Alternative Strategy for Stress Tolerance: Opioids

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Endogenous opioids have been implicated in the pathway of tolerance to stresses. Hibernating tissues tolerate stress. Serum from hibernating woodchucks (hibernation induction trigger [HIT]), from summer nonhibernating animals (summer woodchuck active plasma [SWAP], and potential “hibernation opioid mimics” (D-Ala2 D Leu5 Enkephalin [DADLE]), and Deltorphin D (Delt D) were used as ischemic preconditioning agents (IPC) in an in vivo surgically induced cardiac ischemia rat model. Comparison of the IPC treatment was monitored by the molecular intensity of stress transcripts for polyubiquitin and HSP70 in Northern blot analyses. Delt D and HIT significantly reduced total polyubiquitin transcript expression, 2.1-fold and 1.4-fold, respectively, in ischemic tissue, while SWAP and DADLE did not differ from saline controls. The Delt D effect was sensitive to glibenclamide (Glb), a KATP (potassium adenosine triphosphate) channel blocker. No inducible HSP70 was detected. The demonstration of an opioid IPC modulation of the ubiquitin stress pathway found here may be relevant for development of drug intervention in heart attacks and stroke.

ISCHEMIC heart disease is a leading cause of mortality, and the risk increases with age. It is well established that the stress response is a critical determinant of survival and longevity (1,2). Threshold levels of stress elevate protective repair enzymes, and antioxidant enzymes are known to provide cross-resistance to stress from different stressors (heat, exercise, ultraviolet light, ischemia, toxins). The use of preconditioning agents to activate protective mechanisms, without exposure to potentially damaging toxic agents, is clearly an attractive alternative strategy to improve cell survival, organ preservation, and life quality.

Naturally occurring opioids have been implicated in protective strategies for humans. Frog skin secretions with peptides including deltorphins have been used by Peruvian Indians to increase physical strength, heighten senses, resist hunger and thirst, and induce fearless emotion (3). Profound neurological and cardiovascular responses to the endogenous opioid family members are well documented from the enkephalins with specificity for delta opioid receptors, the endorphins for mu, delta, and kappa receptors, and the dynorphins, which have an affinity for the kappa receptors (4). Opioid receptors have been found both in the central nervous system and periphery to allow rapid changes in physiology and response to stress.

Hibernating mammals’ myocardium tolerates the stress of depleted energy stores, intracellular acidois, hypoxia, hypothermia, and cellular volume shifts for extended periods. The physiology of the hibernating bear is similar to human ischemia and infarction, but is not tolerated in humans and results in a significant proportion of the health problems in the United States today. Recently, hibernating bears were found to maintain muscle strength using a contractile strength assay, which is unlike rodents and humans who lose strength when immobilized for a long time (5). The ability to utilize hibernation factors in the pathway for tissue preservation in aging humans to delay sarcopenia or reduce ischemic damage in cardiovascular or neurological occlusion episodes would have potential clinical application.

Characterization of the hibernation induction trigger (HIT) indicated that it was an 88 KDa peptide with opioid characteristics isolated from the albumin fraction of blood plasma from winter hibernators (6). Evidence of the opioid nature of HIT was indicated by induction of hibernation in summer active squirrels by the winter hibernating woodchuck albumen and by the delta opioid DADLE (D-Ala2 D Leu5-Enkephalin acetate [Tyr-D-Ala-Gly-Phe-D-Leu]), which were reversible by opiate antagonists (7). DADLE is a nonselective delta opioid that binds to both delta1 and delta2 receptors. Both HIT and DADLE were used successfully as ischemic preconditioning (IPC) agents to preserve organs for transplantation or after cardiac ischemia (8–11). Opioid preconditioning was also found to mimic ischemic preconditioning in human heart muscle (12).

