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Proteomic mapping of proteins released during necrosis and apoptosis from cultured neonatal cardiac myocytes

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Marshall KD, Edwards MA, Krenz M, Davis JW, Baines CP. Proteomic mapping of proteins released during necrosis and apoptosis from cultured neonatal cardiac myocytes. Am J Physiol Cell Physiol 306: C639–C647, 2014. First published January 8, 2014; doi:10.1152/ajpcell.00167.2013.—Cardiac injury induces myocyte apoptosis and necrosis, resulting in the secretion and/or release of intracellular proteins. Currently, myocardial injury can be detected by analysis of a limited number of biomarkers in blood or coronary artery perfusate. However, the complete proteomic signature of protein release from necrotic cardiac myocytes is unknown. Therefore, we undertook a proteomic-based study of proteins released from cultured neonatal rat cardiac myocytes in response to H2O2 (necrosis) or staurosporine (apoptosis) to identify novel specific markers of cardiac myocyte cell death. Necrosis and apoptosis resulted in the identification of 147 and 79 proteins, respectively. Necrosis resulted in a relative increase in the amount of many proteins including the classical necrotic markers lactate dehydrogenase (LDH), high-mobility group B1 (HMGB1), myoglobin, enolase, and 14-3-3 proteins. Additionally, we identified several novel markers of necrosis including HSP90, α-actinin, and Trim72, many of which were elevated over control levels earlier than classical markers of necrotic injury. In contrast, the majority of identified proteins remained at low levels during apoptotic cell death, resulting in no candidate markers for apoptosis being identified. Blotting for a selection of these proteins confirmed their release during necrosis but not apoptosis. We were able to confirm the presence of classical necrotic markers in the extracellular milieu of necrotic myocytes. We also were able to identify novel markers of necrotic cell death with relatively early release profiles compared with classical protein markers of necrosis. These results have implications for the discovery of novel biomarkers of necrotic myocyte injury, especially in the context of ischemia-reperfusion injury.

necrosis; apoptosis; cardiac myocyte; proteomics

APOPTOSIS AND NECROSIS REPRESENT TWO IMPORTANT MECHANISMS of cell death during cardiac disease (18, 20). Morphologically, cells undergoing apoptosis or necrosis have distinct characteristics. Apoptotic cell death results in the formation of membrane-enclosed apoptotic bodies that contain intact organelles and cytoplasm, which are engulfed by phagocytic cells (35). Necrotic cells are characterized by organelle swelling, nuclear condensation, and rupture of the plasma membrane (35). Apoptosis is a “clean” form of cell death, in which very little inflammatory activation occurs. In contrast, necrosis is a “messy” form of cell death, releasing factors into the extracellular milieu, which results in activation of the inflammatory response (15, 25).

Previously, necrosis has been shown to result in the release of specific intracellular proteins. The chromatin binding protein high-mobility group B1 (HMGB1) is released preferentially during necrotic cell death, and its release is actually inhibited via chromatin binding during apoptosis (2, 28). Additionally, the cytosolic proteins lactate dehydrogenase (LDH), 14-3-3 proteins, and peptidyl-prolyl cis-trans isomerase cyclophilin A (CypA) are released from necrotic cells (5, 30). Finally, the intracellular protein interleukin-1α is sequestered in the nucleus of apoptotic cells but is released into the extracellular environment by necrotic cells (6). These markers of cell death have been shown to be specific to necrosis in multiple types of cells. Interestingly, while factors released from cells undergoing necrosis are known to stimulate an inflammatory response (28, 29), cells undergoing apoptosis promote survival in neighboring cells via the release of specific factors (36). Apoptotic cell death has been shown to result in the release of specific factors into the surrounding tissue including sphingosine-1-phosphate (36). As a second example, the protein transforming growth factor-β is secreted by apoptotic liver cells and T-cells (4).

Proteomics is a powerful tool in the field of biomarker discovery. The use of proteomic analysis has revolutionized the identification of novel biomarkers for various disease states. Altered protein profiles can indicate the presence or absence of progression and even treatment efficacy of different diseases (1). This technique has been utilized to identify specific markers of many diseases and injuries. For example, traumatic brain injury results in an increase in levels of several 14-3-3 protein isoforms as well as enolase in cerebral spinal fluid (30). Consistent with this, necrosis of cortical neurons also released 14-3-3 proteins alongside fatty acid binding protein (FABP), LDH, and enolase (12). Proteomic analysis of...
snake venom-induced necrotic wound exudate identified many intracellular proteins, the most abundant being titin, creatine kinase, and various glycolytic enzymes. (26).

