JAK/STAT and PI3K/AKT Pathways Form a Mutual Transactivation Loop and Afford Resistance to Oxidative Stress-Induced Apoptosis in Cardiomyocytes

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Key Words

Apoptosis • PI3K/AKT • NF- κ B • JAK2/STAT3 • Cardiomyocytes • Signaling pathway

Abstract

Cardiac tissues contain cells susceptible to and cells resistant to apoptosis, and this difference is important for normal morphogenesis during development and for abnormal loss of cells during pathogenesis such as myocardial infarction and heart failure. While efforts have been made to understand the cellular and intercellular events of apoptotic cells, the signaling mechanisms in cells surviving from apoptotic injuries have been overlooked. Understanding signal transduction processes in cells with apoptosis resistance is of crucial importance to develop better strategies of preserving post-mitotic cells. To this end, we performed studies in neonatal rat ventricular myocytes using oxidative stress (H_2O_2) as an apoptotic

inducer. We identified a population of cells bearing higher resistance to apoptosis and found that the cells that survived from apoptotic insults had markedly higher levels of AKT and STAT3. Inhibition of AKT activity by a dominant negative AKT construct or by a PI3K inhibitor reduced active NF- κ B and STAT3 expression without significantly altering the activity of the latter. Activation of AKT by a constitutively activated AKT construct caused the opposite effects. Direct activation of NF- κ B also enhanced STAT3 expression, an effect abrogated by NF- κ B inhibitor. On the other hand, knockdown of STAT3 by siRNA or inhibition of STAT3 activity by decoy oligodeoxynucleotides or by JAK2 inhibitor diminished AKT expression. In conclusion, cardiomyocytes possess an apoptosis-resistant property as a cytoprotection mechanism which is likely conferred by mutual transactivation between AKT/NF- κ B and JAK2/STAT3, a novel crosstalk between the two signaling pathways within the networking governing the cell fate.

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1015-8987/08/0214-0305\$24.50/0

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Introduction

Apoptosis, literally a self-motivating cell death, is essential for developmental morphogenesis by regulating cell homeostasis and tissue remodeling in organisms and thus is a physiological regulator of cell density. Yet excessive apoptotic cell death also critically participates in the pathogenesis of degenerative diseases like myocardial infarction, failing heart, atherosclerotic arteries and hypertensive vessels, and Alzheimer's disease and other neurodegenerative disorders [1-3]. In tissues composed of post-mitotic cells, like heart, new cells cannot be regenerated and cell loss is a 'one way and no U-turn' traffic. Under such a circumstance, preserving cells that survived from apoptotic injuries becomes one of the keys to delay pathological processes. Indeed, cells of even the same histological origins possess different susceptibility or resistance to apoptotic insults; some can tolerate more severe apoptotic induction and some cannot. This property is important for normal morphogenesis during development and for abnormal loss of cells during pathogenesis. Obviously, understanding signal transduction processes in cells that survived from apoptotic injuries is of crucial importance to develop better strategies of preserving cells with apoptosis resistance. In the past two decades, major efforts have been focused on apoptotic cell death and the related intracellular events in apoptotic cells and much less attention has been paid to cells with better resistance to apoptosis or cells that survived from apoptotic injuries. This study was designed to shed light on this issue from three angles: (1) to identify the population of cells bearing higher resistance to apoptosis in cardiomyocytes; (2) to investigate whether this apoptosis-resistant trait can last for repeated apoptotic insults; and (3) to delineate the signaling mechanisms underlying apoptosis-resistant property of cells.

Materials and Methods

Cell Culture

Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured as described previously [4, 5]. Briefly, 1-3 days old rats were decapitated and their hearts were aseptically removed. Their ventricles were dissected, minced and trypsinized overnight at 4°C. The next day, cells were dissociated with collagenase and pre-plated twice for 60 min at 37°C. The non-adherent cardiomyocytes were removed and plated in 24-well plates in DMEM/F-12 medium (Invitrogen) containing 10% FBS and 10 µM cytosine β-D-arabino-furanoside (Ara-C, Sigma) to inhibit proliferation of non-cardiac cells.

