP/washout, followed by ischemia/reperfusion. Data expressed as % Maximum LDH release accomplished by Triton X-100. $N = 4$ for each treatment. Paired t-test as compared to ischemic (untreated) control. $^{*}P < 0.05$, $^{**}P < 0.0001$. Paired t-test comparing CHE and CHE/OP depicted as $\dagger P < 0.01$.

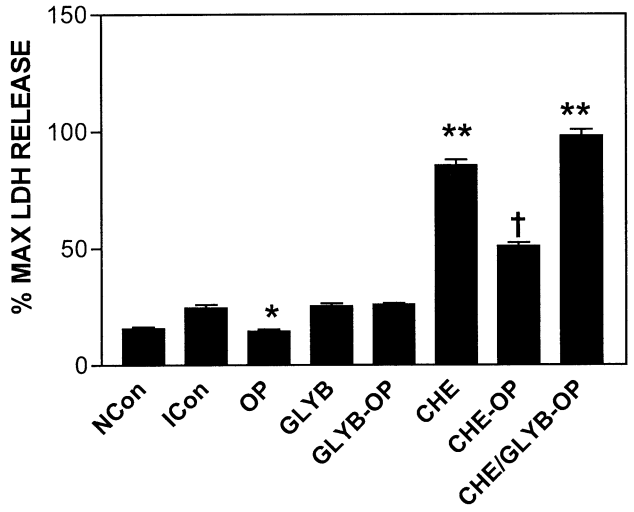

FIG. 4. Glybenclamide Inhibition of delta-opioid HL-1 myocyte cardioprotection. 10 uM DADLE(OP) or 100 uM (GLYB) were administered for 10 min each followed by washout before 60 min simulated ischemia followed by 180 min reperfusion. Additional CHE/GLYB group consisted of 10 min CHE/10 min GLYB/washout, followed by ischemia/reperfusion. Data expressed as % Maximum LDH release accomplished by Triton X-100. $N = 4$ for each group. Students t-test as compared to ischemic(untreated) control. $^{*}P < 0.05$, $^{**}P < 0.0001$. Paired t-test comparing CHE, CHE/GLB-OP versus CHE/OP depicted as $\dagger P < 0.01$.

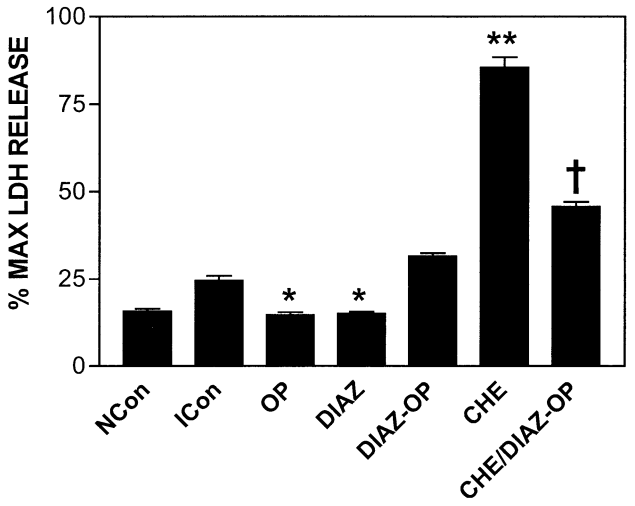

FIG. 5. Diazoxide Mimics delta-opioid HL-1 myocyte cardioprotection. 10 uM DADLE(OP) or 100 uM (DIAZ) were administered for 10 min each followed by washout before 60 min simulated ischemia followed by 180 min reperfusion. Additional CHE/DIAZ group consisted of 10 min CHE/10 min DIAZ/washout, followed by ischemia/reperfusion. Data expressed as % Maximum LDH release accomplished by Triton X-100. $N = 4$ for each group. Students t-test as compared to ischemic (untreated) control. $^{*}P < 0.05$, $^{**}P < 0.0001$. Paired *t*-test comparing CHE and CHE/DIAZ-OP depicted as $\dagger P = 0.01$.