In the context of the myocardium, coronary artery perfuse has been used to identify biomarkers associated with cardiac ischemia-reperfusion injury (8). Proteins included the classical markers LDH, creatine kinase, FABP3, and troponin proteins but also novel candidates such as cysteine and glycine-rich protein 3 (Csp3) and myosin-binding protein C (MyBP-C). A similar study identified 320 proteins released following myocardial infarction, especially MyBP-C and oxidative stress-related proteins such as peroxiredoxins (14).

However, while perfuse from whole heart has been analyzed following injury, the specific contributions of the cardiac myocyte to the postnecrotic extracellular milieu have not been examined. Moreover, the proteomic signature of protein release from necrotic myocytes is not known. The aim of the present study, therefore, was to conduct proteomic analyses of the proteins released by isolated cardiac myocytes during necrosis, and compare that to those released during apoptosis.¹

**MATERIALS AND METHODS**

Reagents. Sytox Green was purchased from Invitrogen; M199 medium, Hanks’ buffered saline solution (HBSS), penicillin/streptomycin, and bovine growth serum (BGS) were purchased from HyClone; the neonatal rat myocyte isolation kit was purchased from Worthington; the silver staining kit was purchased from Thermo Pierce; zVAD-FMK was purchased from Promega; β-lapachone was from Enzo Life Sciences; the TUNEL kit was purchased from Roche; and all other chemicals/reagents were from Fisher and Sigma-Aldrich.

Cell culture. All experiments involving the harvesting of neonatal rat hearts were approved by the University of Missouri-Columbia Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the use and care of animals. Neonatal rat ventricular myocytes were isolated according to the Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the use and care of animals. Neonatal rat ventricular myocytes were isolated according to the manufacturer’s instructions. Briefly, hearts were harvested from 1- to 3-day-old rat pups and digested with trypsin and collagenase. Following differential plating to remove fibroblasts, the cardiomyocytes were plated on gelatin-coated dishes and cultured overnight in M199 medium supplemented with 10% BGS and penicillin/streptomycin. The next day the cells were washed twice with PBS and incubated in serum-free M199 media for a further 2 days.

Cell death assays. Myocytes were cultured in six-well plates at a density of 2.5 × 10⁵ cells per well. Following treatment, necrosis was determined by Sytox Green exclusion. Briefly, treated myocytes on six-well plates were incubated with Sytox in HBSS for 30 min at 37°C. Cells were then fixed in 4% paraformaldehyde and counterstained with bis-benzamide to stain all nuclei. The resultant fluorescence images were then collected using an inverted fluorescent microscope (Olympus IX51) connected to a digital camera. TUNEL staining was performed according to the manufacturer’s instructions.

Sample preparation. Myocytes were cultured in 10-cm plates at a density of 3 × 10⁶ with 5 ml of media. After treatment the media were collected and spun at 10,000 g for 10 min to remove cells and cell fragments. The supernatant was loaded onto an Amicon Ultra centrifugal filter with a molecular mass cutoff of 3 kDa and centrifuged at 10,000 g for 30 min at 4°C. Concentrated media were removed from the filter, and protein was precipitated using 4 volumes of ice-cold 80% acetone. The precipitated protein was separated via centrifugation at 17,000 g for 10 min at 4°C, and the resultant pellet was subjected to either proteomic analyses or SDS-PAGE.