Fig. 1. Diagram illustrating the experimental protocols for H₂O₂ treatment of cells.

1x 10⁵ cells/well were seeded in 24-well plate for further experiments. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observation of cell beating [4, 5].

Experimental Protocols

As illustrated in Figure 1, NRVMs were initially divided into three groups: control, H₂O₂, control-H₂O₂ groups and each group consisted of four wells in a 24-well culture plate. Cells of H₂O₂ group were incubated in culture medium containing 70 µM of H₂O₂ for 30 min. This concentration of H₂O₂ was chosen based on our pilot experiments showing approximately 50% (49.2±4.5%, n=16 batches of cells) apoptotic cell death in NRVMs. The dead cells were removed by rigorously washing with fresh medium and the remaining live cells were then divided into two groups. One group of cells was used for RT-PCR and Western blot experiments and the other was continued to culture in fresh medium. After five days the NRVMs which survived were re-seeded in two wells to maintain the same cell density (1x 10⁵ cells/well) and insulted with H₂O₂ (70 µM) a second time for 30 min and subsequently, the H₂O₂-treated cells were again incubated with H₂O₂ the third time at a higher concentration (150 µM). Cells of control-H₂O₂ group were treated with H₂O₂ only in parallel with the second H₂O₂ treatment for H₂O₂ group. Control group was not treated with H₂O₂ at all. Apoptosis, mRNAs and proteins were then quantified. The parallel control cells were handled in the same manner except that they were mock-treated with water but not with H₂O₂. H₂O₂ was purchased from Sigma Chemical, and 1,000x stocks were freshly prepared in sterile water.

MTT Assay for Cell Viability

Cell Proliferation Kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); Roche Molecular Biochemicals, Laval, PQ, Canada] was used to quantify surviving cells from oxidative stress, as previously described in detail [6].

Enzyme-Linked Immunosorbent Assay (ELISA)

The Cell Death Detection ELISA kit (Roche Molecular Biochemicals) was employed to quantify DNA fragmentation

on the basis of antibody detection of free histone and fragmented DNA, as detailed elsewhere [6-8].

Terminal Deoxyribonucleotide Transferase-Mediated dUTP Nick End Labeling (TUNEL)

DNA fragmentation of individual cells was detected in situ by TUNEL with the In Situ Cell Death Detection kit, Fluorescein (Roche Molecular Biochemicals), as detailed elsewhere [6-8].

Small Interference RNA (siRNA)

The Stealth™ siRNAs targeting STAT3 and a scrambled control siRNA were synthesized by Invitrogen [9]. Cells were transfected with the siRNAs using lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). Before siRNA treatment, cells were starved to synchronize growth by incubating in serum- and antibiotics-free medium for 12 h. Cell lysates were prepared 24 h post-transfection for real-time RT-PCR analysis.

Decoy Oligodeoxynucleotides (dODN)

The procedures were essentially the same as previously described elsewhere [9, 10]. Single-stranded phosphorothioate oligodeoxynucleotides were synthesized by IDT incorporation (Coralville, IA). The ODNs were washed in 70% ethanol, dried and dissolved in sterilized Tris-EDTA buffer (10 mM Tris + 1 mM EDTA). The supernatant was purified using Micro Bio-spin30 columns (BioRad, Hercules, CA) and quantified by spectrophotometry. The double-stranded dODNs were then prepared by annealing complementary single stranded oligodeoxynucleotides by heating to 95°C for 10 min followed by cooling to room temperature (RT) slowly over 2 h. The STAT3 dODN has the following sequences:

GAT CCU TTC CCG GAA UC TCC TGC CUT TTC GGG AAU CTC CTG

CTAGGU AAG GGC CTT UGAGGAGGG AAA GCC CTT GAG GAC (the bold and underlined sequences indicate the consensus binding sites for STAT3).