Another synthetic delta opioid, Deltorphin D (Delt D; Tyr-D-Ala-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH3), a specific delta2 receptor subtype agonist, has been found to be an effective myocardium IPC agent that reduces infarct size (12,13). Delt D, not DADLE, was more effective in the swine in vivo cardiac ischemic model suggesting differences in the protective pathways of the two opioids (14). In addition to cardiovascular effects, neuroprotective roles for delta opioid receptors have also been documented (15).

Mechanisms operative in opioid preconditioning are not well understood. The exogenously supplied opioid agonists, used for in vitro and in vivo cardiovascular protection, mediated reduced infarct size or decreased cell death in...
cardiomyocytes via activation of Gi/o proteins, protein kinase C, or K\textsubscript{ATP} channels (16–20). Both delta and kappa opioid receptors were implicated in cardioprotection using sarcolemma and mitochondrial K\textsubscript{ATP} channel pathways (18–20). The ischemic preconditioning benefit was found sensitive both to opioid antagonists and K\textsubscript{ATP} channel blockers.

Another major pathway operative in cell and organ protection is the stress response. Two known major stress molecular responders are heat shock HSP70 and polyubiquitin (UbB and UbC), which act as molecular chaperones to aid in refolding denatured proteins and degradation of damaged proteins (21).

A direct relation between the amount of heat shock protein induced and the degree of myocardial protection has been found (22). Inducible HSP70 responded to kappa opioid activation by U-50488H pretreatment in rat ventricular myocytes (23).

Ubiquitin has a major role in the proteolytic regulation of cellular control mechanisms, removal of denatured proteins, and participation in end-stage cell death/apoptosis. At least three unique genes coding for ubiquitin are well known. UbA, UbB, and UbC comprise the ubiquitin coding regions (24,25). Constitutively expressed UbA is a monoubiquitin coding sequence fused to a ribosomal protein that has not been shown to be inducible under stress. The stress-inducible polyubiquitin genes, UbB (4 tandem ubiquitin repeats and unique 3' region, 1.2 Kb) and UbC (10 multiple tandem ubiquitin repeats and a unique 3' sequence, 2.5 Kb) have been characterized in the rat (25). Different species show variations in the number of tandem ubiquitin coding units in UbB and UbC genes (24). The differences in transcript sizes make identification of polyubiquitins UbB and UbC easily identified on electrophoretic gels and allow determination of differential expression of the ubiquitin gene transcripts (24). Differences in 3' sequence regions also allow detection of unique polyubiquitin transcript expression in response to ischemic stress during in situ studies (25). Polyubiquitin has been shown to have a heat shock element (26) and was found to be essential for resistance to heat and starvation in yeast (27) as well as in mammalian cells (28). Polyubiquitin expression also has been found in response to ischemic stress both in the heart and brain (29–31). In a time of protein sparing, during hibernation, the ubiquitin–proteosome degradation pathway is suppressed (33). Thus inhibition of the degradation pathway may be a critical pathway in cytoprotective survival strategy.

Of the 3 ubiquitin transcripts, UbC induction was the most pronounced in brain ischemia (30) as well as in response to the stress of ultraviolet irradiation (33), tumor growth (34), during differentiation (35), and anaerobic exercise (36). UbC therefore may be a good indicator of ischemic damage.

The previous data indicate a general polyubiquitin response to stress, and specifically to ischemia stress. The current study is the first to evaluate the effects of opioid IPC on differential ubiquitin and HSP70 response to ischemic stress using HIT and the synthetic delta opioid agonists Delt D and DADLE, each with unique delta subtype receptor agonist specificities. Comparison between stress molecule response to natural HIT with the synthetic delta opioids were therefore examined.

The rat in vivo surgical ischemic model was used since the pathology in the rat has been shown to relate to clinical ischemic heart disease. The progression in the rat to heart failure is similar to what happens when a patient sustains a large myocardial infarction, survives, and goes on to develop heart failure without another ischemic insult (37). The present study employs the surgical constriction of the left coronary artery without reperfusion to induce ischemia. This approach avoids the difficulty of interpreting molecular response due to ischemia versus the additional effects of reperfusion injury (38).