Mass spectrometry. The acetone pellet was resuspended in 10 μl of urea buffer (6 M urea, 100 mM HEPES, pH 7.8, 100 mM DTT). Protein was reduced and alkylated with iodoacetamide and digested overnight with trypsin. The digest was acidified with 1% (final concentration) formic acid. Following centrifugation, 20 μl were removed to an autosampler vial and placed at 4°C in the LC system’s autosampler. A portion of the digest (10 μl) was loaded onto a C8 trap column (C8 captrat, Michrom Bioresources). Bound peptides were eluted from this trap column onto a 10.5-cm, 150-μm ID pulled-needle analytical column packed with Magic AQ C18 reversed phase resin (Michrom Bioreources). Peptides were separated and eluted from the analytical column with a continuous gradient of acetonitrile from 5 to 45% (in 0.1% formic acid) over 80 min. The Proxeon Easy nLC-II HPLC system was attached to an LTQ Orbitrap mass spectrometer. Following a high-resolution FTMS scan of the eluting peptides, each second, the nine most abundant peptides were subjected to peptide fragmentation (CID in iontrap). Data across a total of 100 min of elution were collected and then searched against the NCBI nr DB database using Sorecerer2 IDA with the following criteria: fixed modification of carbamidomethyl cysteine, trypsin cuts KR except before P, with up to 2 missed cleavages, using monoisotopic precursors within a mass range of 400 to 4,200 atomic mass units, precursor mass tolerance 25 ppm, fragment mass tolerance of 0.8 Da, oxidized methionine as a variable modification. A decoy database strategy was used to calculate protein and peptide false discovery rates (FDR). A randomized decoy database was generated using the Decoy-DBCreator V0.1 (http://www.p3db.org/p3db1.0/tools/DecoyDBCreator/) and concatenated to the forward mammalian sequences (FASTA-formatted protein sequences). Identified proteins were examined using Scaffold and were filtered to report only those proteins with >99% confidence of identification with a three peptide minimum (proteins identified by a single peptide were eliminated) and >95% confidence of peptide identification. An additional precursor mass filter of ±10 ppm was also employed. Employing these stringent criteria resulted in a protein FDR of 0.0% and a peptide FDR of 5.2%. Protein scoring and peptide scoring are based on the peptide- and protein-prophet algorithms (17).

Bioinformatics analysis. To identify proteins whose concentrations were correlated in a time-dependent (or dose-dependent) manner, we computed Spearman’s exact test of correlation between the abundance (spectral counts) of each identically protein and time (or dose) for each independent experiment. Spearman’s correlation is a nonparametric test, which makes no distributional assumptions on the data. Within each of the time-dependent and dose-dependent analyses, the resultant P values between independent experiments were combined into a single P value using Fisher’s method (23), which was then adjusted for multiple testing using the Benjamini-Hochberg false discovery rate-based approach, and reported as q values. All computations were made using R statistical software with the pspearman (27a) and MADAM (19a) packages.

Within each of the time-dependent and dose-dependent experiments, it was important to define a reference (aka universe) of proteins, which is a requirement for more complex downstream analyses. For the time-dependent experiment, the reference set was defined to be any protein identified (as described previously) within any of three replicates at any time point, as well as any protein identified at the zero concentration within any of the three replicates from the dose-dependent experiment. Similarly, for the dose-dependent experiment, the reference set was defined to be any protein identified (as described previously) within any of three replicates at any dose, as well as any protein identified at time zero within any of the three replicates from the time-dependent experiment. These reference sets were used in conjunction with Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com) to identify canonical pathways that were enriched for genes associated with the proteins that demonstrated a significant (q < 0.20) time- or dose-dependent relationship. We also used IPA to build novel networks from the significant (q < 0.2) genes in the reference set. GI accession numbers

¹ This article is the topic of an Editorial Focus by Mark A. Knepper (17a).

C640 PROTEINS RELEASED DURING MYOCYTE NECROSIS

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neonatal myocytes with increasing concentrations of H2O2 for protein release in response to oxidative stress. Treatment of ventricular cardiac myocytes treated with increasing concentrations of H2O2 for 3 h. B: after treatment with the different concentrations of H2O2, media were collected and concentrated and proteins were acetone precipitated. The precipitated proteins were then run on an SDS-PAGE gel followed by silver staining. C: Sytox Green staining in neonatal rat ventricular cardiac myocytes treated for increasing times with 250 μM H2O2. D: silver staining of proteins released into the culture media following the different exposure times to H2O2.

Results shown are representative of 3 independent experiments, with the cell death experiments performed in duplicate. Error bars indicate SE; *P < 0.05 vs. control (Con).

(Supplemental Tables S1 and S2; Supplemental Material for this article is available online at the Journal website) from the reference sets were converted to Gene IDs (Entrez) in IPA. In situations where multiple accession numbers mapped back to the same gene, the accession number with the median q value was retained for further analysis. Not all accession numbers (e.g., bovine) could be mapped because IPA only derives its Knowledge Base from human, rat, and mouse data.

