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Review Article

Mechanisms of load dependency of myocardial ischemia reperfusion injury

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Abstract: Coronary artery disease and associated ischemic heart disease are prevalent disorders worldwide. Further, systemic hypertension is common and markedly increases the risk for heart disease. A common denominator of systemic hypertension of various etiologies is increased myocardial load/mechanical stress. Thus, it is likely that high pressure/mechanical stress attenuates the contribution of cardioprotective but accentuates the contribution of cardiotoxic pathways thereby exacerbating the outcome of an ischemia reperfusion insult to the heart. Critical events which contribute to cardiomyocyte injury in the ischemic-reperfused heart include cellular calcium overload and generation of reactive oxygen/nitrogen species which, in turn, promote the opening of the mitochondrial permeability transition pore, an important event in cell death. Increasing evidence also indicates that the myocardium is capable of mounting a robust inflammatory response which contributes importantly to tissue injury. On the other hand, cardioprotective maneuvers of ischemic preconditioning and postconditioning have led to identification of complex web of signaling pathways (e.g., reperfusion injury salvage kinase) which ultimately converge on the mitochondria to exert cytoprotection. The present review is intended to briefly describe mechanisms of cardiac ischemia reperfusion injury followed by a discussion of our work focused on how pressure/mechanical stress modulates endogenous cardiotoxic and cardioprotective mechanisms to ultimately exacerbate ischemia reperfusion injury.

Keywords: Heart, ischemia-reperfusion, pressure, calcium overload, oxidative/nitrosative stress, signaling mechanisms, inflammation, stem cells

Introduction

Systemic hypertension is a common disorder with global prevalence estimates of 1 billion individuals. It accounts for an estimated 7.6 million deaths each year and for 13.5% of total mortalities, more than any other single risk factor [1-4]. In the United States, the data from 2007-2010 indicate that 33% of adults 20 years or older have hypertension which represents about 78 million American adults; the prevalence is nearly equal between men and women although African-Americans are among those with highest prevalence of hypertension (44%) in the world [5]. Of interest is the fact that the population of the United States continues to increase and the Census Bureau projects it to almost reach 440 million by the year 2050 (an increase of about 130 million from 2010) [6, 7]. Importantly, the proportion of patients greater than 65 years of age is increasing at a greater rate than the total population. This is reflected by the data indicating that while the total population increased by 9.7% between 2000 and 2010, those older than 65 years increased by 15.1%. Further, the greatest proportional increases over this 10-year period occurred in the oldest age groups, with a 29.9% increase in those 85-94 years of age and a 25% increase in those greater than 95 years of age [6, 8]. Population projection data spanning 2010-2050 also support the general conclusion that greater proportional increase occurs in those 65 years or older and that among them those older than 85 years of age show the greatest increase [7]. Since the prevalence of hypertension increases with age and hypertension represents an accumulation of years of pressure overload on target organs, hypertension-related clinical sequels (e.g., ischemic

heart disease and myocardial infarction) will become even more pressing challenges for the health care system.

The propensity of the hypertensive heart to ischemic events is multifactorial including a) epicardial coronary stenosis (e.g., due to atherosclerosis) and b) cardiac microvascular disease and endothelial dysfunction, accompanied with ultrastructural remodeling of cardiac microvessels, that can result in progressive impairment of flow-mediated vasodilation. Other factors can be arterial stiffness with long standing hypertension and accompanying increased left ventricular afterload and central pulse pressure; the concomitant fall in central diastolic pressure reduces coronary perfusion, further exacerbating myocardial ischemia [9-14]. Importantly, alterations in energy metabolism of the hypertensive heart also increase susceptibility to ischemia. This notion is supported by the findings that patients with hypertension have measurably lower phosphocreatine to adenosine triphosphate ratios during stress compared to healthy controls [15].

As discussed above, atherosclerosis is the predominant underlying cause of coronary heart disease which can result in myocardial infarction with ischemic death of cardiomyocytes [16, 17]. Reperfusion of the acutely or chronically ischemic myocardium (e.g., via thrombolysis, percutaneous coronary angioplasty and/or coronary bypass) is essential in order to salvage the myocardium [17, 18]; yet, injury to the endothelium and cardiomyocytes occurs upon reperfusion [19, 20]. Reperfusion-induced injury is also a significant clinical problem in cardiac transplantation or during open heart surgery when the myocardium is subjected to global ischemic cardioplegic arrest [21, 22]. The following section provides an overview of some key events in myocardial ischemia reperfusion injury prior to discussion of how pressure overload modulates these mechanisms to exacerbate the outcome of an ischemia reperfusion insult to the heart.

Mechanisms of myocardial ischemia reperfusion injury

Hallmark features of myocardial ischemia reperfusion (IR) injury include marked oxidative/nitrosative stress and intracellular calcium ($[Ca^{2+}]i$) overload (**Figure 1**). During ischemia, a reduction in mitochondrial energy production ensues that is accompanied by decreased intracellular pH (pH_i) due to increased lactic acid production consequent to anaerobic glycolysis. The reduction in pH_i, in turn, causes disruption of ionic homeostasis and subsequent $[Ca^{2+}]i$ overload. This occurs because during ischemia, activation of sarcolemmal Na^+/H^+ exchanger occurs as the cell attempts to restore its pH_i. However, the Na^+ that enters the cell on the Na^+/H^+ exchanger is not pumped out efficiently because a fall in ATP and an increase in phosphate (Pi) inhibit the Na^+/K^+-ATPase. As a result, the Na^+/Ca^{2+} exchanger, that normally extrudes Ca^{2+} from the cell, is inhibited or even reversed thereby raising $[Ca^{2+}]i$. However, it is upon reperfusion that a much greater rise in $[Ca^{2+}]i$ occurs which contributes to the genesis of ventricular arrhythmia and myocardial stunning [20, 23-27] (**Figure 1**). Although the rise in $[Ca^{2+}]i$ is attributed primarily to reversal of the Na^+/Ca^{2+} exchanger and the L-type Ca^{2+} channel [24-27], T-type Ca^{2+} channels have also been implicated in this phenomenon [28]. Further, resumption of ATP synthesis upon reperfusion may activate sarcoplasmic reticulum Ca^{2+} cycling resulting in cytosolic Ca^{2+} oscillations and propagation of Ca^{2+} waves [29]. Consequences of $[Ca^{2+}]i$ overload include activation of degradative enzymes including proteases, phospholipases and nucleases that can cause irreversible tissue injury [27].