The assay of IPC effects at 20 minutes and 24 hours postocclusion were chosen to determine the stress transcripts environment at the onset of the hostile ischemic challenge, which may reflect a prestress environment or endocrine responses (39), and at 24 hours, when a second window of opioid ischemic preconditioning has been reported by some investigators (18).

The hypotheses to be tested are: 1) that IPC will alter the initial ischemic cardiovascular stress environment; and 2) that different naturally occurring (HIT) and synthetic preconditioning agents DADLE and Delt D may differ in their modulation of the stress response.

Methods

Pretreatment of Subjects

Six-to-eight-week-old male rats (~250 g) were randomly divided into groups of 6–11 animals each. Surgical groups included animals pretreated with either saline, SAWP (summer active woodchuck plasma; 1 ml/kg), HIT (hibernating woodchuck serum; 1 ml/kg), Delt D (ZymoGenetics, Inc., Seattle, WA; 1 mg/kg), DADLE (1 mg/kg), and Glibenclamide (Glb; 1 mg/kg Sigma Aldrich). The doses used and regimen of injection in the tail vein was 2 days, 1 day, and 1 hour prior to surgery as had been used previously to improve postischemic myocardial function in rabbit isolated hearts (11). The Gb injections were given 30 minutes prior to HIT or Delt D. Pretreated subjects were randomly divided for coronary artery occlusion into either 20-minute or 24-hour occlusion to obtain an early and late window of molecular response to ischemic stress. Control group rats underwent pretreatment without surgical ischemia.

Ischemia Induction

Aseptic surgical procedures set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals were followed throughout all surgeries. Anesthesia was obtained with 0.15 ml/300 g ketamine and 0.1 ml/300 g Xylazine administered intraperitoneally to surgically induce the myocardial infarction (MI). The animals were intubated and placed on a rodent respirator (Harvard model 683; Harvard Apparatus, Holliston, MA). Briefly, the heart was exposed through a left thoracotomy between the fifth and sixth ribs, and a pericardectomy was performed to allow visualization of the left anterior descending coronary artery, which emerges from beneath the left atrial appendage. A deliberate attempt was made to ligate this vessel 3–4 mm distal to the left atrium to produce small-to-medium-sized infarcts by passing a 6-0 Cardiopoint suture (U.S. Surgical,
Norwalk, CT) under the artery. Previous experience has shown that a larger MI is produced if the artery is occluded within 1–2 mm of the left atrium as opposed to more distally. Successful ligation was verified by an immediate blanching of the affected area as well as the appearance of Q-waves and/or elevated ST segments on the electrocardiogram. Muscle and skin incisions were immediately closed with separate purse string sutures, and the lungs were fully expanded (40).

Tissue Collection

The heart was excised and the right ventricle was dissected away from the left ventricle either 20 minutes or 24 hours postocclusion. The tissue postsurgery was divided into ischemic (infracted) and nonischemic areas identified by both color and akinesia differentiation. The infracted area, distal to the occlusion, was already bulged due to lack of contraction and blanched so that the infracted area is easily detected and dissected from the remaining viable, nonischemic, nonbleached left ventricle free-wall myocardium. Nonsurgical controls had the left ventricle region excised. All tissue samples were promptly flash-frozen in liquid nitrogen and stored at −80°C prior to RNA extraction and Northern blot analysis.