**SDS-PAGE.** Precipitated proteins were resolved by SDS-PAGE using 10–15% acrylamide. For silver staining the gels were fixed in 30% methanol-10% acetic acid and then stained according to the manufacturer’s instructions. For Western blotting the proteins were transferred onto PVDF membranes and blotted using the following commercially available antibodies: LDH (ab52488), HMGB1 (ab18256), and Trim72 (ab88302) from Abcam; pan 14-3-3 (sc-629), commercially available antibodies: LDH (ab52488), HMGB1 (ab18256), and Trim72 (ab88302). Precipitated proteins were run on an SDS-PAGE gel followed by silver staining. Precipitated proteins were then run on an SDS-PAGE gel followed by silver staining. For Western blotting the proteins were transferred onto PVDF membranes and blotted using the following manufacturer’s instructions. For Western blotting the proteins were transferred onto PVDF membranes and blotted using the following manufacturer’s instructions. For Western blotting the proteins were transferred onto PVDF membranes and blotted using the following manufacturer’s instructions.

**Statistics.** Differences in cell death end points were calculated by one-way ANOVA followed by Scheffé’s post hoc test using StatPlus software. P < 0.05 was considered statistically significant.

**RESULTS**

Concentration- and time-dependent induction of necrosis in myocytes. We first characterized the induction of necrosis and protein release in response to oxidative stress. Treatment of neonatal myocytes with increasing concentrations of H2O2 for 3 h induced a dose-dependent increase in necrosis as measured by Sytox Green exclusion (Fig. 1A). Silver staining similarly revealed a concomitant dose-dependent release of proteins into the media following H2O2 exposure (Fig. 1B). Importantly, H2O2-induced cell death was insensitive to the pan-caspase inhibitor zVAD-FMK (data not shown), demonstrating that this was indeed primary necrosis and not merely secondary necrosis after apoptosis. We then took the 250 μM concentration, as this gave us a near maximal necrosis, and examined the temporal response. Incubation of the myocytes with this concentration of H2O2 induced a time-dependent necrosis (Fig. 1C). Again, this was mirrored by a time-dependent release of proteins into the culture media (Fig. 1D).

**Proteomic identification of proteins released during necrosis.** In a parallel set of experiments we subjected the concentrated media from each time point following 250 μM H2O2 treatments to shotgun proteomic analysis. Unweighted spectrum counts for all proteins identified from three independent experiments were collected (Supplemental Table S1), and those proteins that were observed in all three experiments were subjected to further analysis. A total of 147 proteins common to all three experiments were identified (Supplemental Table S1), some of which were present in the control sample, e.g., actin, myosin-7, and collagen, and others that were only detected following H2O2 treatment, e.g., ezrin, MyBP-C, and Trim72.

Pathway analysis of the proteins released during necrosis (Table 1) demonstrated enrichment for the canonical pathways of aryl hydrocarbon signaling (e.g., HSP90, glutathione-S-transferase P, cathepsin-D) and 14-3-3-mediated signaling (e.g., 14-3-3 γ, ζ, and ε). Glycolysis was also very well represented with 13 hits out a size of 41 and included such proteins as LDH, enolase-3, phosphoglucomutase-1, and phosphoglycerate kinase-1. Remodeling of epithelial adherens junctions (e.g., actin-related protein-2/3 complex subunit 4 [ARPC4] 2, α-actinin-2, tubulin-β) and ephrin receptor signaling (e.g., cofilin, ARPC4) were also well represented (Table 1). Identification of novel networks, using a score cutoff of >20, revealed several networks including those involved in RNA posttranscriptional modification, posttranslational modification, and protein folding (Table 2). Not surprisingly, networks covering cell death and survival, cellular compromise, and cardiovascular disease were also significantly represented, as

![Table 1. Significant canonical pathways for proteins released during necrosis (Set 1) sorted by P value](http://ajpcell.physiology.org/)

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathway</th>
<th>P</th>
<th>Hits</th>
<th>Size %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl hydrocarbon receptor signaling</td>
<td>5.75E-03</td>
<td>17</td>
<td>171</td>
</tr>
<tr>
<td>14-3-3-Mediated signaling</td>
<td>1.95E-02</td>
<td>13</td>
<td>121</td>
</tr>
<tr>
<td>Glycolysis I</td>
<td>1.95E-02</td>
<td>13</td>
<td>41</td>
</tr>
<tr>
<td>Remodeling of epithelial adherens junctions</td>
<td>4.07E-02</td>
<td>16</td>
<td>70</td>
</tr>
<tr>
<td>Ephrin receptor signaling</td>
<td>4.90E-02</td>
<td>10</td>
<td>210</td>
</tr>
</tbody>
</table>