The cells were transfected with 100 nM STAT3-dODN using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The concentration of the STAT3-dODN was found to cause around 40% cell death in our previous study [9]. For apoptosis study, cells were seeded in 96-well tissue culture plates. At 50% confluence, the cells were washed with serum-free DMEM/F-12 medium once and then incubated with 50 µl fresh fetal bovine serum (FBS)-free medium. STAT3-dODN and lipofectamine (0.25 µl) were separately mixed with 25 µl of Opti-MEM® I Reduced Serum Medium (Gibco, Grand Island, NY) for 5 min. Then the two mixtures were combined and incubated for 20 min at room temperature. The lipofectamine:dODN mixture was added dropwise to the cells and incubated at 37°C for 5 h. Subsequently, 25 µl fresh medium containing 30% FBS was added to the well and the cells were maintained in the culture until use, either for cell growth assays or for RNA or protein extraction [9].

Gene Transfection

The constructs expressing the constitutively active

mouse AKT in pECE (caAKT), and dominant negative bovine PKB in pCMV6 (dnAKT) were kind gifts from Dr. T. Franke [11] and Dr. M. Greenberg [12]. The cDNA transfection procedures are the same as for siRNA and decoy ODN described above and have also been described in detail elsewhere [9, 13]. For each transfection, 2-3 µg caAKT or dnAKT cDNA was used.

Drug Treatment

For drug treatment, cells were starved in serum-free DMEM/F-12 medium for 12 h and then incubated with the drugs at desired final concentration for 1 h. The drugs used included hydrogen peroxide (H₂O₂, Sigma), wortmannin (Sigma, PI3K inhibitor), ZM449829 (Tocris, putative JAK3 inhibitor) [14], AG490 (Tocris, selective JAK2 inhibitor, 100 µM) [14], heregulin (NF-κB activator, Biovision Research Products, Mountain View, CA), NF-κB inhibitor ligand (Cedarlane Lab Ltd, Homby, ON). For experiments involving co-application of activators and inhibitors, cells were pretreated with inhibitors for 1 h before addition of activators. These agents were dissolved in DMSO, with the final concentration of DMSO in culture medium <0.1%. For more rational comparison, the same concentration of DMSO was also included in culture medium for control groups. For all experiments, cell density was 1 x 10⁵ cells/well in a 24-well plate.

Real-Time RT-PCR

RNA was isolated with RNeasy® Mini Kit (Qiagen), according to the manufacturer's protocols and treated with DNase I to remove genomic DNA. TaqMan quantitative assay of transcripts was performed with real-time two-step reverse transcription PCR (GeneAmp 5700, PE Biosystems), involving an initial reverse transcription with random primers and subsequent PCR amplification of the targets [5, 10]. Human GAPDH control reagents (Applied Biosystems) were used as internal controls.

Western Blot Analysis

Protein sample preparation and Western blotting process were basically the same as previously described [7, 10, 13]. The primary antibodies used were for endogenous levels of total PI3K (phosphatidylinositol 3-kinase, p110-α), AKT (protein kinase B, AKT1/AKT2/AKT3), ERK1/ERK2 (extracellular signal-regulated kinase), JAK1 and JAK2 (the junas activated kinases), STAT1 and STAT3 (the signal transducers and activators of transcription), and NF-κB (p65), and for the active (phosphorylated) phospho-(Tyr) PI3K-P⁸⁵, JAK1-P^{Tyr1022/1023}, JAK2-P^{Tyr1007/1008}, STAT1-P^{Tyr701}, STAT3-P^{Ser727} and -P^{Tyr705}, AKT-P^{Ser473}, ERK1/2-P^{Thr202/Tyr204}, and NF-κB (p65)-P^{Ser536}. All primary antibodies were purchased from Cell Signaling and diluted 1:1000 in TBS containing 3% BSA. The secondary antibody used was anti-rabbit, purchased from Bio-Rad and diluted 1:5,000. Protein loading was controlled by probing all Western blots with anti-GAPDH antibody.