Reactive oxygen species (ROS), on the other hand, are generated primarily through mitochondrial respiratory chain, NAD(P)H oxidase and xanthine oxidase during myocardial IR injury [25-27]. Detrimental consequences of ROS which contribute to tissue injury include: a) impairment of respiratory chain activity (e.g., complex I), b) plasma membrane damage with subsequent impairment of ion pumps thereby exacerbating the effects of ATP deprivation on cellular ionic homeostasis and c) peroxidation of unsaturated fatty acid components of the membrane phospholipids; this will render them more susceptible to attack by phospholipase A_2 whose activity may already be elevated by $[Ca^{2+}]i$ overload [25-28, 30, 31].

The large burst of ROS and $[Ca^{2+}]i$ overload upon reperfusion of the ischemic heart are major triggers for the mitochondrial permeability transition (MPT) pore [25, 27, 30, 31]. The

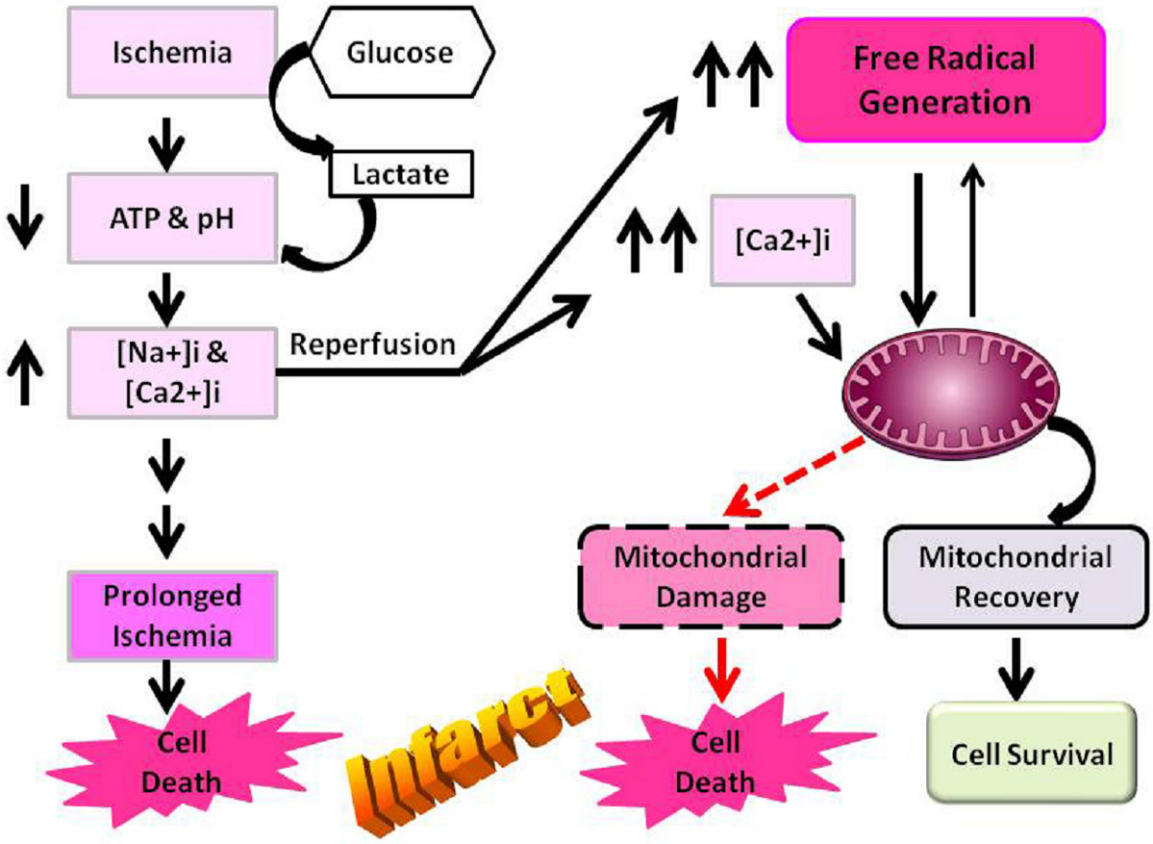

Figure 1. Diagram depicts critical events in cardiac ischemia reperfusion injury [27].

MPT pore is a non-specific conduit that is formed at the site of contact between mitochondrial inner and outer membranes which allows for solute flux of less than about 1.5 kDa size. The exact molecular composition of the MPT pore remains controversial although use of genetically modified mice suggest an important regulatory role for cyclophilin D; loss of cyclophilin D reduces the sensitivity of MPT pore to activation by calcium or during ischemia and reperfusion [32, 33]. Induction of MPT pore by $[Ca^{2+}]i$ overload and ROS is facilitated by decreased mitochondrial membrane potential and increased Pi levels, conditions that are present during myocardial IR injury. In addition, restoration of pH_i at reperfusion also triggers MPT pore induction [20, 23, 27, 34-37]. Opening of the MPT pore allows solutes and water to enter the mitochondria thereby increasing matrix volume. As a result, mitochondrial outer membrane ruptures facilitating release of cytochrome c which, in turn, promotes apoptosis. In addition, MPT pore induction uncouples the mitochondria, leading to inhibition of ATP synthesis and hydrolysis of the ATP that is derived from glycogen breakdown eventually causing cell death by necrosis [25, 34, 36-39]. Thus, both necrotic and apoptotic cell death occur during IR injury. The pivotal roles of these processes, in mediating myocardial IR injury, are underlined by numerous studies indicating that cardioprotections of both ischemic preconditioning and postconditioning are associated with reductions in generation of ROS, calcium overload and MPT pore opening [26, 31, 37, 39-44]; ischemic preconditioning describes the phenomenon whereby several brief bouts of ischemia and reperfusion prior to a more prolonged ischemic phase (i.e., index ischemia) confers significant protection to the ischemic-reperfused heart while postconditioning refers to cardioprotection conferred by restoration of coronary circulation to the ischemic myocardium in a stuttering fashion [31, 40-51] (**Figure 2**). While considerable attention has focused on cardioprotection of ischemic preconditioning and postconditioning maneuvers [31, 40-51], a novel and intriguing paradigm has emerged which advocates that targeted modulation of autophagy could exert beneficial effects in