Size indicates number of molecules that comprise a canonical pathway in the Ingenuity Knowledge Base, and hits indicates number of the significantly correlated genes that are members of that pathway. GI protein numbers were mapped to molecules (mostly genes) using Ingenuity Pathway Analysis (IPA). P represents hits/size. P is based on Fisher’s exact test and indicates pathways that are enriched for significant proteins/genes from Set 1 protein, with Set 1 universe as the reference set (see MATERIALS and METHODS for details). Pathways with P < 0.05 and at least 2 hits were considered significant.
were networks involved in redox status (glutathione depletion and free radical scavenging).

In terms of necrotic release, the archetypal necrosis indicators LDH and HMGB1 exhibited the expected time-dependent increases in response to H$_2$O$_2$ (Supplemental Table S1). Other proteins previously reported to be released during necrosis, such as myoglobin, MyBP-C, FABP3, and 14-3-3 proteins, were also identified as being progressively released. The majority of proteins that were also released during necrosis have not been previously identified. These included the elongation factor proteins, various nucleus-associated proteins (importins and Ran GTPase), membrane repair proteins (Trim72), vesicular proteins [Vps35, transitional ER (TER)-ATPase], lysosomal proteins (cathepsin-D, prosaposin), and cytoskeletal/contractile proteins (α-actinin, filamin, coflin, and moesin). However, not all cytoskeletal/contractile proteins were released and no significant alterations in the media levels of myosin heavy and light chains and cytoplasmic actin were observed. Interestingly, although multiple mitochondrial proteins were identified (aspartate aminotransferase, ATP synthase-β, cytochrome c, cytochrome-c oxidase 6B1, malate dehydrogenase), none of these exhibited a time-dependent release in response to H$_2$O$_2$.

Western analyses of proteins released during necrosis. To confirm some of the proteins identified as being released during H$_2$O$_2$-induced necrosis, we performed Western blotting on the samples from the temporal studies. The proteins chosen were either classical necrosis markers such as LDH and HMGB1, or novel proteins that exhibited the largest changes in spectrum counts in the proteomic analyses such as Trim72 and eEF1α2. As shown in Fig. 2, H$_2$O$_2$ elicited a time-dependent release of LDH, HMGB1, myoglobin, FABP3, enolase, and 14-3-3 proteins into the culture media. Trim72, HSP90, TER-ATPase, eEF1α2, and α-actinin were also released in a similar pattern, thereby corroborating the proteomic data.

We next wanted to test whether the release of these proteins was specific to H$_2$O$_2$ or whether they were general markers of necrosis. To this end we treated the neonatal myocytes with β-lapachone (20 μM) induced a time-dependent increase in necrosis as measured by Sytox Green exclusion (Fig. 3A). This was associated with a concomitant release of proteins into the culture media (Fig. 3B). Western analyses revealed the significant release of LDH, HMGB1 myoglobin, FABP3, enolase, 14-3-3, Trim72, HSP90, TER-ATPase, eEF1α2, and α-actinin (Fig. 3C), similar to that seen with H$_2$O$_2$ treatment.

Proteomic identification of proteins released during apoptosis. We next compared the protein release during necrosis against that of apoptosis. Treatment of neonatal myocytes with increasing concentrations of staurosporine for 18 h led to a dose-dependent increase in apoptosis as measured by TUNEL staining (Fig. 4A). Silver staining of the media following treatment revealed a modest increase in proteins that was equivalent over

