Postnatal shifts in ischemic tolerance and cell survival signaling in murine myocardium

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The myocardial capacity to recover from I/R is governed in part by intracellular recruitment of prosurvival signaling pathways that modify metabolic activity, energy utilization, mitochondrial viability, intracellular repair mechanisms, autophagy, and oncotic and apoptotic cell death processes. This involves phosphorylation of key effectors including Akt (20), p38 mitogen-activated protein kinase (p38MAPK; Ref. 34), glycogen synthase kinase-3β (GSK-3β; Ref. 16), heat shock protein 27 (HSP27; Refs. 16, 34, 36), and connexin-43 (Cx43; Ref. 2, 37), among others. Hypoxia-inducible factor-1α (HIF-1α), a transcriptional regulator responsive to cellular Po2 (21), is also crucial in mediating adaptive resistance to stresses such as I/R, while caveolae and caveolin-3 (Cav-3) are key to membrane lipid-raft integrity and cell survival signal transduction from the plasma membrane (5, 39).

In contrast to adult hearts, the immature heart has been characterized as highly resistant to I/R stress, with enhanced functional tolerance, reduced cell death, and also evidence of enhanced metabolic or bioenergetic tolerance (25, 31). However, the mechanisms underlying this I/R resistance are not well defined, particularly with regard to prosurvival signaling and associated regulation of apoptosis and autophagy. Thus our study aims were to measure 1) postnatal shifts in myocardial I/R tolerance, and 2) the expression and phosphorylation of key proteins involved in regulating cell survival and autophagy vs. apoptosis. This is the first study to determine in the immature murine heart, postnatal changes in key prosurvival signal kinases and effectors (Akt, p38MAPK, GSK-3β, HSP27, Cx43, HIF-1α, and Cav-3), in addition to apoptotic regulators (Bax and Bcl-2; Refs. 12, 26), and controllers of autophagosome or final autolysosome formation (Beclin1, Parkin, light-chain 3β, or LC3β; Refs. 14, 17).

MATERIALS AND METHODS

Animal ethics. All animal experiments were approved by the Murdoch Childrens Research Institute and Griffith University Animal Ethics Committees and were performed in accordance with the Prevention of Cruelty to Animals Act (1986), under the guidelines of the National Health and Medical Research Council of Australia’s Code of Practice for the Care and Use of Animals for Experimental Purposes 7th Edition (2004).

Myocardial protein expression. Male C57Bl/6 mice (n = 6–8 per group) aged ~3 days (neonates) and 2, 4, 8, or 12 wk were killed by cervical dislocation, and hearts were rapidly excised and immediately frozen in liquid N2. These defined, critical periods of maturation were chosen based on ontogenetic studies in rats where the suckling period (transition from predominantly carbohydrate to fatty acid diet and the opening of eyes) occurs within 2 wk; the weaning period (transition from maternal milk consumption to solid food/water intake) at 4 wk;...
sexual maturation between 4 and 8 wk; and young adulthood from 8 wk (32).

Mice were handled at a similar time each day by a single experimenter to account for variability, and proteins were extracted under stringent conditions. Frozen hearts were ground to a fine powder in liquid N₂ using a prechilled mortar and pestle. Tissue (100 mg) was vortexed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA, 0.1% SDS, and 1% NP-40, with a cocktail of protease and phosphatase inhibitors; Sigma-Aldrich) and kept at 4°C for 45 min. Samples were then homogenized with a TissueRuptor (Qiagen) and centrifuged at 2,000 g at 4°C for 10 min to pellet cellular debris. The supernatant was transferred to a fresh tube, and the protein concentration was estimated via the bicinchoninic acid assay (Sigma-Aldrich). Western blotting of extracted proteins was performed with antibodies for Akt, p38MAPK, GSK-3β, HIF-1α, Bax, Bcl-2, Beclin1, Parkin, and LC3B.