primary ventricular myocyte models and isolated heart models. As shown in the GLYB-only group in Fig. 4, K_{ATP} channel antagonism alone was not cytotoxic versus untreated ischemic control, which supports findings in whole heart using non-selective K_{ATP} channel closer GLYB [31] and selective, mitochondrial K_{ATP} closer 5-hydroxydecanoate (5-HD) [32–35]. Yet, pretreatment of HL-1 with GLYB eliminated the benefit of subsequent OP administration, and GLYB-OP resembled ischemic control. This result indicates that DADLE cytoprotection of HL-1 myocytes is K_{ATP} channel dependent. Although the most deleterious combination, CHE/GLYB-OP was not significantly cytotoxic versus CHE alone, indicating that relative to the impact of GLYB, PKC antagonism was largely responsible for cytotoxicity.

Figure 5 shows that the selective $MitoK_{ATP}$ channel opener DIAZ closely mimicked OP-only treatment reduced cytotoxicity, which supports the importance of the $MitoK_{ATP}$ channel in HL-1 opioid cytoprotection. Cytoprotection from DIAZ is well documented in both ischemic and pharmacologic preconditioning studies [11, 36], though DIAZ has been shown to also open $SarcK_{ATP}$ in conditions of high ADP levels, as occurs during severe or prolonged ischemia [37]. The relative cardioprotective contributions of sarcolemmal and mitochondrial K_{ATP} channel opening continue to be revealed. The consequences of Sarc/Mito channel opening impact different measures of cardioprotection. $MitoK_{ATP}$ opening primarily affects mitochondrial integrity and aerobic capacity during reperfusion, and contributes to redox signaling mechanisms of cardioprotection [38]. $SarcK_{ATP}$ channel increases potassium efflux from the cell, hastening repolarization and shortening action potential duration [39]. Hyperpolarization reduces Ca^{2+} entry through the L-type calcium channel, reducing Ca^{2+} overload and protecting osmotic balance. However, opening of the sarcolemmal K_{ATP} channel may also promote opening of the mitochondrial channel. As demonstrated by Waring, hyperpolarization by $SarcK_{ATP}$ channel opening may trigger increased phospholipase D activity [40]. Phospholipase D activity is proposed to contribute to pre-conditioning [41, 42] by increasing diacyl-glycerol formation and PKC activation, which phosphorylates and accelerates $MitoK_{ATP}$ channel opening [43].

The results of the current study do not differentiate the relative contribution of the mitochondrial versus sarcolemmal K_{ATP} channel. The cytoprotective impact of K_{ATP} channel openers may depend upon oxygen conditions and the model employed. Though diazoxide shows a 2000-fold preference for the $MitoK_{ATP}$ channel in normal oxygen conditions [44], this preference is reduced in ischemic conditions and permits the opening of $SarcK_{ATP}$ channels. In isolated hearts undergoing ischemia/reperfusion, Tanno demonstrated that selective $SarcK_{ATP}$ channel blockers were able to attenuate, though not abolish the infarct reducing effect of diazoxide [45]. This result suggests that diazoxide had opened both $MitoK_{ATP}$ and $SarcK_{ATP}$, and that $SarcK_{ATP}$ opening contributed to cytoprotection. However, in the isolated myocyte model, Sato *et al.* showed that the cytoprotective benefits of diazoxide were independent of $SarcK_{ATP}$ status [46]. Further investigations of our group are underway to ascertain the HL-1 preconditioning impact of both channel types, by using specific $SarcK_{ATP}$ closer HMR 1098 and $MitoK_{ATP}$ closer 5-HD.

Interestingly, the benefits of OP and DIAZ mediated K_{ATP} opening were not additive. As shown in Fig. 5, DIAZ/OP combination increased death versus either agent alone, so that DIAZ/OP resembled untreated control. Supportive work by Liu, Marban, Kowaltkowski, and Garlid has shown that excessive opening of mitochondrial K_{ATP} channels leads to increased mitochondrial membrane potential, reduced reperfusion aerobic respiration capacity, and increased mitochondrial calcium uptake [47], concluding with mitochondrial osmotic swelling and cell death [48]. However, in the CHE/DIAZ-OP group, the addition of DIAZ-OP reduced death by approximately 53% as compared to CHE alone. The current data demonstrate that the addition of mitochondrial K_{ATP} openers(DIAZ or OP) downstream of PKC partly counters the toxic effects of PKC antagonism by CHE. Therefore, PKC activation appears to be an essential upstream event in HL-1 opioid pre-conditioning.