![Fig. 2. H$_2$O$_2$-induced release of specific proteins from cardiac myocytes. Western blotting was performed for lactate dehydrogenase (LDH), high-mobility group B1 (HMGB1), myoglobin, fatty acid binding protein 3 (FABP3), enolase, Trim72, 14-3-3 proteins, HSP90, transitional ER (TER)-ATPase, eukaryotic elongation factor eEF1α2, and α-actinin in concentrated media from cardiac myocytes exposed to 250 μM H$_2$O$_2$ for increasing times. Results shown are representative of 3 independent experiments.](http://ajpcell.physiology.org/ by 10.2032.246 on July 8, 2017 http://ajpcell.physiology.org/ Downloaded from)
pared with the necrosis studies, considerably fewer proteins were identified overall in the apoptotic samples (79 vs. 147). Of the 79 hits, 26 were unique to apoptosis including such proteins as biglycan, carboxypeptidase-E, filamin-α, laminin, periostin, transferrin, and vimentin. However, reanalysis of the necrosis data revealed that 25 of these proteins were identified in at least one if not two of the runs, with transferrin being the only protein identified unique to the apoptotic experiments. However, the levels of transferrin in the media did not change with staurosporine treatment. The remaining 53 proteins were in common with those identified in all three of the H_2O_2 experiments.

Similar to transferrin, the majority of these proteins did not significantly change with increasing concentrations of staurosporine, except at the highest concentration, e.g., enolase-3, LDH, and myoglobin. Many instead followed the pattern exhibited in the silver stain, such that there was a modest, if any, increase in counts for all concentrations, e.g., α-actinin-4, filamin-α, HSP90, and vimentin. Despite the small changes, pathway analysis revealed a significant number of canonical pathways enriched by the proteins released during apoptosis (Table 3). However, these were for the most part poorly represented, with only two to three hits for each pathway (Table 3). Consistent with this, only two novel networks (again using a cutoff of >20) were enriched for in the apoptosis study, one covering cellular assembly and organization and the other lipid metabolism (Table 4).

The proteomic data were confirmed by Western blotting for the same panel of proteins tested in the necrosis experiments (Fig. 4C). As with the proteomic studies, no major increases in the amount of protein in the media were seen for LDH, HMGB1, myoglobin, etc., except at the highest staurosporine concentration. The exceptions were TER-ATPase and α-actinin, which demonstrated elevated levels in response to staurosporine, although this effect was not dose dependent (Fig. 4C). To better compare the apoptosis vs. necrosis data, we reran the experiments in parallel, treating the neonatal myocytes with either 1 μM staurosporine for 18 h or 250 μM H_2O_2.
Table 3. Significant canonical pathways for proteins released during apoptosis (Set 2) sorted by P value

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathway</th>
<th>P</th>
<th>Hits</th>
<th>Size</th>
<th>%</th>
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<tr>
<td>Axonal guidance signaling</td>
<td>4.14E-03</td>
<td>2</td>
<td>20</td>
<td>10%</td>
</tr>
<tr>
<td>Protein kinase A signaling</td>
<td>7.37E-03</td>
<td>3</td>
<td>26</td>
<td>12%</td>
</tr>
<tr>
<td>Cardiac hypertrophy signaling</td>
<td>8.03E-03</td>
<td>2</td>
<td>10</td>
<td>21%</td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>9.22E-03</td>
<td>2</td>
<td>28</td>
<td>7%</td>
</tr>
<tr>
<td>Thrombin signaling</td>
<td>9.48E-03</td>
<td>2</td>
<td>41</td>
<td>5%</td>
</tr>
<tr>
<td>Leukocyte extravasation signaling</td>
<td>9.52E-03</td>
<td>2</td>
<td>47</td>
<td>4%</td>
</tr>
<tr>
<td>RhoGDI signaling</td>
<td>9.95E-03</td>
<td>2</td>
<td>13</td>
<td>16%</td>
</tr>
<tr>
<td>Endothelin-1 signaling</td>
<td>1.05E-02</td>
<td>2</td>
<td>6</td>
<td>32%</td>
</tr>
<tr>
<td>Tight junction signaling</td>
<td>1.20E-02</td>
<td>2</td>
<td>27</td>
<td>8%</td>
</tr>
<tr>
<td>Synaptic long-term depression</td>
<td>1.24E-02</td>
<td>2</td>
<td>15</td>
<td>13%</td>
</tr>
<tr>
<td>Cellular effects of sildenafil (Viagra)</td>
<td>1.29E-02</td>
<td>2</td>
<td>25</td>
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<td>Epithelial adherens junction signaling</td>
<td>1.30E-02</td>
<td>2</td>
<td>114</td>
<td>2%</td>
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<tr>
<td>Sperm motilify</td>
<td>1.40E-02</td>
<td>2</td>
<td>22</td>
<td>9%</td>
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<tr>
<td>p70S6K signaling</td>
<td>1.52E-02</td>
<td>2</td>
<td>62</td>
<td>3%</td>
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<tr>
<td>14-3-3-Mediated signaling</td>
<td>1.65E-02</td>
<td>2</td>
<td>68</td>
<td>3%</td>
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<tr>
<td>Antioxidant action of vitamin C</td>
<td>1.82E-02</td>
<td>2</td>
<td>32</td>
<td>6%</td>
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<tr>
<td>Dendritic cell maturation</td>
<td>1.90E-02</td>
<td>4</td>
<td>40</td>
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<td>Regulation of actin-based motility by Rho</td>
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<td>133</td>
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<td>9</td>
<td>23%</td>
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<td>5</td>
<td>60</td>
<td>8%</td>
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<tr>
<td>Glycolysis I</td>
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<td>2</td>
<td>19</td>
<td>10%</td>
</tr>
<tr>
<td>Intrinsic prothrombin activation pathway</td>
<td>8.11E-02</td>
<td>3</td>
<td>63</td>
<td>5%</td>
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<tr>
<td>Pentose phosphate pathway</td>
<td>8.70E-02</td>
<td>2</td>
<td>23</td>
<td>9%</td>
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</table>