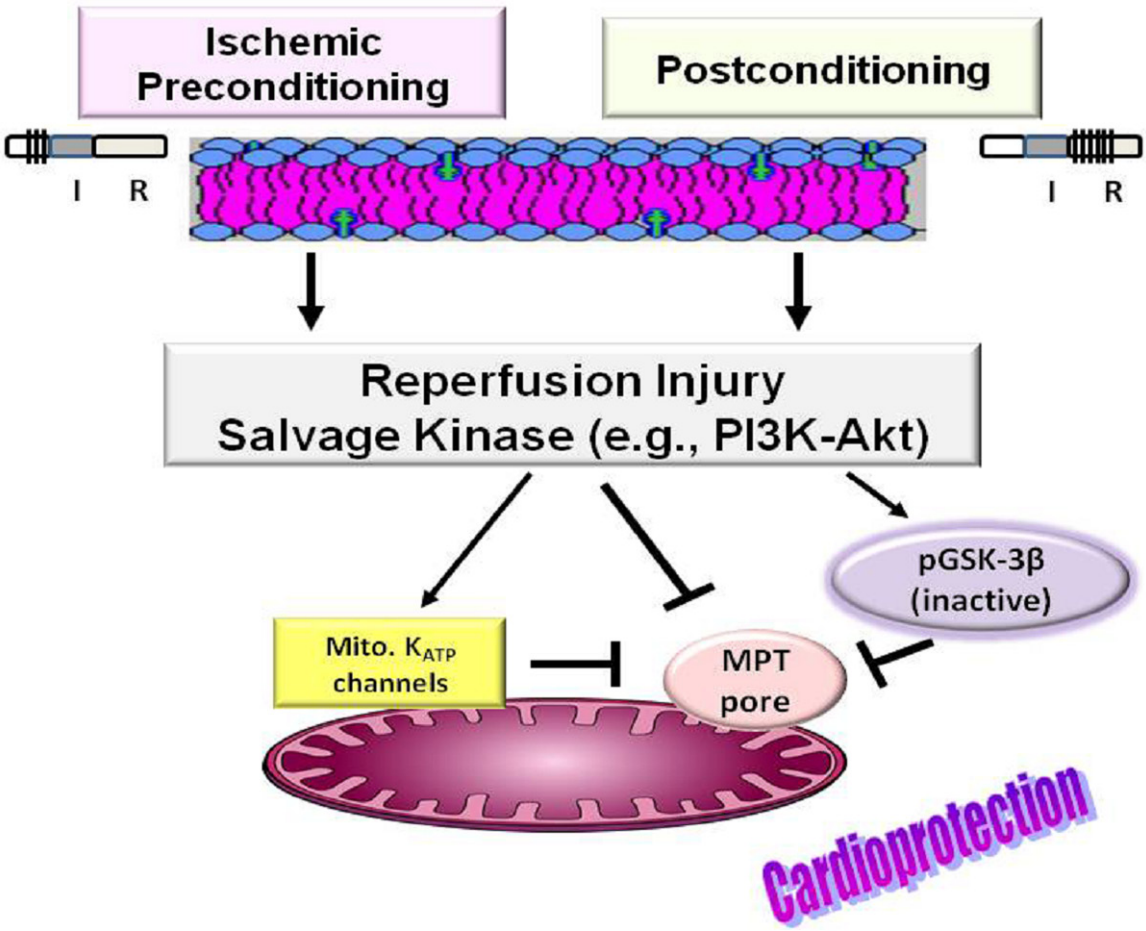

Figure 2. Diagram shows that cardioprotection of ischemic preconditioning (e.g. 3 bouts of ischemia (I) and reperfusion (R), each for 5 min, before a prolonged ischemia phase) and postconditioning (e.g., 6 cycles of I and R, each for 30 sec, at reperfusion) is associated with activation of the reperfusion injury salvage kinase (RISK) pathway. An important outcome relates to upregulation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway which, in turn, leads to phosphorylation and inactivation of glycogen synthase kinase-3β (GSK-3β) culminating in inhibition of the mitochondrial permeability transition (MPT) pore. Also, shown is mitochondrial (mito.) K_{ATP} channels whose activation confers cardioprotection, likely through inhibition of MPT pore induction.

stressful conditions such as IR injury; autophagy is a highly-regulated cellular "housekeeping" process for the degradation and disposal of protein aggregates and dysfunctional/damaged organelles (e.g., mitochondria in a process referred to as mitophagy). Indeed, recognition of the dichotomous "life-or-death" patho (physiological) role of autophagy has led to considerable research focused on harnessing its cardioprotective potential [52]. In support of this notion, upregulation of autophagy has been suggested to play a causal role in infarct-sparring effect of both ischemic-preconditioning and postconditioning [53-55].

The earlier discovery of ischemic preconditioning led to a surge of interest in unraveling cardioprotective mechanisms [37, 45-49]; this well-studied phenomenon is also referred to as early or classical preconditioning to distinguish it from the delayed phase or second window of protection [23]. Also the more recently discovered phenomenon of postconditioning has generated much interest in understanding of its underlying mechanisms because it is a more clinically relevant and amenable maneuver than ischemic preconditioning [50-52]. While the exact mechanism(s) of either ischemic preconditioning or postconditioning remains elusive, considerable progress has been made towards a better understanding of the signal transduction pathways that convey the extracellular signal generated by these cardioprotective maneuvers to intracellular targets [30]. As

a result, it is now known that cardioprotection involves activation of a diverse array of prosurvival signaling pathways collectively referred to as the reperfusion injury salvage kinase (RISK) pathway [19, 30] (**Figure 2**). Components of RISK include protein kinases C, G, and A, members of the mitogen activated protein kinase family (e.g., extracellular-regulated kinase 1/2, P38, c-*jun*-N terminal kinase) and the phosphatidylinositol-3 kinase-protein kinase B/Akt (PI3K-PKB/Akt) cascade [30]. While the contribution of individual protein kinases is often the subject of intense debate, it is increasingly recognized that certain survival protein kinases (e.g., PI3K-Akt) are shared by both ischemic preconditioning and postconditioning protocols [19, 30], likely accounting for the observation that combination of both protocols does not further reduce infarct size than either maneuver alone [51]. Tyrosine kinase is a well-recognized upstream activator of the PI3K-Akt cascade [30]. In turn, many of the downstream targets phosphorylated by Akt activate various anti-apoptotic pathways (e.g., epsilon isoform of protein kinase C and nitric oxide synthase to generate nitric oxide) [30]. The involvement of these prosurvival pathways is further substantiated by the demonstration that several endogenous cardioprotective agents, such as adenosine, mimic ischemic preconditioning by activating a pathway that is modulated by certain isozymes (e.g., epsilon) of protein kinase C in rabbit or rat heart [47, 56-59]. Indeed, generation of adenosine during postconditioning also causes eventual activation of PI3K-Akt and subsequent downstream activation of protein kinase C epsilon [30]. However, debate prevails regarding the nature of the "end effector" of the signaling pathway, with some investigators suggesting the mitochondrial ATP-sensitive K^+ (mito. K_{ATP}) channels while others further indicate that the opening of mito. K_{ATP} channels leads to mild oxidative stress, activating one or more protein kinases that stimulate an "unidentified" end effector [19, 30, 45-48]. Activation of mito. K_{ATP} channels is believed to result in a number of effects including reduction in mitochondrial Ca^{2+} accumulation and prevention of cytochrome C loss from the intermembrane space [42, 43, 49]. The importance of this cascade of events is illustrated by the effectiveness of mito. K_{ATP} channel inhibitors (e.g., glibenclamide) in blocking the beneficial effects of a wide range of cardioprotective agents and protocols (e.g., adenosine, opioids, ischemic preconditioning and postconditioning) [28, 47, 48, 51]. On the other hand, mito. K_{ATP} channel openers (e.g., diazoxide) mimic the effect of preconditioning [47-49, 60, 61]. Further, studies utilizing isolated mitochondria indicate that pharmacological activation of protein kinase C protects against MPT pore opening under the same conditions in which diazoxide is protective [49]. While the targets of ischemic (or pharmacologic) preconditioning and postconditioning are likely to be multiple (e.g., protein kinase C/mito. K_{ATP} channels), the key signaling pathways ultimately must converge to prevent MPT pore induction (e.g., during reperfusion) to reduce infarct size [19, 20, 27, 30, 42-46, 62-64].