**SDS-PAGE and Western blotting.** Samples (5–20 μg of protein) were loaded and separated on 10 or 15% SDS-PAGE. They were then electrotransferred to polyvinylidene fluoride membranes (100 V, 1.5 h) and blocked with 0.5% skim milk solution with Tris-buffered saline/Tween20 (TBST) using the Snap i.d. system (Merck-Millipore). Membranes were subsequently incubated with either total (1:1,000) or phosphorylated Akt (1:2,000), total (1:1,000) or phosphorylated p38MAPK (1:500), total (1:1,000) or phosphorylated GSK-3β (1:1,000), total (1:1,000) or phosphorylated HSP27 (1:1,000), total (1:1,000) or phosphorylated Cx43 (1:1,000), and total levels of Cav-3 (1:1,000), HIF-1α (1:500), Bcl-2 (1:1,000), Bax (1:1,000), Parkin (1:1,000), Beclin1 (1:1,000), and LC3B (1:1,000) in 0.5% skim milk solution for 1 h at 23°C on a tube roller. Membranes were returned to Snap i.d. cassettes and washed and probed with appropriate secondary antibodies (1:2,000). A final round of washing was followed by enhanced chemiluminescent detection (Perkin-Elmer) using medical imaging film (Amersham). Film was scanned at 600 dpi, and relative density calculations were performed by ImageJ (National Institutes of Health). Phosphorylated and total protein data were normalized to either GAPDH (1:10,000), β-tubulin (1:1,000), or histone H3 (1:1,000). All primary antibodies were purchased from Cell Signaling Technology, except for Cav-3 (BD Biosciences) and histone H3 (1:1,000). Secondary antibodies were purchased from Bio-Rad.

**I/R injury in perfused immature and adult hearts.** Isolated hearts from male C57Bl/6 mice (n = 6–8 per group) of young (4 wk) and adult (12 wk) groups were perfused in Langendorff-mode, as previously described (35, 41), and allocated to either: 1) normoxic perfusion; 2) 10 min of total global zero-flow ischemia (10IS); or 3) 25 min of ischemia followed by 10 min of aerobic reperfusion (25IR). The rationale for utilizing 4 wk animals was as a trade-off between their relative immaturity and physical heart size permitting for aortic cannulation, balloon insertion, and isolated perfusion. In addition, rodent weaning from maternal milk ceases at postnatal day 28 (32). Coronary arteries were perfused at a constant pressure of 80 mmHg with modified Krebs-Henseleit perfusion fluid (in mM: 119 NaCl, 11 glucose, 22 NaHCO₃, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 0.5 EDTA, and 2.6 CaCl₂), equilibrated with 95%O₂-5%CO₂ at 37°C, pH 7.4. The apex of the heart was vented with a polyethylene apical drain, and a fluid-filled balloon connected to a pressure transducer was inserted into the left ventricle (LV) via the mitral valve and inflated for continuous measurement of left ventricular pressure (LVP). All hearts were immersed in perfusion fluid to maintain temperature at 37°C. Changes in LVP were recorded on an 8-channel data acquisition system (AD Instruments) and digitally processed to determine heart rate, index of contractility (±dP/dt), peak systolic, end-diastolic, and developed (systolic – diastolic) pressures. Hearts were excluded from study if contractile function was unstable or cardiac arrhythmias persisted after 30 min of steady-state equilibration. After a 10-min baseline period of assessment at spontaneous heart rate, hearts underwent ventricular pacing at 420 beats/min (Grass S9 stimulator). At the end of experiments hearts were snap-frozen in liquid N₂ and stored at −80°C until sample preparation and analysis for protein expression as outlined above.

**Infarct size measurement.** An additional subgroup of mice (4 wk, n = 12: 12 wk, n = 6) underwent 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) infusion post-25I/R to determine infarct size, as previously described (28). Briefly, 1% TTC in PBS was infused into the coronary circulation via the aortic cannula. Hearts were fixed in 10% formalin for 24 h, sectioned into 1-mm slices, and weighed. Slices were scanned at a resolution of 600 dpi, and digital area analysis was performed by ImageJ (National Institutes of Health). Infarct size was normalized to slice weight and expressed as a fraction of the total area at risk (%AAR). Statistical analyses. Results are presented as means ± SE. Differences in baseline expression of proteins between age groups were assessed by one-way ANOVA. Age- and I/R-dependent differences in protein expression, and in cardiac responses were assessed via two-way ANOVA and Bonferroni post hoc tests. Correlations between age and protein expression were assessed by Spearman’s rho rank correlation test. Two-tailed statistical significance was accepted for P < 0.05.