Size indicates the number of molecules that comprise a canonical pathway in the Ingenuity Knowledgebase and hits indicates the number of the significantly correlated genes that are members of that pathway. GI protein numbers were mapped to molecules (mostly genes) using IPA. % represents hits/size. P is based on Fisher’s exact test and indicates pathways that are enriched for significant proteins/genes from Set 2, with Set 2 universe as the reference set (see MATERIALS AND METHODS for details). Pathways with P < 0.05 and at least 2 hits were considered significant.

for 3 h. Cells left untreated for 18 h were used as control. The media were then collected, concentrated, and subjected to immunoblotting for the same proteins shown in Figs. 3 and 4C. Marked release of LDH, HMGB1, myoglobin, FABP3, enolase, Trim72, and the elongation factor eEF1α also increased over control conditions in necrotic myocyte media as early as 30 min. Importantly, the elongation factor eEF1α also increased over control conditions in necrotic myocyte media as early as 30 min. Importantly, the release of these proteins was not restricted to necrosis induced by oxidative stress. The endoplasmic reticulum protein TER-ATPase, eEF1α, and α-actinin, a modest release was seen in the staurosporine samples (Fig. 5). However, this was still considerably less than that observed in response to H2O2.

DISCUSSION

The aim of the present study was to map the proteins released during cardiac myocyte necrosis, with the goal of identifying novel markers of necrotic injury that could have potential use as clinical biomarkers. Proteins identified in this study were mainly from the cytosolic compartment. However, endoplasmic reticulum proteins, cytoskeletal components, and nuclear proteins were identified as well. Interestingly, mitochondrial proteins were poorly represented in the protein milieu released from necrotic cells (see below). We confirmed several proteins that have been previously identified markers of necrotic tissue injury in a wide variety of disease states and in vitro cell culture models of necrosis. For example, we identified the known necrotic injury markers 14-3-3 (30), myoglobin (21), enolase (30), FABP3 (10, 21), creatine kinase (26), HMGB1, (2, 28), MyBP-C (14), and LDH (5). In addition, we also found that CypA, a recently reported necrotic marker (5), was also time-dependently released during necrosis induced by oxidative stress.

Our analyses also revealed novel markers of necrotic cell death. For example, Trim72 (also known as Mutsugumin-53), a muscle-specific, membrane repair protein (3), showed a time-dependent increase in relative expression after induction of necrosis by oxidative stress. The endoplasmic reticulum protein TER-ATPase (37) showed a similar expression pattern to Trim72, increasing in relative abundance early and remaining elevated out to 4 h. Relative levels of the chaperone protein HSP90, the cytoskeletal protein α-actinin, and the elongation factor eEF1α also increased over control conditions in necrotic myocyte media as early as 30 min. Importantly, the release of these proteins was not restricted to necrosis induced by oxidative stress. Necrotic death induced by activation of the PARP1-dependent necrosis pathway using β-lapachone also resulted in the significant release of the myoglobin, 14-3-3,

Table 4. Novel networks for proteins released during apoptosis (Set 2) sorted by significance score