Given the pivotal role of the MPT pore in myocardial IR injury, intense research has focused on mechanisms regulating the MPT pore; these studies have identified glycogen synthase kinase-3β (GSK-3β) as a critical regulator of the MPT pore (**Figure 2**). GSK-3β is a serine-threonine kinase which is best known for its regulation of glycogen metabolism. However, GSK-3β is now recognized as a multifunctional kinase responsible for phosphorylation of more than 20 substrates. GSK-3β is primarily localized in the cytosol and is constitutively active. However, multiple kinases (e.g., Akt/protein kinase B) can phosphorylate it at serine 9 residue, rendering it inactive. Indeed, phosphorylation of GSK-3β by multiple signaling pathways (e.g., components of RISK) is believed to increase the activation threshold of MPT pore thereby conferring cardioprotection [63]. For example, Juhaszova and colleagues [64] showed that the threshold for ROS-induced MPT pore opening was elevated by GSK-3β inactivation or by its knockdown using siRNA in isolated cardiomyocytes. Further, Gomez et al. [65] showed that MPT pore opening by $[Ca^{2+}]i$ overload was suppressed in mitochondria isolated from postconditioned wild type mice but not those from mice expressing a mutated form (i.e., GSK-S9A) which is insensitive to phosphorylation at serine 9. Additional evidence in support of phosphoGSK-3β-mediated inhibition of MPT pore opening in response to ROS and calcium overload comes from numerous studies including a) those using pharmacological inhibitors of GSK-3β (e.g., LiCl, SB216763, SB415286), b) those utilizing δ-opioid receptor agonists, adenosine

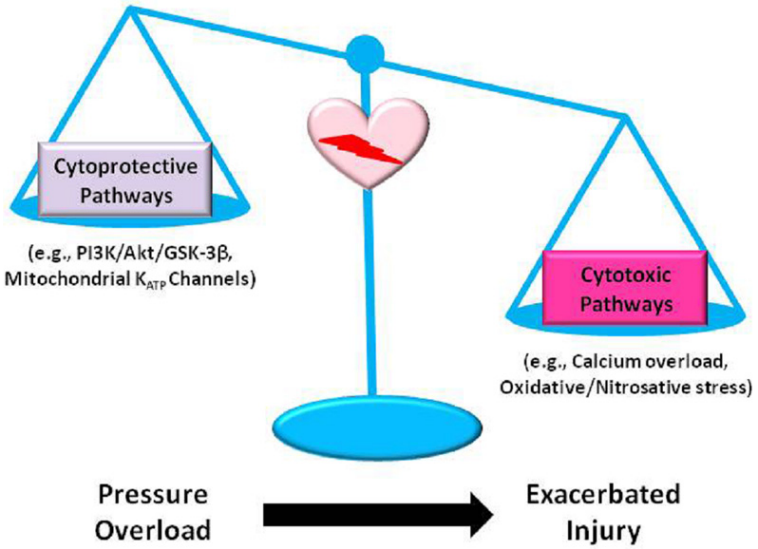

Figure 3. Diagram summarizes our working hypothesis that pressure overload accentuates cardiotoxic but attenuates cardioprotective mechanisms thereby causing exacerbation of myocardial ischemia-reperfusion injury.

A2b receptor agonists or erythropoietin and c) studies using cardioprotective maneuvers of ischemic preconditioning or postconditioning, among others [63]. Although the role of GSK-3β in regulation of MPT pore is indisputable, it is not yet clear how its inactivation causes MPT pore inhibition. Nonetheless, a number of possibilities have been proposed including mitochondrial translocation of phosphoGSK-3β and complex formation with cyclophilin D ultimately leading to cardioprotection [66]. Importantly, gene targeting studies indicate that cyclophilin D is a component of the MPT pore [67] and cyclosporine A-induced inhibition of cyclophilin D or genetic deletion of cyclophilin D significantly limits infarct size [34, 37, 63, 68].

With that background in mind, the following section describes our studies focused on the impact of pressure overload on myocardial IR injury and the underlying mechanisms.

Impact of myocardial load on the outcome of myocardial IR insult

As mentioned earlier, systemic hypertension is an established risk factor for coronary heart disease and it also adversely affects the outcome of acute myocardial infarction. The latter is corroborated by reports indicating that acute elevation in blood pressure increases while acute reduction in blood pressure reduces susceptibility to IR injury [69-76]. Nonetheless, the interpretation of these studies is confounded by neurohumoral adaptations that accompany changes in blood pressure, some of which are known to impact the outcome of IR injury independent of the blood pressure [77-79]. For our studies focused on pressure-related effects on cardiac IR injury, we have used the isolated heart preparation in order to avoid the confounding influences of neurohumoral changes that accompany chronic or acute elevation of blood pressure. These studies were based on the premise that increase in myocardial load is a common denominator of systemic hypertension of various etiologies. It is noteworthy that for determination of pressure-related effects on the outcome of IR injury we have adjusted the pressure-head of the Langendorff-perfused heart (e.g., 80 or 160 cmH_2O). This maneuver significantly increases both the coronary flow and the contractile parameters of the heart. In order to decipher the impact of the increase in coronary flow rate, per se, we carried out additional experiments using the constant flow perfusion protocol as detailed previously [80]. Comparison of data from experiments whereby the pressure-head is adjusted against the heart with those whereby coronary flow rate is adjusted indicates that the primary determinant of the outcome of an IR insult, in our studies, relates to the pressure and associated mechanical stress/load on the myocardium [80].

