ONLINE SUPPLEMENT

Pressure Overload Regulates Expression of Cytokines, γH2AX and GADD153 via GSK-3β in Ischemic-Reperfused Hearts

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Methods

Male Sprague-Dawley rats (9-11 weeks of age; Harlan Laboratories, Indianapolis, IN) were obtained and housed in a room maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C) and light cycle (0600-1800h). The use of animals for this study was approved by the GHSU institutional animal care and use committee.

For all isolated heart perfusion experiments, the animals were heparinized (1000 U/kg) and decapitated prior to removing the hearts and perfusing them on a Langendorff apparatus. The perfusion medium was standard Krebs-Henseleit buffer (37°C) containing 11 mM glucose and equilibrated with 95% O2-5% CO2; the perfusion pressure was set at either 80 or 160 cm H2O (i.e., 59 or 118 mmHg, respectively). We have established that hearts perfused at 160 than 80 cmH2O, under baseline conditions, display significant increases in coronary flow rate, rate-pressure product, ventricular developed pressure and indices of myocardial contractility and relaxation (i.e., maximum ± dP/dt); further, following an ischemia reperfusion insult, hearts subjected to the high pressure display greater reduction in myocardial function in association with increased infarct size/cell death compared to those subjected to low pressure.1-4 The perfusion buffer lacked or contained lithium chloride (LiCl, 1 mM) which was used as an inhibitor of GSK-3β; the marked infarct-sparing effect of LiCl is similar to SB216763, another GSK-3 β inhibitor.1,5 Following a period of stabilization (25 min), each heart was subjected to 40 min of ischemia followed by 15 min of reperfusion (n=5 hearts/group/condition). Time-controlled normoxic hearts served as controls (n=4 hearts/group). Thereafter, cardiac tissue was filtered through a 100 µm cell strainer (BD Biosciences, Bedford, MA) and centrifuged (1500 rpm, 10 minutes) to obtain single cell suspension for flow-cytometry based assays or cytoospin
preparation for immunofluorescence staining.\textsuperscript{3,6} Also, myocardial tissue was fixed in buffered formalin for subsequent immunohistochemical studies.\textsuperscript{7}

**Analytical Flow Cytometry**

Commercially available antibodies against each protein of interest were used coupled with the use of a \textit{FACSCalibur} flow cytometer (BD BioSciences, San Diego, CA) as described previously.\textsuperscript{6,8}

**Immunostaining Protocols**

Cytospin preparations from each experimental group were subjected to immunofluorescence staining for IL-10, IL-17 and IL-23; the nuclear counterstain 6-diamino-2-phenylindole (DAPI) labels DNA and was used for detection of cells. Further, formalin-fixed paraffin-embedded heart tissue was cut in 4 µm sections and processed for immunohistochemical assessment of γH2AX, GADD153 or annexin V according to previously described protocols.\textsuperscript{6-8} In addition, as an initial attempt to identify cellular source (e.g., cardiomyocytes) of the pro-inflammatory cytokines (e.g., IL-17), co-immunostaining protocol was carried out using brain natriuretic peptide (BNP) as a marker of cardiomyocytes.

**Assessment of Mitochondrial Membrane Potential ($\psi_m$)**

For determination of $\psi_m$, we used JC-1 assay which is a cationic dye that accumulates in mitochondria. Monomers of JC-1 dye fluoresce in the green range while JC-1 aggregates fluoresce in the red range; therefore, an increase in green fluorescence intensity represents mitochondrial swelling. Since accumulation of JC-1 in the mitochondria is dependent on the $\psi_m$, loss of $\psi_m$ indicates loss of JC-1 aggregates and is manifested by decrease in red fluorescence.\textsuperscript{9,10} Flow cytometry application of the JC-1 technique can be used effectively as an index of $\psi_m$ and as a surrogate marker of mitochondrial permeability transition (MPT) pore opening, which is a critical event in cell death.\textsuperscript{3,9-10} Accordingly, heart cells were incubated for 15 min in the presence of 2 µM JC-1 at 37°C, 5% CO\textsubscript{2}, washed twice and resuspended in DMEM (1x10\textsuperscript{6} cells/ml). Thereafter, labeled cells were analyzed and quantified by flow cytometry with excitation at 488 nm and emission at 530 nm (green) or 590 (red).\textsuperscript{9-10}

**Assessment of Cell Death**

Assessment of necrosis and apoptosis was achieved using the flow-cytometry-based Annexin V/7-Amino-Actinomycin D (7-AAD) protocol.\textsuperscript{11} Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis; 7-AAD is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Staining was performed according to the manufacturer's instructions (BD Biosciences, Bedford, MA). In brief, cells were washed twice with cold PBS and then resuspended in Binding Buffer and gentle vortex prior to incubation with PE Annexin V and 7-AAD. Cells were analyzed by flow cytometry within 1 hour after adding binding buffer.