<table>
<thead>
<tr>
<th>Network</th>
<th>Top Diseases and Functions</th>
<th>Score</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellular assembly and organization, cellular function and maintenance, connective tissue disorders</td>
<td>45</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Lipid metabolism, small molecule biochemistry, developmental disorder</td>
<td>24</td>
<td>11</td>
</tr>
</tbody>
</table>

Score was calculated as $-\log_{10}(P)$, where $P$ is calculated from Fisher’s exact test; higher numbers imply lower $P$ values. Molecules indicate the number of significant genes or gene products that comprise the network; the maximum was capped at 35 (default setting). The networks were built using an algorithm provided in Ingenuity Pathway Analysis (IPA) software and were built in part using relationships curated from the literature and contained in the Ingenuity Knowledge Base. A description of the network was derived by considering the most common functions and diseases associated with the molecules that comprise the network.

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would suggest that release of S100 proteins during ischemia-reperfusion (8). Again, the lack of this protein in our analyses may be from noncardiac cell types such as smooth muscle cells or fibroblasts.

Several recent studies have reported that the release of mitochondrial components during injury, especially mtDNA and formyl peptides, is a powerful stimulator of the inflammatory and immune responses (19, 38). In our study we identified several mitochondrial proteins in the conditioned media. However, to our surprise there was no additional release of any of these components in response to either H$_2$O$_2$ or staurosporine. This is in contrast to previous studies where the release of carbamoyl phosphate synthetase-1, mitochondrial transcription factor A, and cytochrome c has been reported (7, 9, 32). It is not entirely clear why we did not see necrotic release of mitochondrial proteins, and it raises the question of whether there is a specific release mechanism for a mitochondrion’s nucleic material but not its proteinacious componentry. It may be that during necrosis the mitochondria rupture to an extent that allows the escape of DNA and peptides but not the larger proteins.

Perhaps not unexpectedly we did not find an apoptosis-specific marker. Proteins were released during apoptosis; however, they were not unique to this form of cell death nor did their expression significantly change with increasing doses of staurosporine. Increased protein release did occur at the highest dose of staurosporine; however, this protein release was likely due to secondary necrosis. These proteins are therefore more associated with necrotic cell death and not apoptotic cell death. The only protein unique to the apoptotic samples was transferrin and its expression did not change, at least when measured by spectrum count. Its presence in the basal “apoptotic” media versus “necrotic” media is most likely due to longer incubation time (18 h vs. 3 h). In fact, many proteins were found in the media at baseline. This may be partly due to the fact that we do see a basal level of cell death (~5–10%) in the myocyte culture. However, a previous proteomic study demonstrated that neonatal rat cardiomyocytes in fact secrete many proteins (31). Indeed, many of the proteins we identified here are consistent with those identified in the previous report, including α-actin, the β-subunit of ATP synthase, various collagens, eEF1α2, myosin heavy chains, and transgelin-2 (31). Thus the proteins identified at baseline are likely a combination of cell death and secretion.

Currently, cardiac troponin is the gold standard biomarker for necrotic cardiac injury (24, 34). However, cardiac troponin might not be specific to necrotic injury, and it is actually released during ischemia without the presence of accompanying necrosis (13). Another potential biomarker, FABP3, is released during exercise (22). Other biomarkers of cardiac injury such as creatine kinase are detected only several hours after the initial injury and are less sensitive (16, 34). Thus, current clinical markers used to identify necrotic cardiac injury have delayed release profiles and may not be specific to necrotic myocytes. To the best of our knowledge, our study is the first to analyze the components of the protein milieu released in a time-dependent manner from necrotic and apoptotic cardiac myocytes. These data identify several possible protein biomarkers released during necrosis that can be utilized to detect both the presence and the extent of necrosis in future studies of cell death, perhaps in the form of an antibody array. More importantly, they may allow earlier and more specific

**Fig. 5.** Comparison of proteins released from cardiac myocytes following staurosporine or H$_2$O$_2$ exposure. Western blotting for LDH, HMGB1, myoglobin, FABP3, enolase, Trim72, 14-3-3 proteins, HSP90, TER-ATPase, eEF1α2, and α-actin in concentrated media from cardiac myocytes exposed to either 1 μM staurosporine (Stauro) for 18 h or 250 μM H$_2$O$_2$ for 3 h. Control media were incubated with the cells for 18 h. Results shown are representative of 3 independent experiments.
detection of necrotic cardiac injury, as well as enable the extent of injury to be determined noninvasively in the patient.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