**RESULTS**

**Postnatal growth and normoxic protein expression.** Figure 1A shows that body weight and total heart mass increase markedly during postnatal maturation. Notably, total heart mass in neonates (0 wk) more than doubles within 2 wk and achieves a plateau at 4 wk (~3-fold increase compared with neonate), while body weight progressively increases by ~10-fold from 0 to 12 wk. Figure 1 also shows postnatal changes in basal expression of total and phosphorylated Akt, p38MAPK, GSK-3β, HSP27, and Cx43. Phosphorylation is represented as a ratio of phosphorylated to total protein (P:T). Postnatal expression patterns for Akt, p38MAPK, GSK-3β, HSP27, and Cx43 are shown in Figure 1B. During postnatal maturation, Akt phosphorylation progressively increased with age to 8 wk and then decreased at 12 wk (Fig. 1). Postnatal changes in basal expression of Akt, p38MAPK, GSK-3β, HSP27, and Cx43 are shown in Figure 1C. Phosphorylation levels were initially high at 0–2 wk and then decreased to a minimum at 2–8 wk before rising again to 12 wk of age (Fig. 1). Expression of Cx43 progressively increased with age from a minimum at 0 wk.

In terms of phosphorylation status, Akt phosphorylation progressively increased with age to 8 wk and then decreased at 12 wk (though remaining elevated compared with the neonate). The phosphorylation of p38MAPK, GSK-3β, HSP27, and Cx43 tended to be initially high and declining from 2 wk (Fig. 1). A significant correlation was apparent between age and the P:T ratio for Akt, GSK-3β, HSP27, and Cx43 (see Table 2).

**Figure 2** presents data for postnatal changes in expression of apoptosis and autophagy regulatory proteins. Neonatal hearts expressed the highest levels of proapoptotic Bax (Fig. 2A) and antiapoptotic Bcl-2 (Fig. 2B), and the autophagy proteins LC3B (Fig. 2D) and Beclin1 (Fig. 2E), with protein expression levels all declining with postnatal maturation. Parkin levels were stable during postnatal maturation. Notably, total heart mass in neonates (0 wk) more than doubles within 2 wk and achieves a plateau at 4 wk (~3-fold increase compared with neonate), while body weight progressively increases by ~10-fold from 0 to 12 wk. Figure 1 also shows postnatal changes in basal expression of total and phosphorylated Akt, p38MAPK, GSK-3β, HSP27, and Cx43. Phosphorylation is represented as a ratio of phosphorylated to total protein (P:T). Postnatal expression patterns for Akt, p38MAPK, GSK-3β, HSP27, and Cx43 are shown in Figure 1B. During postnatal maturation, Akt phosphorylation progressively increased with age to 8 wk and then decreased at 12 wk (Fig. 1). Postnatal changes in basal expression of Akt, p38MAPK, GSK-3β, HSP27, and Cx43 are shown in Figure 1C. Phosphorylation levels were initially high at 0–2 wk and then decreased to a minimum at 2–8 wk before rising again to 12 wk of age (Fig. 1). Expression of Cx43 progressively increased with age from a minimum at 0 wk.

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I/R tolerance in immature vs. adult hearts. In assessing changes in I/R tolerance, isolated-perfused hearts from immature (4 wk) and adult (12 wk) groups were studied. Table 1 gives functional data for isolated hearts, during preischemic (normoxic) perfusion and 25-min global ischemia with 10-min reperfusion (25I/R). Figure 3, A and B, shows representative individual LV pressure traces for immature and adult hearts, respectively. Hearts were reperfused for 10 min to examine protein expression and phosphorylation changes in early reperfusion (and to acquire sufficient protein yields from viable myocardial tissue). Compared with adult hearts, immature hearts exhibited improved postischemic recoveries for LV developed pressure traces for immature and adult hearts, respectively. Hearts were reperfused for 10 min to examine protein expression and phosphorylation changes in early reperfusion (and to acquire sufficient protein yields from viable myocardial tissue). Compared with adult hearts, immature hearts exhibited improved postischemic recoveries for LV developed pressure traces for immature and adult hearts, respectively.  

Table 1. Left ventricular function in perfused hearts of immature and adult mice

<table>
<thead>
<tr>
<th></th>
<th>Immature (4 wk old)</th>
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<th>Adult (12 wk old)</th>
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<tr>
<td></td>
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<td>Postreperfusion</td>
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<tr>
<td>CF, ml/min</td>
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<td>LVDP, mmHg</td>
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<td>EDP, mmHg</td>
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<td>+dP/dt_{max}, mmHg/s</td>
<td>3,943 ± 491</td>
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Values are means ± SE; n = 6 hearts per group. Measurement of the left ventricular function parameters in hearts sampled immediately before onset of global ischemia (preischemia) and after 10 min of reperfusion (postreperfusion). Measurements of coronary flow (CF), left ventricular developed pressure (LVDP), end diastolic pressure (EDP), rate of left ventricular contraction (+dP/dt_{max}), and rate of left ventricular relaxation (−dP/dt_{max}) were sampled. Body weights were 4 wk, 16.1 ± 1.0 g, and 12 wk, 28.6 ± 0.5 g (P < 0.001). P values shown reflect preischemia vs. postreperfusion comparisons. *P < 0.05 vs. 4 wk.
Fig. 2. Basal markers of cell death in development. Baseline myocardial expression of Bax (A), Bcl-2 (B), Bax:Bcl-2 (C), LC3B (D), Beclin1 (E), and Parkin (F). Levels were assessed Western blotting of heart extracts across the specified developmental ages. Data are presented as means ± SE. *P < 0.05 vs. 0 wk; ^P < 0.001 vs. 0 wk.