Immunoperoxidase cell staining

The purity of cardiomyocytes in the culture before and after H₂O₂ treatment was verified using ABC staining systems (Santa Cruz) according to the manufacturer's instructions. Cells after treatment were placed on coverslips and fixed in 4%

Fig. 2. Survival from oxidative stress-induced apoptosis of cultured neonatal rat ventricular myocytes. (A) Cell viability determined by MTT assay, expressed as percentage of surviving cells ($n=6$ independent samples). Shown in the left panel is the extracellular H_2O_2 concentration ($[\text{H}_2\text{O}_2]_0$)-dependent reduction of cell viability after an initial H_2O_2 insult and after a second H_2O_2 exposure, respectively. The symbols are experimental data, and the lines represent the best-fit to the Hill equation: $E = 1/[1+(IC_{50}/H)^n]$, where E is % surviving cells at a given $[\text{H}_2\text{O}_2]_0$, IC_{50} is the $[\text{H}_2\text{O}_2]_0$ that produces 50% cell death (right panel), and n is the Hill coefficient. Note that around 50% cells were resistant to apoptosis induced by $70\ \mu\text{M}$ H_2O_2 and the surviving cells were 85% tolerant to the second H_2O_2 insult at the same concentration. $*p<0.05$, 1st H_2O_2 vs. 2nd H_2O_2 . (B) Verification of the cell death induced by 30 min exposure to H_2O_2 ($70\ \mu\text{M}$) via the apoptotic mechanism. Apoptosis was identified by morphological examination under a light microscope (magnification $\times 100$, upper panels), by TUNEL nuclear staining superimposed on the phase contrast image of the same scope to view the contour of the cells (middle panels), and by mean optical density (OD) values obtained from ELISA ($n=6$, lower panels) as a function of H_2O_2 concentrations. The arrows in the upper right panel indicate the shrinking cells with blebbing. In the lower left panel, the symbols are the experimental data, and the line represents the best-fit to the Hill equation as described above (E here represents % OD values at a given $[\text{H}_2\text{O}_2]_0$). $*p<0.05$, 1st H_2O_2 vs. 2nd H_2O_2 .

paraformaldehyde, following by incubation in 0.3% hydrogen peroxide in PBS for 30 min to quench endogenous peroxidase activity. The cells were then exposed to the primary antibody (1:200 diluted monoclonal anti-sarcomeric α -actin antibody) for 30 min, followed by incubation with biotin-conjugated secondary antibody for 30 min and then with avidin-biotinylated horseradish peroxidase complex for another 30 min. The cells were incubated in peroxidase substrate DAB until desired stain was developed. The cells were examined under a light microscope.

Statistical Analysis

Group data are presented as mean $\pm$ SEM. Unpaired t -tests were used for single comparisons. A two-tailed $p<0.05$ was taken to indicate a statistically significant difference.

Results

Incubation of neonatal rat ventricular myocytes (NRVMs) with H_2O_2 produced concentration-dependent cell death which was of primarily apoptosis, based on morphological changes (cell shrinkage, membrane

blebbing and nuclear condensation), and the data from TUNEL and ELISA (Fig. 2). Under our experimental conditions, $70\ \mu\text{M}$ H_2O_2 caused around 50% ($49.2\pm4.5\%$, $n=16$ batches of cells) apoptotic cell death as determined by TUNEL (same below) and control non-treated cells had $<1\%$ cell death.

The cells that survived from the initial H_2O_2 challenge were maintained in the normal culture medium for five days. The cells were then insulted again by $70\ \mu\text{M}$ H_2O_2 and in parallel, cells of control- H_2O_2 group were also treated in the same way. Intriguingly, while control- H_2O_2 cells showed around 50% ($50.2\pm5.7\%$) apoptosis as the H_2O_2 group cells with the first round of H_2O_2 treatment, the cells of H_2O_2 group demonstrated only $<1\%$ cell death. Elevation of H_2O_2 concentration to $150\ \mu\text{M}$ resulted in nearly 80% apoptosis in control- H_2O_2 group but only around 40% ($41.0\pm4.6\%$) apoptosis in H_2O_2 group. These results indicate that the cells that survived from the initial H_2O_2 insult had intrinsic resistance to apoptosis and this resistance was boosted up five days after the initial insult. Immunostaining of cells with antibody against the cardiac