Our initial studies, using 36-week-old hypertensive and glucose intolerant rats, revealed that isolated hearts subjected to a high pressure (i.e., 160 cmH_2O or about 118 mmHg) display a significant increase in infarct size in response to an IR insult compared to those subjected to a low pressure (i.e., 80 cmH_2O or about 59 mmHg) [81]. We reasoned that the disease states in the aging rat and pre-existing cardiomyopathy, per se, may have rendered the heart susceptible to the impact of elevated pressure. In order to prevent the confounding influences of disease states and aging, we carried out

subsequent IR protocols using healthy adult rats (9-11 weeks of age). Accordingly, we established that pressure overload significantly increases infarct size in association with poorer functional recovery following an IR insult [82]. Subsequent studies explored potential contributing mechanisms to the adverse impact of high pressure on the ischemic-reperfused heart. Thus, we tested the hypothesis that elevated pressure, and associated mechanical stress, accentuates the contribution of cytotoxic pathways (e.g., calcium overload, oxidative stress/nitrosative stress, etc.) and/or attenuates the contribution of cardioprotective pathways (e.g., mito. K_{ATP} channels, PI3K-Akt signaling pathway); the net effect of these changes would be increased MPT pore opening and consequent cell death (**Figure 3**). The details of these studies are presented below.

Effect of myocardial load on calcium overload

As described earlier, a hallmark feature of cardiac IR injury is $[Ca^{2+}]i$ overload which exerts multiple effects including induction of MPT pore thereby contributing to cell death [82]. One consequence of increased myocardial load is activation of the angiotensin II type 1 receptor and nonspecific cation channels with subsequent Ca^{2+} accumulation via the Na^+/H^+-Na^+/Ca^{2+} exchanger combination and the T-type or L-type Ca^{2+} channels. Since $[Ca^{2+}]i$ overload is cytotoxic, in part, by inducing the MPT pore, we also explored the effect of cyclosporine A-induced inhibition of MPT pore in pressure overloaded hearts. Accordingly, the effect of candesartan (angiotensin II type 1 receptor antagonist), cariporide (inhibitor of the Na^+/H^+ exchanger), mibefradil (T-type Ca^{2+} channel blocker), diltiazem (L-type Ca^{2+} channel blocker), and cyclosporine A (inhibitor of MPT pore) were examined. The elevation in perfusion pressure, from 80 to 160 cmH_2O, increased baseline myocardial performance but caused larger infarcts and further reduced recovery of mechanical function after ischemia reperfusion. Whereas mibefradil abrogated the effect of high pressure on infarct size, the other agents reduced infarct size at both perfusion pressures. Hearts exposed to mibefradil, diltiazem, or cariporide displayed greater functional recovery than those exposed to candesartan or cyclosporine A, revealing that an uncoupling exists between reduced cell death and recovery of mechanical function of the viable portions of the myocardium. Collectively, the data suggested an important link between pressure-mediated worsening of infarct size and exacerbation of $[Ca^{2+}]i$ overload (e.g., via T type channels). Nonetheless, it is noteworthy that the contribution of sarcoplasmic reticulum to $[Ca^{2+}]i$ overload in the ischemic-reperfused heart is now established [29]. Accordingly, resumption of ATP synthesis upon reperfusion activates sarcoplasmic reticulum Ca^{2+} cycling. Sarcoplasmic reticulum Ca^{2+} cycling is promoted by cytosolic Ca^{2+} overload and consequent Ca^{2+} uptake through the sarco(endo)plasmic reticulum Ca^{2+}-ATPase followed by Ca^{2+} release through the ryanodine receptors when the Ca^{2+} storage capacity of the organelle is exhausted. These changes cause Ca^{2+} oscillations that propagate as Ca^{2+} waves and are believed to facilitate partial mitochondrial permeabilization due to close anatomic proximity between the two organelles thereby favoring hypercontracture and cell death [29]. In light of the profound impact of pressure overload on the ischemic-reperfused heart, potential pressure-related regulation of sarcoplasmic reticulum Ca^{2+} cycling should be established.

Effect of myocardial load on oxidative/nitrosative stress

Excessive ROS generation is a critical event in myocardial IR injury. During ischemia, low levels of ROS are generated which can damage the electron transport chain thereby causing inefficient transfer of electrons with consequent increase in ROS generation. With availability of oxygen during early reperfusion, a large burst of ROS occurs which plays a pivotal role in the genesis of reperfusion-induced injury. Important cardiac sources of ROS (e.g., superoxide) include the mitochondrial respiratory chain distal to complex I (NADH dehydrogenase), xanthine oxidase and NAD(P)H oxidase [26, 27, 31, 83]. While the myocardium possesses endogenous antioxidant defenses, such as the superoxide dismutase (SOD) and catalase, these mechanisms can be overwhelmed following ischemia and reperfusion. In turn, these conditions are conducive to the interaction of superoxide with nitric oxide to produce peroxynitrite, a potent oxidant. Consequently, exacerbated oxidative/nitrosative stress serves as a major trigger for the MPT pore opening and subsequent cell death [25, 83].

In light of the pivotal role of oxidative/nitrosative stress in cardiac IR injury, we sought to determine whether pressure overload exacerbates oxidative/nitrosative stress due to increased generation of reactive substances or reduced ability to scavenge ROS thereby promoting greater MPT pore opening with consequent exacerbation of cell death via necrosis and/or apoptosis. Pressure overload decreased the level of reduced glutathione but increased that of nitrotyrosine (a stable footprint of peroxynitrite) level in ischemic-reperfused hearts. Further, pressure overload increased DNA injury as demonstrated by increased 8-hydroxydeoxyguanosine (an index of oxidative DNA damage) and γH2AX (a sensitive marker of double strand DNA breaks, the most severe form of DNA injury) [80, 83]. The activity of catalase, but not SOD, was lower in ischemic-reperfused hearts perfused at higher pressure. Mitochondria isolated from ischemic-reperfused hearts subjected to higher perfusion pressure displayed significantly greater [^{3}H]-2-deoxyglucose-6-Pi entrapment suggestive of greater MPT pore opening and this was consistent with greater necrosis and apoptosis as determined by flow cytometry. Tempol (SOD mimetic) reduced infarct size in hearts subjected to low or high perfusion pressure but it remained greater in the higher pressure group. By contrast, uric acid (peroxynitrite scavenger) markedly reduced infarct size at higher pressure, effectively eliminating the differential between the two groups. Inhibition of xanthine oxidase, with allopurinol, reduced infarct size but did not eliminate the differential between the low and high pressure groups. However, amobarbital (inhibitor of mitochondrial complex I) or apocynin (inhibitor of NAD(P)H oxidase) reduced infarct size at both pressures and also abrogated the differential between the two groups. Consistent with the effect of apocynin, pressure-overloaded hearts displayed significantly higher NAD(P)H oxidase activity. Furthermore, pressure-overloaded hearts displayed increased nitric oxide synthase activity which, along with increased propensity to superoxide generation, may underlie uric acid-induced cardioprotection. Collectively, these observations indicate that increased oxidative/nitrosative stress, coupled with lack of augmented SOD and catalase activities, contributes importantly to the exacerbating impact of pressure overload on MPT pore opening and cell death in ischemic-reperfused hearts [83].