The time to ischemic contracture in 4-wk-old immature hearts was not significantly different compared with adult hearts (395 ± 41 vs. 362 ± 63 s; P = 0.15). However, the mean amplitude of contracture in immature hearts was significantly smaller than the adult (61 ± 8 vs. 84 ± 8 mmHg; P = 0.02). Due to the model and limited severity of I/R, the present study was not designed to quantitatively examine reperfusion-induced arrhythmias. However, immature hearts had only occasional expression of arrhythmias, predominantly bigeminy waveforms. In contrast adult hearts exhibited reperfusion-induced arrhythmias ranging from single premature beats to brief, reversible episodes of ventricular fibrillation. Myocardial infarct size was greater in adult compared with immature hearts (Fig. 3G). Despite some variability in infarct sizes, younger myocardium exhibited a greater capacity to survive I/R.

Maturation-related changes in early protein responses to brief ischemia. Maturation-related differences in protein expression and kinase phosphorylation were apparent in preischemic myocardium (Fig. 4). Preischemic levels of Cav-3 and phosphorylation of Akt were unaltered by maturation, whereas p38MAPK phosphorylation declined and phospho-GSK-3β, phospho-HSP27, and HIF-1α all increased with maturation (Fig. 4). A 10-min ischemic insult exerted select age-dependent effects on protein phosphorylation. The proportion of phosphorylated Akt was reduced by ischemia in immature but not adult hearts (Fig. 4B). GSK-3β phosphorylation declined marginally (though significantly) with ischemia in both age groups (Fig. 4C). Phosphorylation of HSP27 was unaltered by ischemia (Fig. 4D). The phosphorylation state of Cx43 was profoundly augmented by ischemia, and was greater in immature vs. mature hearts (Fig. 4E). Cav-3 expression was increased by ischemia in adult but not immature hearts (Fig. 4F), while HIF-1α levels were relatively stable with ischemia, remaining greater in adult than in immature hearts (Fig. 4G).

Ischemia also modified the expression of apoptosis and autophagy proteins (Fig. 5). Bax expression decreased similarly in immature and adult hearts (Fig. 5A), while Bcl-2 levels declined in immature but not mature hearts (reaching similar levels in both groups; Fig. 5B). As a result of these changes, the Bax:Bcl-2 ratio (considered an indicator of apoptotic potential) was greatly diminished to a similar extent in both immature and adult hearts after 10 min of ischemia (Fig. 5C). LC3B expression increased significantly with ischemia in immature hearts while significantly decreasing in adult hearts (Fig. 5D). After ischemia, immature hearts had a marginal increase in Beclin1 (Fig. 5E), whereas adult hearts had a slight increase in Parkin expression (Fig. 5F). Thus immature and mature hearts exhibit similar shifts and levels of apoptotic proteins during ischemia, whereas immature hearts express higher levels of autophagy proteins during ischemia.

Maturation-related changes in early protein responses to reperfusion. Reperfusion after 25-min ischemia (25I/R) further modified protein phosphorylation and expression in an age-dependent manner (Figs. 4 and 5). The phosphorylation states...
of Akt, p38MAPK, GSK-3β, HSP27, and Cx43 all increased above normoxic levels during reperfusion in both age groups (Fig. 4). Reperfusion P:T ratios for Akt, p38MAPK, and Cx43 did not differ between immature and adult hearts. However, reperfusion P:T ratios for GSK-3β (Fig. 4C) and HSP27 (Fig. 4D) were higher in adult than in immature hearts. Cav-3 expression remained markedly elevated during reperfusion in adult but not immature hearts (Fig. 4F). Expression of HIF-1α was not further modified by reperfusion and remained significantly higher in adult compared with immature hearts (Fig. 4G).