Collectively, our LDH assay results indicate that in the HL-1 cultured myocyte model, delta-opioid preconditioning enhanced viability in a PKC and K_{ATP}-channel dependent manner. HL-1 opioid cytoprotection appears to be exclusively enacted through the delta-opioid receptor. Previous work by our group [27] used saturation binding and competition binding assays to determine the presence of delta-receptor binding on HL-1 membranes (using DADLE, DPDPE, reversible by naltrindole), and to demonstrate the lack of selective mu- and kappa-receptor binding (using DAMGO, CTAP, and U69,593). Furthermore, HL-1 demonstrated broad stimulation delta-opioid mediated (^{35}S)GTPγS binding, with an efficacy ranking of DADLE > SNC80 > DPDPE > DSLET > deltorphin II at a 10 uM concentration, the concentration used in this study. DADLE cytoprotection occurs at doses from 10 uM to 10 pM (data not shown), however, the higher dose was used in the current study due to previously confirmed 10 uM and 1 uM DADLE stimulation of GTPγS binding (+26.6% and +18.4% above control, respectively). The results of the current study indicate that HL-1 opioid cardioprotection is accomplished through similar pathways of other primary isolate myocyte preconditioning models, including rat [11, 36], chicken [12, 49], rabbit [16, 50–52], pig [53–55], and human [18, 21, 22].

Primary Myocyte Culture *versus* HL-1 Cell Culture

The use of an immortalized cell line has several advantages over primary isolates. Primary isolations of ventricular myocytes inevitably yield heterogeneous cell populations, including fibroblasts, endothelial cells, and leukocytes [56]. Attempts to promote cellular homogeneity include the use of fibroblast inhibitors, differential gradients during isolation, and pre-plating methods to selectively remove fibroblasts. Immunological detection of myocyte-specific markers is required to verify their percentage among the isolated cells [57]. Viability and resilience of the isolated myocytes is dependent upon the isolation procedure, species, and age of animal utilized.

In addition to variable yields, primary cell isolation depletes myocytes of the endogenous antioxidant reduced glutathione (GSH), rendering them susceptible to oxidative injury. Reiners and colleagues showed that adherent monolayer cultures exposed to trypsin digestion required approximately 24 h to recover basal levels of reduced glutathione [58], which were decreased by 40–95% from standard passaging techniques. Because the isolation of primary myocytes from whole heart requires considerable mechanical manipulation and enzymatic digestion, one can speculate that the GSH loss in viable cells would be similar if not elevated from adherent cell passaging techniques. The depleted antioxidant reserve would likely impact the results of preconditioning mechanisms that rely, in part, on redox signaling. Under this consideration, investigations affected by free radical dynamics must allow sufficient recovery time between isolation and the induction of experimental interventions. This lag time unpredictably reduces the population of viable myocytes; this reduction may impact spontaneous [Ca^{2+}]i transients and contractile activity [59] that are affected by the density of viable myocytes [60–62].

In contrast, immortalized cells preclude animal use, are more economical, have predictable yields, have high-throughput capability, and are homogeneous. Depletion of GSH upon passage is remedied by the 3 to 4 day period between plating and experimentally appropriate confluency. HL-1s can be successfully restored from frozen cultures, allowing extended passage. As Claycomb and colleagues have described previously, HL-1 cells can be serially passaged while maintaining differentiated cardiomyocyte morphological, biochemical, and electrophysiological properties [28]. As we demonstrated previously, HL-1s possess functional delta-opioid receptors, the dominant opioid receptor sub-type in human myocardium [22]. As such, HL-1 myocytes may offer an alternative model of delta opioid modification of ischemia/reperfusion injury.

CONCLUSIONS

Our laboratory previously characterized the presence of functional delta-opioid receptors on the HL-1 atrial myocyte. Current findings indicate a PKC and K_{ATP} channel-dependent delta-opioid specific cytoprotection from simulated, in vitro ischemia/reperfusion injury. The HL-1 cell line presents an economical, high-throughput alternative for elucidating the elusive effectors of delta opioid cardioprotection.

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