As alluded to above, exacerbated oxidative/nitrosative stress in pressure overloaded ischemic-reperfused heart augments DNA injury. In turn, DNA injury leads to activation of poly (ADP-ribose) polymermase-1 (PARP) in order to facilitate DNA repair in a process which consumes NAD^+ [84]. Importantly, however, hyperactivation of PARP has been linked to mitochondria-mediated necrosis although the precise mechanism remains elusive. Nonetheless, a recent study proposes that oxidative stress, MPT pore and PARP activity contribute to a single death pathway in the ischemic-reperfused heart. Accordingly, a provocative mechanism has been proposed whereby PARP-mediated prolongation of mitochondrial depolarization contributes significantly to cell death via an energy crisis (e.g., consequent to depletion of NAD^+ thereby limiting ATP generation) rather than by mitochondrial outer membrane rupture. In addition, PARP activity could directly inhibit mitochondrial transport of adenine nucleotides, preventing cytosolic ATP access to the matrix where it could facilitate repolarization. Consequently, ATP depletion would result in sustained depolarization ultimately causing mitochondrial failure and plasma membrane rupture without affecting the integrity of the outer membrane of the mitochondria. In support of the important contribution of PARP hyperactivation to cell death, its inhibition has been shown to exert significant cardioprotection [85-87]. In light of exacerbated DNA injury in pressure overloaded ischemic-reperfused hearts, in a pilot study, we tested the hypothesis that inhibition of PARP would confer greater protection under the high pressure condition. Interestingly, however, our initial observations suggest that while treatment with 4-hydorxyquanozoline (a PARP inhibitor) significantly reduces infarct size of the ischemic-reperfused heart subjected to the low pressure, the treatment seemingly does not confer significant protection under the high pressure condition (unpublished data); the dose-related possibility for this observation is under investigation. Thus, establishing pressure-related regulation of PARP in the ischemic-reperfused heart and its relation to MPT pore status is a fertile ground for exploration.

Effects of myocardial load on cardioprotective mechanisms

The MPT pore may serve as the end-effector of cardioprotective mechanisms, namely the mito-

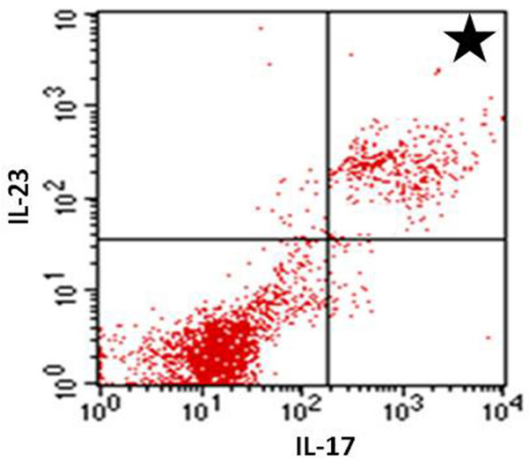

Figure 4. Panel shows a dot matrix from flow cytometry-based assessment of cardiac cells from ischemic-reperfused hearts that co-express IL-17 and IL-23 (upper right quadrant; indicated by asterisk).

chondrial K_{ATP} channels and GSK-3β [19, 20, 30, 60, 61, 63-66, 68]. Therefore, in light of our demonstration that augmented MPT pore induction contributes to pressure overload-induced exacerbation of infarct size [82, 83], we sought to determine whether elevation in perfusion pressure attenuates cardioprotection associated with activation of mitochondrial K_{ATP} channels or inhibition of GSK-3β. Further, we also determined whether perfusion pressure modulates the regulation of the MPT pore by mitochondrial K_{ATP} channels and/or GSK-3β. These studies used diazoxide (a mitochondrial K_{ATP} channel opener), glibenclamide (inhibitor of K_{ATP} channels), lithium chloride (LiCl, a non-selective inhibitor of GSK-3β), SB-216763 (a selective inhibitor of GSK-3β), cyclosporine A (inhibitor of MPT pore induction) and the combination of cyclosporine A and glibenclamide or the combination of glibenclamide and LiCl. As expected, the increase in perfusion pressure in the absence of a drug caused larger infarcts, an effect associated with poorer recovery of function following ischemia reperfusion. Treatment with either diazoxide or cyclosporine A reduced infarct size at both perfusion pressures but cyclosporine A was more protective, than diazoxide, at the higher pressure. On the other hand, LiCl and SB-216763 reduced infarct size at both pressures, with the effect more marked at the higher perfusion pressure. Glibenclamide did not affect infarct size but eliminated the cardioprotective effect of cyclosporine A while having no effect on LiCl-induced cardioprotection [68]. Collectively, the results indicate that perfusion pressure primarily affects GSK-3β-mediated regulation of MPT pore formation in the ischemic reperfused heart.

As mentioned earlier, GSK-3β is downstream of the PI3K/protein kinase B (Akt) pathway. Indeed, the cardioprotection of postconditioning and insulin relates to activation of the PI3K/Akt pathway [88]. Thus, we conjectured that pressure overload attenuates postconditioning- and insulin-induced cardioprotection, an effect caused by reduced PI3K-Akt signaling. The contribution of PI3K/Akt pathway was assessed in the context of determining the levels of relevant proteins and their phosphorylation status including the 3'-phosphoinositide dependent kinase 1 (PDK-1) and phosphatase and tensin homolog on chromosome ten (PTEN); PDK-1 and PTEN are positive and negative regulators of the PI3K/Akt signaling pathway, respectively. To further establish the role of myocardial load, we also determined whether pressure unloading (i.e., switchover from high to low pressure immediately upon reperfusion of the ischemic heart) confers cardioprotection comparable to either postconditioning or insulin treatment [88].