In terms of apoptotic proteins, Bax remained repressed during reperfusion (Fig. 5A) and was modestly higher in adult than in immature hearts. Expression of Bcl-2 decreased during reperfusion in immature hearts only, and was significantly lower than in adult hearts (Fig. 5B). The Bax:Bcl-2 ratio remained comparably repressed in reperfused hearts from both age groups (Fig. 5C). LC3B (Fig. 5D) declined slightly from ischemic levels in immature hearts (not adult), achieving similar levels (slightly elevated above preischemia) in both age groups. Beclin1 (Fig. 5E) increased with reperfusion in both age groups, with slightly higher levels apparent in immature hearts. No changes were evident in Parkin expression during reperfusion, with levels slightly higher in immature hearts than in adult hearts (Fig. 5F).

**DISCUSSION**

The present study for the first time characterizes the concurrent expression profile for key proteins involved in prosurvival phospho-regulation and signaling in response to ischemic stress, including subsequent apoptosis and autophagy, in neonatal and maturing murine myocardium. In the early, upstream prosurvival signaling cascade, neonatal hearts exhibit greater Akt reserve available for phospho-activation compared with mature hearts. Downstream at one of the major effectors, these neonatal hearts also show the greatest degree of phospho-inhibition of GSK-3β. Moreover, neonatal tissue exhibits the highest baseline expression of HIF-1α and Cav-3 and of LC3B and Beclin1. These adaptations likely render neonatal myocardium highly resistant to I/R stress, permitting posts ischemic recovery of myocardial contractile function and limiting infarct size, as was confirmed in the present study (Fig. 3), and support previous observations of posts ischemic contractile function recovery in rats reported by others (reviewed in Refs. 30–32). We examined early reperfusion-related functional recovery (10 min) to observe the early onset of molecular responses. Our prior studies show that age-matched adult murine hearts achieve ~50% recovery of LV developed pressure after more prolonged reperfusion (45–60 min), compared with 20% here at 10 min (35, 41).

Postnatal maturation induces changes consistent with age-related reductions in I/R tolerance, including decreased levels of total Akt, Bcl-2, LC3B, and Beclin1 expression; reduced I/R responsiveness of Cx43, LC3B, and Beclin1; and increased levels of dephosphorylated GSK-3β. Notably, Cav3 falls in the initial 2 wk postpartum, Cx43 is initially high and then declines at ~4 wk. Akt remains low in the initial 4 wk of life, Beclin1 is high initially and falls at week 2. Thus complex/biphasic protein changes underlie the previously reported biphasic changes in I/R tolerance and recovery of posts ischemic contractile function with early maturation (30–32).

**Postnatal changes in body and heart weight.** Marked and progressive postnatal body growth was evident, increasing by ~3-fold within 2 wk and ~10-fold by 12 wk (Fig. 1A). On the other hand, heart mass increased ~3-fold early in life but reached a plateau after 4 wk (Fig. 1A). Weismann et al. (41) report that maturation in C57Bl/6 mice from neonate to adult (12~16 wk) is associated with increases in both LV mass and body weight of ~3-fold in 2 wk, 5-fold in 4 wk, and ~10-fold by 16 wk, with a decline in ratio of heart to body weight. Although these data are comparable to body weight changes in the present study, heart mass is curiously static between 4 and 12 wk in the present study, leading to a greater postnatal decline in the ratio of heart to body weight. Interestingly, the expression of Akt, key to the control of cellular growth and metabolism, was highest at 2 wk and progressively declined with maturation (Fig. 1B), suggestive of early promotion of growth followed by downregulation of this signaling with maturation.

**“Survival” and “death” protein expression profile for neonatal myocardium.** The neonate is highly tolerant of hypoxic or ischemic insult (25, 31), an advantageous remnant from the uterine environment where requirements for nutrients and metabolic waste clearance are critical during rapid growth periods. This greater resilience may be facilitated by cardioprotective signaling impacting on energetic and metabolic viability. Intracellular quantities of signaling proteins available for engagement and phospho-regulation may be a fundamental factor governing transduction (thus efficacy) of cardioprotective signaling. Prosurvival Akt was not particularly high in neonatal hearts (maximal at 2 wk), although much less Akt was phospho-activated at baseline, and fell further with ischemia, thus leaving a greater proportion available for subsequent recruitment (phospho-activation). This may promote resistance to stress in the neonatal heart. In contrast to Akt, the degree of phospho-inhibition of GSK-3β was highest in the neonate.