Northern Blot Technique

RNA was isolated using Tri-Reagent protocol (Molecular Research Center, Cincinnati, OH), which uses a phenol-guanidinethiocyanate monophase reagent. Northern blots were prepared and assayed as described previously (36). Electrophoresis using 1% formaldehyde-agarose gel separated RNA fragments. RNA was stained with ethidium bromide, and only those samples with the characteristic 18S and 28S ribosomal bands were analyzed. Transfer to blots, hybridization, and washes followed the protocol (Amerham, Arlington, IL). The DNA probes were for ubiquitin pBR130, which contains the ubiquitin coding sequence obtained from Dr. Rohan Baker (Canberra, Australia) and inducible HSP70 (Sressgen Biotechnologies, San Diego, CA, or Sigma Biochemicals, St. Louis, MO). Since the two inducible polyubiquitins UbB and UbC differ in fragment size, the separated fragments on an electrophoretic gel are easily detected with the pBR130 probe. Control probes, glyceraldehyde 3-phosphate dehydrogenase, 18S ribosomal RNA (Ambion, Inc., Austin, TX), or 18S ethidium densities were used as nonchanger standards to normalize RNA concentrations in each sample by dividing the inducible probe density by the density of the standard after subtraction of background density of the film. The blots were hybridized with the ubiquitin probe, analyzed for signal intensity, stripped, and reprobed with control or HSP70 probes. Within each experiment, all experimental and control groups were exposed to the same radioactive probe, development time, and noninducible probes. Levels of gene expression were quantified by densitometric scanning of the radioactive signals from the specific inducible and control probes. The ratio-inducible transcript/noninducible control density represent the experimental values. The mean values from each control and experimental samples were obtained and subjected to statistical analyses for significance between treatments.

All probes were labeled with 32P-dCTP (New England Nuclear, Boston, MA) using the Random Primers Labeling system (Gibco BRL, Invitrogen, Carlsbad, CA) according to manufacturer’s specifications. The signals were assayed by taking a computer image of the ethidium bromide-stained gel and the autoradiograph film using Gel Doc software (BioRad Corp., Hercules, CA). The computer image was then used to determine the respective density of the samples by using Molecular Analyst software (BioRad). The software allows drawing areas around signals from each sample, which then receive numerical density values.

Statistical Analysis

All data are expressed as a mean value ± the standard error (SE) of the mean. Statistical differences were detected by analysis of variance (ANOVA) using SPSS (Chicago, IL) with least significant difference (LSD) post hoc analyses. P values less than .05 were considered significant. Repeated measures analyses were used for analysis of the 24-hour postocclusion time interval.

RESULTS

20-Minute Ischemic Interval Postocclusion: Alteration of Stress Response

Figure 1 shows sample results of pretreatment with the IPC agents on Northern blots in surgically treated animals. Tables 1 and 2 provide the means and standard errors for the ratios of ubiquitin signal normalized for RNA concentration (ubiquitin density/control probe density). Figure 2 shows the fold changes in ubiquitin transcript expression from surgical and nonsurgical animals relative to their respective saline controls (ischemic Delt D vs ischemic saline).

20-Minute Postocclusion: Total Ubiquitin

Ischemic tissue.—The total ubiquitin expression in ischemic tissue in saline-pretreated animals was not significantly elevated relative to nonsurgical animals (Table 1 and Table 2) at this 20-minute occlusion window, 1.4-fold (3.66/2.66, Table 2). Pretreatment with either HIT or Delt D, not DADLE or SWAP, showed significantly reduced (> .05) mean total ubiquitin (UbB + UbC), 1.4 and 2.0, respectively (3.66/2.56, 3.66/1.71) relative to saline-only ischemic tissue (Tables 1 and 2).

Nonsurgical tissue.—The nonischemic region did not show statistically significant differences in total ubiquitin transcripts relative to saline as a function of IPC treatment.

Nonsurgical Controls.—In nonsurgical controls, HIT treatment significantly reduced total ubiquitin transcripts (p = .031) 1.8-fold (2.66/1.49; Table 2). SWAP pretreatment did not reduce total ubiquitin expression. Delt D-pretreated nonsurgical controls were just below significance (p = .055), a 1.7-fold decrease (2.66/1.57; Table 2) compared with saline. IPC treatment with DADLE did not significantly reduce total or differential ubiquitin transcript levels (Figure 1, Table 1). Indeed, ubiquitin expression from
DADLE-pretreated animals exhibited higher ubiquitin transcript expression than saline-treated animals in all comparisons (Table 1), although the increase was not significant using the post hoc LSD statistic.