Fig. 3. Western blot analysis of changes of various proteins critical to cell survival in response to oxidative stress with whole cell lysates extracted from cultured neonatal rat ventricular cells. (A) Examples of Western blot bands. Left panels: total protein levels (including both non-active and active forms) with antibodies targeting both non-phosphorylated and phosphorylated proteins. Right panels: levels of active form of the proteins with antibodies targeting only phosphorylated proteins. The names of the proteins are indicated above (for the upper row) or below (for the lower row) the bands and the numbers within the brackets indicate the molecular masses of the proteins in kDa. For ERK1/2 (p44/p42), the molecular masses are 44 and 42 kDa. The target bands are shown in the upper part of each panel and the GAPDH bands in the lower part of each panel. Columns are labeled with 1, 2 and 3, with 1 for control band, 2 for H₂O₂ (70 μ M, 30 min), and 3 for H₂O₂ with antibodies pretreated with their respective antigenic peptides. (B) Mean values of protein levels in the cells survived from the initial apoptotic insult, with each bar representing a pooled sample from 5 independent batches of cells. The densities of all target bands were first normalized to their corresponding GAPDH signals and the normalized data were then further normalized to control data to obtain relative levels of proteins. Dash line indicates the normalized protein levels under control conditions. (C) Mean values of protein levels in the cells on 5 days after surviving from the initial apoptotic insult. The values were normalized to the parallel control cells. (D) Mean values of protein levels in the cells survived from two apoptotic insults. 'Protein name-T' and 'protein name-P' indicate the total (T) and phosphorylated (P) forms of the proteins, respectively. * $p < 0.05$ H₂O₂ vs. control.

marker α -actin indicates that the surviving cells from H₂O₂ insults remained ~95% cardiomyocytes as before the treatment (data not shown).

Apoptosis-Resistant Cells Had High Levels of AKT and STAT3

It is conceivable that the apoptosis-resistant NRVMs have abundant expression of survival factors. To test this notion, we performed studies on several candidate

cytoprotective pathways or molecules, including AKT, ERK1/ERK2, HSP90, HSP72, STAT3, and NF- κ B in cells that survived from the initial H₂O₂ insult, as compared with non-treated control cells. We found that only AKT and STAT3 levels were remarkably higher (2.4 folds and 2.8 folds, respectively) in the surviving cells and other molecules were either not significantly different or merely slightly higher (~20-30%) (Fig. 3A & 3B). This difference was consistently seen in day five after surviving from the

Fig. 4. Changes of selected molecules critical to the apoptosis-resistance property at the protein level or mRNA level with whole cell lysates or total RNA samples extracted from cultured neonatal rat ventricular cells. Western blot was used to determine protein levels and real-time RT-PCR was used to quantify transcript levels. ‘Protein name-T’ and ‘protein name-P’ indicate the total (T) and phosphorylated (P) forms of the proteins, respectively, assayed by Western blot, and ‘gene name-m’ indicates the mRNAs, assayed by real-time RT-PCR. (A) Effects of wortmannin (100 nM), a PI3K inhibitor, on the levels of selected proteins. Dash line indicates the normalized protein levels under control conditions (same below). Mean data (n=6 independent samples) of band densities were obtained first by normalizing the target band to its corresponding GAPDH band and then the normalized data were further normalized to control data to obtain relative levels of proteins (same below). Note that the total STAT3 and active NF-κB were reduced along with the decreases in active PI3K and AKT. (B) Effects of AG490 (100 μM), a selective JAK2 inhibitor, on the levels of selected proteins. Note that AG490 reduced AKT-T and AKT-P as well as NF-κB-P along with the decreases in active JAK2-P and STAT3-P. (C) Effects of transfection of cells with either constitutively active AKT construct (caAKT) or dominant negative AKT construct (dnAKT) on the levels of selected proteins. Note that caAKT increased STAT3 at total and active protein levels as well as at mRNA level, whereas dnAKT produced the opposite. (D) Regulation of AKT transcription by STAT3, as revealed by the effects of the siRNA (50 nM) or the decoy oligodeoxynucleotides (dODN, 100 nM) directed to target STAT3 gene or protein. Note that transfection with STAT3-siRNA downregulated the AKT transcription along with STAT3 mRNA knockdown whereas the negative control (NC, scrambled siRNA) failed to affect STAT3-m and AKT-m. Also note that the dODN decreased both AKT total protein and transcript levels, but it did not affect STAT3 levels (data not shown). (E) Regulation of STAT3 transcription by AKT and NF-κB. Treatments are specified by the labels within the brackets. For example, NF-κB-P (caAKT) represents the effect of caAKT on the active form of NF-κB. HR: heregulin (30 ng/ml), a selective NF-κB activator; NIL: NF-κB inhibitor ligand (30 ng/ml). Note that activation of NF-κB by heregulin upregulated STAT3 mRNA (STAT3-m) and total protein (STAT3-T) levels, even in cells transfected with dnAKT, effects prevented by co-application with NIL (HR+NIL). **p*<0.05 H₂O₂ vs. control.