Notably, neonatal myocardium expresses the greatest levels of Cav-3 and autophagy proteins LC3B and Beclin1 and

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<tr>
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Ages were 0, 2, 4, 8, and 12 wk. P:T, ratio of phosphorylated to total protein; GSK-3β, glycogen synthase kinase-3β; HSP27, heat shock protein 27; Cx43, connexin-43; Cav-3, caveolin-3; HIF-1α, hypoxia-inducible factor-1α.
relatively high levels of HIF-1α. This profile is also likely to contribute to enhanced neonatal I/R tolerance because Cav-3 is critical to myocardial protective responses and signaling (5, 13, 33, 39), autophagy induction protects against I/R injury and improves postischemic outcomes (14, 42), and HIF-1α is also critical to cell protection and metabolic adaptation during I/R (21). Curiously, neonatal myocardium also expressed high levels of proapoptotic Bax together with antiapoptotic Bcl-2, with the ratio of the proteins relatively stable. Thus the potential for apoptosis may be high (elevated Bax) but balanced by antiapoptotic Bcl-2 in neonatal tissue.

The neonate expressed the lowest levels of Cx43 (Fig. 1F), crucial in coupling and transmission of electrical excitation between cardiomyocytes and to protection against I/R injury in adults (2, 37). Although Cx43 is reportedly important in cardiogenesis and early development, cardiac expression appears to predominantly increase later in postnatal development, possibly related to when rates of myocyte growth are diminished and stable cell coupling and gap junctions are greater. While apparently key to protective signaling in adults, it is possible other prosurvival adaptations in neonatal tissue preclude or balance a necessity for Cx43-dependent protective signaling. Overall, the expression profile for neonatal myocardium is generally consistent with I/R tolerance as a result of enhanced expression of HIF-1α, Cav-3, LC3B, and Beclin1, a greater reserve of Akt available for phospho-activation, and enhanced phospho-inhibition of GSK-3β.

Postnatal changes in expression and phospho-regulation of survival proteins. Cardioprotective signaling activated by sarcolemmal G protein-coupled receptors (GPCR) and other stimuli is thought to involve phospho-modulation of kinase cas-

Fig. 3. Left ventricular (L.V) recovery and infarct size. The relative recovery of postischemic contractile function (%preischemic baseline values) after 10-min reperfusion following 25-min ischemia in immature (4 wk) and adult (12 wk) hearts (n = 6 per group). Left ventricular pressure (LVP) traces are shown for a 4-wk-old heart (A) and a 12-wk-old heart (B). Mean data are shown for functional recoveries during 10-min reperfusion for L.V developed pressure (LVDP; C), end-diastolic pressure (EDP; D), rate of left ventricular contraction (+dP/dt max; E), and rate of left ventricular relaxation (−dP/dt min; F) (n = 6/group). G: in a separate subgroup, infarct size assessment was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining, with infarct expressed as a proportion of the total area at risk (%AAR), following ischemia-reperfusion of hearts aged 4 wk (n = 12) and 12 wk (n = 6). Representative slices from each heart undergoing TTC staining are also presented. Data are means ± SE.
cades involving elements, such as phosphoinositide 3-kinase, Akt, ERK1/2, and p38MAPK, converging on potential effector molecules, such as HSP27, Cx43, and GSK-3β. Agonism of GPCRs, implicated in conditioning responses, rapidly modifies the activities of kinases such as Akt (42). As noted in the present study, Akt expression progressively declines with age while the extent of phosphorylation increases. A progressive reduction in total Akt, together with a reduced reserve of un-phosphorylated Akt, may well limit capacity to respond effectively to injurious insult in more mature hearts (Fig. 1B). In terms of responsiveness to I/R in immature and adult hearts, Akt phosphorylation was comparably elevated by I/R in both ages (Fig. 4A), despite a decline in immature hearts during ischemia itself.

Depending on the timing of activation, p38MAPK may exert both protective and injurious actions during I/R (8, 9, 11, 15, 27). On phospho-activation, p38MAPK translocates from cytoplasm to nucleus, and also phospho-activates MAPK-activated kinase 2 (MAPKAPK2). Via such signaling p38MAPK modulates a host of cellular functions including inflammation, cell growth, differentiation, wound healing, energy metabolism, contractility, hypertrophy, and apoptosis (8, 9, 11, 15, 27). This signaling also targets effectors of cardioprotection including HSP27, Cx43, and αβ-crystallin. The current data show that p38MAPK levels initially rise then fall with maturation, with the extent of p38MAPK phosphorylation progressively declining. During ischemia, p38MAPK phosphorylation was reduced in adult but not in immature hearts and after
reperfusion was comparably activated in both groups. Although preischemic p38MAPK activity may be critical to the induction of protection, intrinsic activation during ischemia is without effect (27), thus a pattern of declining preischemic p38MAPK phosphorylation (Figs. 1C and 4B) may contribute to postnatal reductions in I/R resistance.