UbB

UbB transcript expression in cardiac ischemic tissue was significantly reduced from animals pretreated with both HIT and Delt D ($p = .023$, $p = .00$) relative to saline, 1.9-fold and 2.6-fold ($2.92/1.13, 2.92/1.55$), respectively. The nonhibernating plasma, SWAP and DADLE, did not induce any significant UbB transcript suppression in the ischemic heart (Figure 1, Tables 1 and 2, Figure 2B).

In nonischemic tissue and nonsurgical controls, opioid preconditioning did not reduce UbB significantly with respect to saline controls (Figure 1, Figure 2B, Tables 1 and 2).

UbC

Ischemic tissue.—Only Delt D pretreatment significantly suppressed the UbC transcript ($p = .02$) in cardiac ischemic tissue 2.4-fold ($1.37/0.588$; Table 2). Treatment with HIT, SWAP, saline, or DADLE did not significantly reduce UbC in ischemic tissue. No IPC significantly reduced UbC in nonischemic tissue.

Nonsurgical controls.—Although HIT reduced UbC transcript expression relative to saline 2.5-fold ($1.28/0.52$; Table 2), the reduction was not significant using the post hoc analyses. Delt D was 1.6-fold less ($1.28/0.82$; Table 2) than saline.

24-Hour Interval Postocclusion

At the 24-hour interval, no significant differences were detected between treatment groups using post hoc LSD analyses. The regions of the heart in surgical animals were pooled per treatment groups for repeated measure ANOVA. In surgical tissue, only Delt D was significantly reduced

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**Table 1. Differential Ubiquitin Transcript Expression 20 Minutes Postocclusion; Effects of DADLE on Ubiquitin Expression**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue (N)</th>
<th>Total Ubiquitin</th>
<th>UbB</th>
<th>UbC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IPC Surgical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DADLE</td>
<td>Ischemic (5)</td>
<td>$1.70 \pm 0.24$</td>
<td>$0.93 \pm 0.18$</td>
<td>$0.77 \pm 0.11$</td>
</tr>
<tr>
<td>Saline</td>
<td>Ischemic (5)</td>
<td>$1.20 \pm 0.19$</td>
<td>$0.56 \pm 0.08$</td>
<td>$0.64 \pm 0.18$</td>
</tr>
<tr>
<td>DADLE</td>
<td>Non-I (11)</td>
<td>$1.76 \pm 0.24$</td>
<td>$0.85 \pm 0.15$</td>
<td>$0.91 \pm 0.01$</td>
</tr>
<tr>
<td>Saline</td>
<td>Non-I (12)</td>
<td>$1.02 \pm 0.23$</td>
<td>$0.49 \pm 0.10$</td>
<td>$0.53 \pm 0.14$</td>
</tr>
<tr>
<td><strong>Nonsurgical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DADLE</td>
<td>Control (5)</td>
<td>$1.77 \pm 0.46$</td>
<td>$0.84 \pm 0.21$</td>
<td>$0.93 \pm 0.25$</td>
</tr>
<tr>
<td>Saline</td>
<td>Control (4)</td>
<td>$1.27 \pm 0.23$</td>
<td>$0.58 \pm 0.08$</td>
<td>$0.70 \pm 0.15$</td>
</tr>
</tbody>
</table>

*Note: DADLE = D-Ala$_2$ D Leu$^5$ Enkephalin; UbB = ubiquitin B; UbC = ubiquitin C; IPC = ischemic preconditioner.*
ischemia was monitored at 20 minutes and after 24 hours in the environment, and 2) the synthetic opioids may modulate the early differential ubiquitin stress response in a receptor-specific manner. In the present study, the early molecular response of IPC to ischemia was monitored at 20 minutes and after 24 hours when a second window of preconditioning had been found (18). It is known that coronary artery occlusion initiates a deleterious sequence of events, including the rapid cessation of contraction and conversion from aerobic to anaerobic metabolism, depletion of myocardial energy phosphate stores, and within 15–20 minutes, the onset of myocyte necrosis. Without opioid pretreatment, maximal HSP70 and ubiquitin expression have been detected 2 hours post ischemia/reperfusion (18,30). Initial stress response delay is presumably due to initial lack of energy supply. Therefore, the protective mechanisms induced by IPC, prior to onset of the hostile ischemic challenge, may represent the major cytoprotective resources available to the myocardium. In the present study, the ischemic tissue expression of total ubiquitin mirrored their respective nonsurgical controls.