**Antibodies and Kits**

The primary antibodies against IL-10 and IL-17 (affinity purified goat polyclonal antibodies), IL-23 (a rabbit polyclonal antibody) and GADD153 (a mouse monoclonal antibody) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); IL-10 antibody from Santa Cruz was used for immunostaining studies. On the other hand, Alexa Fluor® 647Mouse Anti-Rat IL-10 for flow cytometry application was purchased from BD Pharmingen (Bedford, MA). A rabbit monoclonal antibody for phospho-histone H2AX (i.e., γH2AX, serine 139) was
purchased from Cell signaling Technology (Danvers, MA). Annexin V: PE Apoptosis Detection
Kit was obtained from BD Biosciences (Bedford, MA).

**Constant flow perfusion studies:**

We previously suggested that increasing the height of the buffer jar above the
Langendorff-perfused heart exerts the dual effects of increasing myocardial load and perfusion
pressure, two hallmark features of systemic hypertension.\(^1\) This contention was based on the
observations that hearts perfused at 160 than 80 cmH\(_2\)O, under baseline conditions, display
significant increases in coronary flow rate (about 2 fold), ventricular developed pressure and
indices of myocardial contractility and relaxation (i.e., maximum ± dP/dt).\(^1,2\) Further, following
an ischemia reperfusion insult, hearts subjected to the high pressure display greater reduction in
myocardial function in association with increased infarct size/cell death.\(^1,3\) In light of the
marked adverse impact of pressure overload (e.g., perfusion at high vs. low pressure head) on the
ischemic-reperfused heart\(^1,3\), we sought to also establish the effects of increased coronary
perfusion achieved via the constant flow perfusion protocol for comparison to effects described
for hearts subjected to 80 or 160 cmH\(_2\)O pressure head (i.e., Figures 1-5).

It is important to note that the “slow-flow” or “no-reflow” phenomenon is a well-
described feature associated with the reperfusion of the ischemic heart which depends, in part, on
the duration of ischemia.\(^12\) Our studies have utilized an ischemia duration of 40 min and
established the time course of changes in coronary flow in the regional model of ischemia
reperfusion injury.\(^2\) The present studies utilized the global model of ischemia reperfusion injury.
In pilot studies, we established that coronary flow rate decreases an average of about 50% (from
baseline values) during the initial 15 min of reperfusion in the global model of ischemia
reperfusion injury. Therefore, in order to simulate coronary flow changes that the globally
ischemic-reperfused heart experiences and apply those changes to the constant flow perfusion
protocol, we carried out the following protocol: a) hearts were perfused with constant coronary
flow rate of either 16 or 8 ml/min during the initial 25 min stabilization phase\(^2\), b) following the
stabilization period, the hearts were subjected to 40 min of global ischemia and c) the reperfusion
phase was carried out for 15 min with coronary flow rate reduced to 50% of the stabilization
phase for high and low flow rates (i.e., 8 and 4 ml/min, respectively; n=3 hearts/condition).
Thereafter, as described under Methods, cardiac cells were prepared and subjected to flow
cytometry-based assays. Data were analyzed using Student’s t-test (p<0.05).

As shown in Figure S1, the percent of cells that were positive for γH2AX (A), IL-10 (B)
and IL-17 (C) were similar between the two groups. On the other hand, the percent of
GADD153 positive cells was greater for ischemic-reperfused hearts subjected to the high than
the low constant flow perfusion (Figure S1, panel D). Increased GADD153 expression was
associated with a small increase in percent of apoptotic and necrotic cells in hearts subjected to
the high than low constant perfusion (Figure S1, panel E). Further, cell preparations of hearts
perfused at the high, than low, flow rate showed a tendency for increased JC-1 monomers but
reduced JC-1 aggregates (Figure S1, panel F).

In conclusion, comparison of data, from experiments whereby the pressure head is
adjusted against the heart (i.e., Figures 1-5) with those whereby coronary flow rate is adjusted
(Figure S1), indicates that the primary determinant of the outcome of an ischemia reperfusion
insult, under the conditions of the present study, relates to the pressure and associated
mechanical stress/load on the myocardium.\(^1,3\)
References:
Figure S1. Panels show percent of cells that were positive for γH2AX (A), IL-10 (B), IL-17 (C) and GADD153 (D) in cardiac cell preparations of hearts subjected to low and high flow rate as described in the text. Also shown are percent of apoptotic, necrotic and normal cells (E) as well as percent of JC-1 monomers and aggregates (F) for the experimental groups. * p<0.05 compared to the low flow rate group.