Under normal physiological conditions, HSP27 is localized proximal to the cytoskeleton and operates as a molecular chaperone in response to varied stressful stimuli. HSP27 is implicated in cardioprotective responses to GPCR agonism (3), overexpression protects against oxidative stress and ischemic injury in cardiomyocytes and myocardium (22, 24), and impaired GPCR protection in aged hearts may involve failed phospho-activation of HSP27 (34). Here we show that HSP27 levels initially increase and then decline with maturation, while the degree of phosphorylation generally declines (Fig. 1E). The latter is consistent with reduced stress resistance. Conversely, reduced phosphorylation of HSP27 during I/R in immature myocardium is inconsistent with greater I/R tolerance in these hearts (Fig. 4D).

The expression of the gap-junction protein Cx43, implicated in cardioprotection (2, 37), increased with maturation in agreement with prior observations of developmental and maturation-related changes in cardiac expression (6). However, the degree of phosphorylation of Cx43 is very high in neonates and progressively falls with maturation. Thus Cx43 is increasingly inactivated despite increased expression in maturing hearts. Importantly, phosphorylation of Cx43 was profoundly increased during ischemia to a much greater extent in immature hearts (with similar postischemic phosphorylation in both groups; Fig. 4E). This pattern of reduced baseline and ischemic phosphorylation in adult hearts may be relevant to I/R intolerance with age, as phosphorylation of Cx43 is key to its protective function.

Postnatal changes in expression and phospho-regulation of GSK-3β. The GSK-3β protein is implicitly involved in glyco- gen synthesis, and numerous studies also support a role in promotion of the mitochondrial permeability transition that occurs with loss of the mitochondrial membrane potential and respiratory chain dysfunction (16). Maintenance of the mitochondrial membrane potential inhibits release of cytochrome c and subsequent proapoptotic caspase activation (15). Phosphorylation of GSK-3β at Ser9, via Akt, p70s6K, or PKC, for example, inactivates GSK-3β. Elevations in GSK-3β expres-
Phosphorylation state. Relative levels of active GSK-3β may thus increase with maturation, contributing to I/R intolerance (Fig. 1D). Conversely, the extent of posts ischemic phospho-inhibition was slightly greater in adult than in immature hearts (Fig. 4C).

Postnatal changes in Cav-3. Caveolins and caveolae are essential to cardioprotective signaling responses (5, 13, 33, 39). Patel and colleagues (40) demonstrated an association between protective GPCRs and signaling with caveolae and Cav-3, abrogation of protection via preconditioning following membrane lipid raft disruption, and elimination of protection via opioid receptor and anesthetic preconditioning with Cav-3 knockout (13, 39). Their more recent study indicates that Cav-3 translocates from the plasma membrane to mitochondria in response to precondioning stimuli, which may facilitate preservation of the mitochondrial membrane potential, bioenergetic function, and cellular integrity (5). Our present work demonstrates a progressive increase in Cav-3 expression from 2 to 12 wk, suggestive of maturation in membrane signaling. Additionally, Cav-3 expression was apparently enhanced with ischemia and reperfusion in adult but not immature hearts. The basis and relevance of this latter response is unclear.

Postnatal changes in apoptosis-related proteins. Programmed cell death is crucial in the developing heart as it undergoes myocardial growth and remodeling, which is suggestive of an overall increased capacity for cellular removal in immature heart. At the birth transition, the right ventricle loses its in utero functional dominance while the LV assumes its postnatal role in supporting systemic cardiac output. At this time the neonatal right ventricle undergoes remodeling that is accompanied by enhanced apoptosis that dramatically declines with maturation (4). This may be reflected here by substantially higher Bax levels in neonates, although this is effectively balanced by increased Bcl-2 expression and thus a slightly lower Bax:Bcl-2 ratio (Fig. 2). These changes are suggestive of an increased capacity for apoptosis (high Bax) that under baseline conditions is countered by antiapoptotic molecules. Upon ischemia with or without reperfusion, Bax, Bcl-2, and the Bax:Bcl-2 ratio all decline in immature and adult hearts (Fig. 5). This may appear counterintuitive, although it is possible that repressed expression of these regulators aids in limiting cell death driven by other proapoptotic processes during I/R (i.e., caspase activation via mitochondrial de-energization and cytochrome c release).