**DISCUSSION**

Evidence was obtained for the first time in this study, which supports our hypothesis that 1) the use of opioid IPC agents will modulate the early differential ubiquitin stress environment, and 2) the synthetic opioids may modulate the molecular stress response in a receptor-specific manner. In the present study, the early molecular response of IPC to ischemia was monitored at 20 minutes and after 24 hours when a second window of preconditioning had been found (18). It is known that coronary artery occlusion initiates a deleterious sequence of events, including the rapid cessation of contraction and conversion from aerobic to anaerobic metabolism, depletion of myocardial energy phosphate stores, and within 15–20 minutes, the onset of myocyte necrosis. Without opioid pretreatment, maximal HSP70 and ubiquitin expression have been detected 2 hours post ischemia/reperfusion (18,30). Initial stress response delay is presumably due to initial lack of energy supply. Therefore, the protective mechanisms induced by IPC, prior to onset of the hostile ischemic challenge, may represent the major cytoprotective resources available to the myocardium. In the present study, the ischemic tissue expression of total ubiquitin mirrored their respective nonsurgical controls.

**Table 2. Differential Ubiquitin Transcript Expression 20 Minutes Postocclusion; Effects of Delt D, HIT, and SWAP on Ubiquitin Expression**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue (N)*</th>
<th>Total Ubiquitin</th>
<th>UbB</th>
<th>UbC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delt D</td>
<td>Ischemic (3)</td>
<td>1.71 ± 0.39</td>
<td>1.13 ± 0.18</td>
<td>0.58 ± 0.55</td>
</tr>
<tr>
<td>HIT</td>
<td>Ischemic (8)</td>
<td>2.56 ± 0.38</td>
<td>1.55 ± 0.25</td>
<td>1.03 ± 0.16</td>
</tr>
<tr>
<td>SWAP</td>
<td>Ischemic (6)</td>
<td>3.19 ± 0.67</td>
<td>1.94 ± 0.38</td>
<td>1.20 ± 0.18</td>
</tr>
<tr>
<td>Saline</td>
<td>Ischemic (5)</td>
<td>3.66 ± 0.45</td>
<td>2.92 ± 0.29</td>
<td>1.37 ± 0.18</td>
</tr>
<tr>
<td>Delt D</td>
<td>Non-I (3)</td>
<td>2.35 ± 0.36</td>
<td>1.35 ± 0.17</td>
<td>1.00 ± 0.19</td>
</tr>
<tr>
<td>HIT</td>
<td>Non-I (8)</td>
<td>2.11 ± 0.23</td>
<td>1.41 ± 0.20</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>SWAP</td>
<td>Non-I (5)</td>
<td>2.41 ± 0.44</td>
<td>1.45 ± 0.22</td>
<td>0.96 ± 0.23</td>
</tr>
<tr>
<td>Saline</td>
<td>Non-I (6)</td>
<td>2.98 ± 0.25</td>
<td>1.82 ± 0.20</td>
<td>1.16 ± 0.10</td>
</tr>
</tbody>
</table>

**Note:** Delt D = Deltorphin D; UbB = ubiquitin B; UbC = ubiquitin C; IPC = ischemic preconditioner; HIT = hibernation induction trigger; SWAP = summer woodchuck active plasma.