Pressure overload increased infarct size in association with changes in protein levels consistent with reduced PI3K-Akt signaling (i.e., ischemic reperfused vs. normoxic hearts). Postconditioning and insulin treatment reduced infarct size but it was greater in hearts perfused at the higher, than the lower, pressure. Wortmannin (a PI3K inhibitor) partially reversed postconditioning-induced cardioprotection, with infarct size being greater in the high-pressure group. Pressure unloading during reperfusion caused the most marked reduction in infarct size whereas pressure loading abolished postconditioning-induced cardioprotection. Nonetheless, the phospho-Akt/total Akt ratio and phospho-GSK-3beta levels were unaffected by perfusion pressure in insulin-treated or postconditioned hearts. Moreover, protein levels were similar in pressure-unloaded and pressure-loaded hearts. Collectively, these observations indicate that pressure overload reduces PI3K-Akt signaling following IR. However, a differential in PI3K-Akt signaling was not observed in ischemia-reperfused, insulin-treated, and postconditioned hearts, suggesting involvement of pathways other than PI3K-Akt

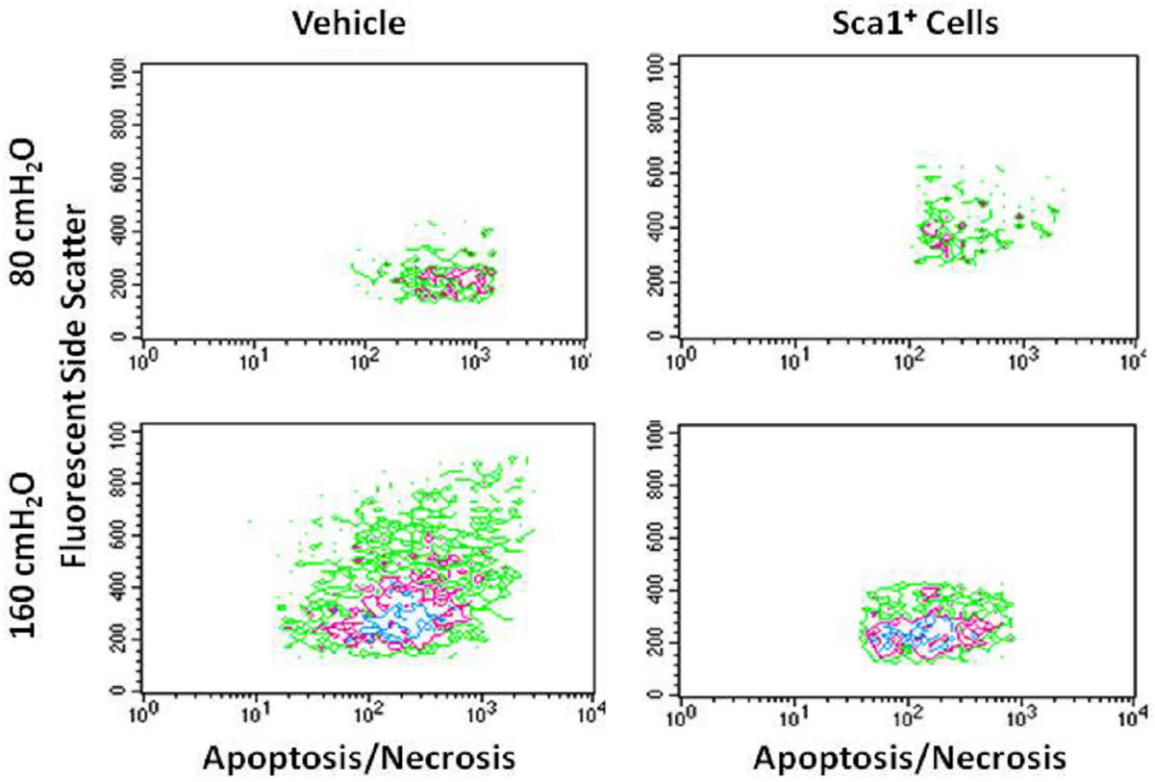

Figure 5. Scatter plots depict early apoptotic (green), late apoptotic (blue) and necrotic (red) cell death in cardiac cell preparations of ischemic-reperfused hearts that were subjected to either 80 or 160 cmH_2O. Immediately before the ischemic phase, hearts were transplanted (through the coronary arteries) with $Sca1^+$ cells.

for the effect of pressure on infarct size under these conditions. Potential players include members of mitogen-activated protein kinases which remain to be explored. Importantly, however, these studies also revealed that pressure unloading at reperfusion represents a novel and effective cardioprotective maneuver [88].

Effect of myocardial load on inflammation and the role of GSK-3β

The contribution of systemic immune and inflammatory mechanisms to the outcome of myocardial IR injury is well-established [89-94]. Importantly, however, it is increasingly recognized that the myocardium can mount a robust inflammatory response to an IR insult [95, 96]. The growth arrest- and DNA-damage inducible protein 153 (GADD153) regulates both apoptosis and inflammatory response [97, 98]. Importantly, GSK-3β may provide a mechanistic link for cellular expression of GADD153, inflammatory response and cell death [99, 100]. In light of our demonstration that pressure overload exacerbates myocardial IR injury associated with significant reduction in phosphorylated (inactive) GSK-3β level, we conjectured that pressure overload, through a GSK-3β-dependent mechanism, increases GADD153 expression, thereby upregulating inflammatory cytokine production and contributing to worsening of myocardial IR injury [80]. In the ischemic-reperfused hearts, pressure overload reduced the anti-inflammatory cytokine, interleukin (IL)-10, but increased pro-inflammatory cytokine, IL-17 without affecting IL-23 (a pro-inflammatory cytokine). Subsequent immunofluorescent labeling studies showed colocalization of IL-17 immunostaining with the brain natriuretic peptide indicating that the cardiomyocyte is a major source of IL-17. Subsequently, using flow cytometry, we have shown co-expression of IL-17 and IL-23 suggestive of cardiomyocyte generation of IL-23 too (**Figure 4**). These observations substantiate the robust ability of endogenous cardiac mechanisms to mount an inflammatory response following an IR insult. Other effects of the pressure overload in the ischemic-reperfused heart included increased expression of GADD153, decreased JC-1 aggre-