Postnatal changes in autophagy proteins. Intracellular repair or quality control via autophagy plays a critical role during the fetal-to-neonatal transition, and in response to the oxidative stress associated with birth and early growth of the heart (19). The transient (3–12 h), but marked, initial upregulation of autophagy within the heart due to amino acid production to simultaneously provide energy and maintain developmental processes was demonstrated in Arg5−/− transgenic mice inhibiting autophagosome formation (19, 32). This trend was similarly observed in the present study with maximum levels of LC3B and Beclin1 (but not Parkin) evident in neonates (0 wk), accompanied by a rapid decline to basal levels at 2 wk (Fig. 2). Autophagy is also beneficial in I/R, implicated in cardioprotective responses to GPCRs (43) and other stimuli (14). Precise signaling control of cardiac autophagy remains to be elucidated. The current data suggest a reduced capacity for autophagy in adult vs. immature hearts, with postnatal reductions in basal expression of LC3B and Beclin1 (Fig. 2, D and E), and reduced or delayed expression of these proteins in response to I/R in adult hearts (Fig. 5, D and E). However, Parkin expression was largely invariant with maturation and relatively insensitive to I/R in both immature and adult hearts (Figs. 2F and 5F). These changes are consistent with the idea of enhanced autophagic potential in neonates (19) and involvement in cardioprotection (14, 42). Lack of change in Parkin levels during I/R contrasts the findings of Huang et al. (14), who recently documented increased Parkin expression in adult mice in response to ischemic preconditioning stimuli.

Study limitations. The current work did not permit assessment of potential shifts in subcellular Parkin localization (or other proteins subject to translocation between organelles after ischemic stimulus, such as Beclin1, LC3B, p38MAPK, and HSP27 proteins) due to limited tissue availability for cellular fractionation and organelle isolation. However, such work appears warranted in more detailed prospective mechanistic studies in neonatal and maturing hearts.

The excision, handling, cannulation, balloon insertion, and perfusion of isolated hearts partake in time-dependent alterations to phoso-regulatory survival protein expression (38). These effects may account for the discrepancies observed between baseline (Figs. 1 and 2) and normoxic (Figs. 4 and 5) P:T protein expression levels. Such handling could not be performed identically in very small hearts from very young mice. However, for each series the same experimenter performed the work, with the same materials and at the same time each experimental day.

Perspectives and Significance

The current study reveals a unique expression profile for the neonatal heart, which exhibits relatively high basal levels of survival proteins HIF-1α and Cav-3, autophagic proteins LC3B and Beclin1, and apoptotic regulators Bax and Bcl-2. Additionally, baseline phospho-activation of Akt is low, whereas phospho-inhibition of GSK-3β is high. In terms of postnatal changes and emergence of myocardial I/R intolerance with increased age, data reveal a complex series of changes in prosurvival and prodeath proteins with maturation. A number of these are consistent with impaired tolerance to I/R (i.e., reduced Akt, Bcl-2, LC3B, and Beclin1 expression; reduced responsiveness of Cx43, LC3B, and Beclin1 to I/R; and increased expression of dephosphorylated GSK-3β with maturation), whereas others appear paradoxical or may reflect adaptations to maturation (i.e., increased Cx43 levels) and reduced intrinsic stress resistance (i.e., increased HIF-1α and reduced Bax expression).

Conclusions

While the present study confirms earlier studies of marked neonatal resistance to hypoxic or I/R stress, our work now specifically reveals an underlying expression profile for prosurvival and proautophagy proteins that may prerreruit “default” stress resistance in neonates, whereas this changes with
postnatal maturation and adaptation, in line with emerging I/R intolerance evident in advanced age.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.Y.L., J.N.P., J.P.H., and S.P. conceived and designed the experiments; N.Y.L., J.N.P., J.P.H., and S.P. performed the experiments; N.Y.L., J.N.P., J.P.H., and S.P. analyzed the data; N.Y.L., J.N.P., J.P.H., M.M.C., and S.P. interpreted the results of experiments; N.Y.L., J.N.P., J.P.H., and S.P. drafted manuscript; N.Y.L., and S.P. reviewed and edited the manuscript; N.Y.L., J.N.P., J.P.H., M.M.C., and S.P. approved final version of manuscript.

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