**Table 3. Ubiquitin Transcript Values Pooled at 24 Hours Postocclusion Using HIT and Opioid Pretreatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Total Ubiquitin</th>
<th>UbB</th>
<th>UbC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delt D</td>
<td>9</td>
<td>0.558 ± 0.113</td>
<td>0.391 ± 0.081</td>
<td>0.167 ± 0.037</td>
</tr>
<tr>
<td>HIT</td>
<td>15</td>
<td>0.798 ± 0.183</td>
<td>0.434 ± 0.067</td>
<td>0.364 ± 0.127</td>
</tr>
<tr>
<td>Saline</td>
<td>15</td>
<td>0.918 ± 0.140</td>
<td>0.515 ± 0.060</td>
<td>0.404 ± 0.116</td>
</tr>
<tr>
<td>SWAP</td>
<td>10</td>
<td>1.055 ± 0.387</td>
<td>0.817 ± 0.301</td>
<td>0.238 ± 0.086</td>
</tr>
<tr>
<td>Glb-Delt</td>
<td>14</td>
<td>1.660 ± 0.338</td>
<td>0.939 ± 0.197</td>
<td>0.721 ± 0.166</td>
</tr>
<tr>
<td>Glb-HIT</td>
<td>12</td>
<td>0.572 ± 0.066</td>
<td>0.352 ± 0.352</td>
<td>0.220 ± 0.029</td>
</tr>
</tbody>
</table>

**Note:** HIT = hibernation induction trigger; Delt D = Deltorphin D; SWAP = summer woodchuck active plasma; Glb-Delt = Glubenclamide-Deltorphin.

1.6-fold relative to saline only (1.37/0.58; Table 3). The Glb-Delt was 4.7-fold higher than Delt D (.72/0.17). No other comparisons were statistically significant.

Eight of 42 animals did not survive 24 hours after occlusion without reperfusion in Delt D only (3), Glb/Delt D (2), and Glb/ HIT (2).

**HSP70**

No significant inducible HSP70 was detected in this model at the time points used.

**Figure 2.** Ischemic preconditioning (IPC) agent effects on ubiquitin transcripts 20 minutes postocclusion. The fold reduction transcript expression after IPC was calculated by comparison to the respective saline control in ischemic or nonsurgical controls (from Tables 1 and 2; i.e., saline ischemic/Delt D (Deltorphin D) ischemic is 3.66/1.77 = 2.1-fold. The nonsurgical controls are also presented. Note that the fold reduction in total ubiquitin and UbB (A and B) in ischemic tissue mirrors the nonsurgical controls. The correlation is not as obvious in UbC (C) transcripts. HIT = hibernation induction trigger; SWAP = summer woodchuck active plasma; DADLE = D-Ala<sup>2</sup> D-Leu<sup>5</sup> Enkephalin.

**OPIOIDS**
treated with the synthetic or natural opioids. The ischemic tissue from animals treated with HIT and Delt D showed significantly reduced total ubiquitin as did the nonsurgical controls. Likewise, DADLE pretreatment did not exhibit reduced total ubiquitin in ischemic tissue or in nonsurgical control cardiac tissue. Ischemic tissue at the onset of ischemic stress showed at least partial maintenance of the prestress opioid-induced molecular environment.

Opioids did not increase HSP70 transcripts in nonsurgical or surgically treated animals in the present study, although a kappa opioid altered HSP70 expression in myocytes (23). Perhaps the response to HSP70 to opioids is receptor specific. In addition, HSP70 was not seen at the second window of preconditioning at 24 hours using opioid IPC here or when heat was used as the preconditioning agent (41).

In contrast to nonsurgical controls, the precondition treatment with both HIT and Delt D showed significantly reduced UbB transcript expression. Since SWAP serum did not alter the ubiquitin response, the effect of HIT serum was not due to a nonspecific serum response.