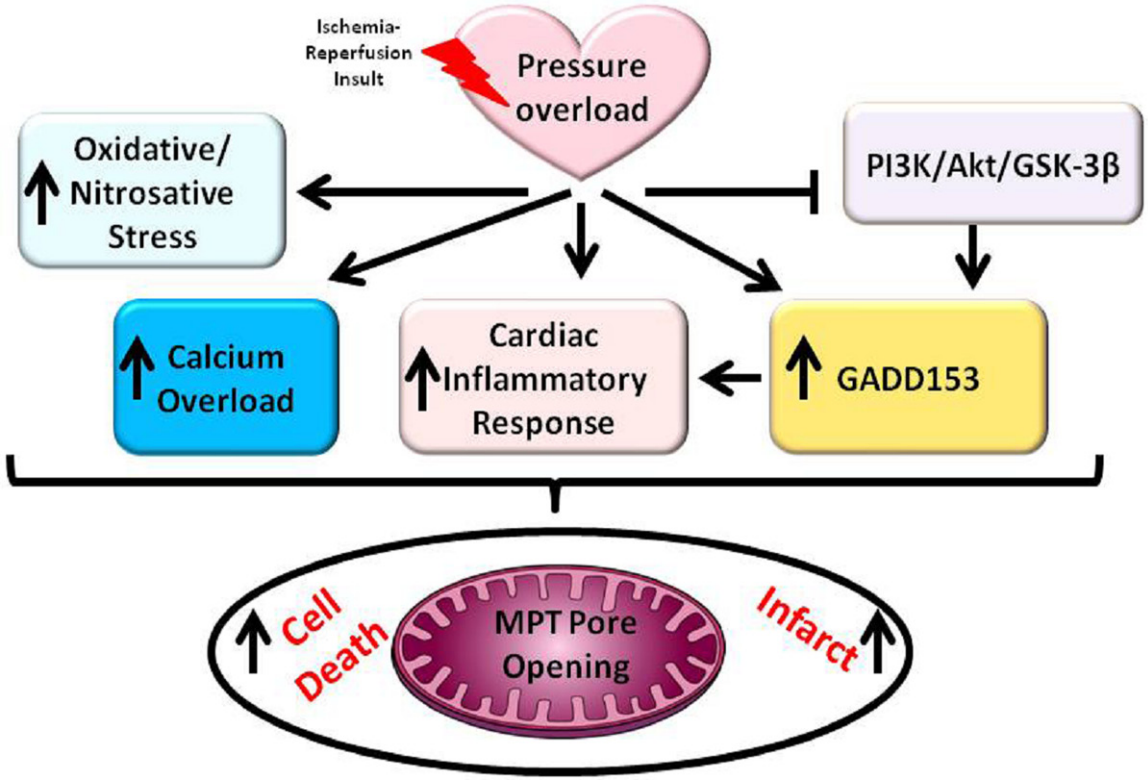

Figure 6. Schematic diagram showing major relevant pathways involved in the effect of pressure overload on the ischemic reperfused heart. An ischemia reperfusion insult exerts multiple and diverse effects including a) increased oxidative/nitrosative stress, b) intracellular calcium overload, c) downregulation of cardioprotection of PI3K-Akt/GSK-3β pathway and d) enhanced inflammatory responses, in part, through a GSK-3β-dependent mechanism involving increased GADD153 expression. Consequently, dysregulation of mitochondrial membrane potential leads to induction of the MPT pore. These changes are augmented by pressure overload, culminating in exacerbation of cell death/infarct size.

gates but increased JC-1 monomers (suggestive of reduced mitochondrial membrane potential (ψ_m)) in association with increased annexin V immunostaining as well as apoptotic and necrotic cell death. Importantly, treatment with LiCl (an inhibitor of GSK-3β) caused a robust increase in IL-10, preserved ψ_m and markedly decreased other parameters (e.g., IL-17 and GADD153) with the effect being most prominent for hearts perfused at the high pressure. Collectively, these observations indicate that pressure overload, via a GSK-3β-dependent mechanism, exacerbates cell death in the isolated ischemic-reperfused heart involving regulation of GADD153 expression and inflammatory response [80]. It is noteworthy that while both an IR insult and pressure overload regulate cardiac cytokine production, the link and mechanisms between cytokine production and cell death remain to be established. However, of interest is a recent report suggesting synergistic interaction between IL-17 ad tumor necrosis factor-α (TNF-α) in augmenting oxidative stress and apoptosis of oligodendrocytes [101]. TNF-α generation also increases in the isolated ischemic-reperfused heart [102]. Therefore, further studies should explore pressure-related regulation of mitochondrial death pathway by pro-inflammatory cytokines in the ischemic-reperfused heart.

Effect of bone marrow-derived stem cells on the outcome of IR injury

The heart is now known to have resident stem cells; yet, the endogenous reparative capacity of the myocardium seems unable to replenish marked loss of cardiomyocytes which occur following acute myocardial infarction [103, 104]. Consequently, in view of the prevailing ethical considerations about use of embryonic stem cells, attention has focused on the potential

usefulness of adult stem cells particularly given the demonstration that bone marrow-derived stem cells (BMDSCs) can be recruited into the heart and transdifferentiate into cardiomyocytes and cells of vascular lineage [105-110]. Indeed, the therapeutic usefulness of BMDSCs in the setting of acute myocardial infarction is now well-established. Nonetheless, it is increasingly recognized that the principal mechanism underlying the beneficial effects of BMDSCs does not relate to their ability to transdifferentiate to cardiomyocytes, smooth muscle and endothelial cells. Rather, BMDSCs release a whole host of cytokines, chemokines and growth factors which then exert their effects via paracrine fashion [106, 109, 110]. As a result, they promote a local microenvironment and cytokine milieu conducive to reducing initial damage to the injurious stimulus (i.e., ischemia and/or reperfusion insult) and also promote repair and recovery of the damaged tissue.

In light of the above, we have carried out pilot studies to determine whether administration of BMDSCs would confer protection to the pressure-overloaded ischemic-reperfused heart. We utilized a protocol which has been extensively used by Meldrum and colleagues whereby Langendorff-perfused rat heart is transplanted via intracoronary administration of stem cells prior to induction of global ischemia [111-114]. As expected, pressure overload increased cell death in vehicle-treated ischemic-reperfused hearts. Treatment with $Sca1^+$ cells reduced cell death with the effect more prominent for hearts subjected to high, than low, perfusion pressure (**Figure 5**). Thus, our ongoing studies are focused on establishing the impact of BMDSCs on pressure-related cardiac cytokine production along with assessment of mitochondrial status and cell death. In this context, it is of interest to establish whether empowering BMDSCs (through up- or down-regulation of relevant genes) would abrogate the adverse impact of pressure overload on the ischemic-reperfused heart.

Conclusion

Systemic hypertension is a very common disorder worldwide. Further, since the prevalence of systemic hypertension increases with age, it represents an accumulation of years of pressure overload on target organs. Thus, hypertension-related clinical sequels, such as ischemic heart disease and myocardial infarction, will continue to present pressing challenges. While the myocardium can develop adaptive mechanisms to cope with stress, such mechanisms usually fail in the long-term thereby increasing its venerability to insults including ischemic events. Surprisingly, however, the vast majority of studies focusing on mechanisms of ischemia reperfusion injury have not taken into consideration the impact of myocardial load/mechanical stress. Our studies over the last decade indicate critical dependency of key components of endogenous cardiac mechanisms on myocardial load. Accordingly, reduced contribution of cardioprotective pathways coupled with augmented activity of cardiotoxic pathways predispose the pressure overloaded heart to exacerbated ischemia reperfusion injury (**Figure 6**). Further elucidation of mechanisms that are differentially regulated by myocardial load should lead to identification of novel therapeutic target(s).

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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