A Delt D-specific UbC transcript reduction was seen at 20 minutes and at 24 hours after occlusion. UbC elevation has been associated with damage levels (30), suggesting that reduction of UbC reflects reduced stress in the ischemic-challenged heart by opioid-specific IPC treatment. Differences in HIT and Delt D differential UbC response seen here may reflect separate modulation pathways or simply a difference in threshold opioid concentrations required for effect. The half-life of Delt D is 2 hours (42) and is more stable than HIT. In the hibernating animal, the production versus degradation process may favor serum with higher HIT concentrations.

In contrast to the reduction of ubiquitin expression by preconditioning with HIT and Delt D, DADLE did not reduce ubiquitin expression. Indeed, in all DADLE-treated animals, stressed or controls, inducible ubiquitin was increased. The difference in the ubiquitin response may reside in the differences in the specificity of the delta opioids since DADLE is a nonspecific subtype agonist, while Delt D is specific for subtype_2 receptors.

Previous studies have shown that DADLE, Delt D, HIT, and Tan 67, a delta subtype_1, can all reduce infarct size (14). Perhaps suppression of ubiquitin has a greater prominence in swine cardiovascular benefit than stimulation of ubiquitin pathways to promote cell death.

In the present study, ubiquitin suppression by Delt D after 24 hours was released by the K_{ATP} channel blocker glibenclamide implicating ubiquitin in the opioid K_{ATP} pathway at the 24-hour second window of protection. HIT was no longer effective after 24 hours perhaps due to decreased biological stability. Theoretically, the opening of K_{ATP} channels known to prevent intracellular Ca^{2+} overload (44–49) could alter the activity of the 26S proteosome required for the ubiquitin–adenosine triphosphate (ATP) proteosome degradation pathway, which is active in response to ischemia (43,50). Preconditioning with synthetic opioids may interfere with the implementation or progression of the ubiquitin-ATP proteosome pathway. Indeed, during hibernation, the proteosome degradation pathway is suppressed (32). The reduction in UbC at the 24-hour second window of protection may be a symptom of less damage.

A specific role for the stress-inducible UbB and UbC transcripts has been to replenish the free ubiquitin pool from the multiple tandem ubiquitin repeat gene products to target denatured or regulatory proteins for the degradation pathway. However, ubiquitination, like phosphorylation, is used in a wide variety of regulatory mechanisms that can be used to alter functional states including the reversible covalent modifications of histones, calmodulin, and cell membrane receptors. The polyubiquitin response must be crucial for survival since it is triggered by multiple stressors including heat, starvation (27), exercise (36), radiation (33), ischemia (30,31), and degenerative diseases (43), and has been conserved in such phylogenetically diverse organisms from yeast (27) to mammalian cells (28). It is also possible that the decline in the heat shock response of elderly people (51) may be ameliorated by pharmaceutical agents that stimulate tolerance to stress.

Differences in ubiquitin response to synthetic opioids as preconditioning agents may be influenced by multiple factors including: 1) opioid receptor specificity, since DADLE is a nonspecific delta agonist while Delt D is a delta_2 opioid receptor agonist subtype; 2) in vivo versus in vitro assay, since isolated organs and cells are not subject to the same neuroendocrine conditions as intact animal; 3) the half-life of the IPC agent; 4) species-specific responses, since swine responded to Delt D and not DADLE (14); 5) differences in transcript expression at different time windows postocclusion; 6) the sensitivity of the assay to detect significant differences; and 8) differences in the occlusion versus occlusion/reperfusion model.

**Conclusion**

The present study is the first to demonstrate an altered, suppressed ubiquitin response by opioid IPC using the naturally occurring HIT serum and the synthetic delta opioid subtype_2 Delt D. The nonspecific delta receptor opioid receptor agonist DADLE did not reduce ubiquitin transcript expression. Differential ubiquitin response may be a valuable diagnostic tool to monitor IPC drug effects on degradation
and restorative pathways. These findings potentially have implications for clinical medicine because hibernation and opioid-induced cellular protection impact on the stress response and thus protein regulation/conservation for survival of damaged tissue. Further studies are required to determine how changes in inducible ubiquitin expression impact the survival of stressed organisms.

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