apoptotic insult; AKT and STAT3 protein levels were 2.1- and 2.4-fold higher than those in parallel control cells (Fig. 3C). Intriguingly, the cells that survived from the second H₂O₂ insult had even higher levels of AKT (3.5 folds) and STAT3 (3.9 folds) (Fig. 3D). Correspondingly, the active forms of AKT (AKT-P) and STAT3 (STAT3-P) were also significantly higher in these apoptosis-resistant

cells compared to control cells. Intriguingly, the level of active form of NF-κB (NF-κB-P) was also markedly enhanced, despite that the total amount of NF-κB (75 kDa) was unaltered (Fig. 3B, 3C and 3D).

The JAK/STAT signaling pathway involves two protein families, the janus activated kinases (JAKs) and the signal transducers and activators of transcription

Fig. 5. Regulation of cell viability by AKT and STAT3. (A) Effects of various treatments on $[H_2O_2]_0$ -dependent reduction of cell viability determined by MTT assay. SiRNA: STAT3-siRNA; caAKT (dnAKT)+siRNA: co-transfection with caAKT (dnAKT) and STAT3-siRNA; caAKT+NIL: treatment with NF- κ B inhibitor ligand (30 ng/ml) in cells transfected with caAKT; dnAKT+HR: treatment with heregulin (NF- κ B activator, 30 ng/ml) in cells transfected with dnAKT. The symbols represent the experimental data and the lines represent the best-fit to the Hill equation: $E = 1/[1+(IC_{50}/H)^n]$. (B) Correlation between AKT level (left panel) or STAT3 level (right panel) and cell viability in the cells that survived from the two H_2O_2 treatments. The cells that survived from the initial oxidative challenge were treated with varying concentrations of H_2O_2 (ranging from 10 to 200 μ M) and the cells that survived from this second oxidative insult were used for protein extraction subjected to Western blot analysis of AKT and STAT3 total protein levels (AKT-T and STAT3-T, respectively). The symbols are the experimental data and the straight lines represent linear regression of the data points, and r^2 stands for correlation coefficient. (C) Verification of the specificity of STAT3-siRNA towards STAT3 at both mRNA (left) and protein (right) levels. Note that STAT3-siRNA failed to affect STAT1 expression. (D) Schematic depiction of dual crosstalks between PI3K/AKT/NF- κ B and JAK2/STAT3 pathways: the positive transactivation loop that maintains higher levels of AKT and STAT3 and the negative feedback mechanism that keeps balanced AKT and STAT3 activities. Solid lines indicate positive actions and dashed lines indicate negative actions.

(STATs). Upon tyrosine receptor phosphorylation, JAKs are activated and in turn phosphorylate the tyrosine motifs in the cytoplasmic tail of the receptor, which allows for STAT binding [14-20]. The prerequisite for STAT transcriptional activities lies in its phosphorylation. Phosphorylation of STAT3 on Tyr 705 is mediated by Janus Kinases, which can result in its homo- or heterodimerization that enables nuclear localization and DNA binding [14].

Treatment of cells with wortmannin (100 nM), a PI3K inhibitor [13], decreased the level of active AKT but did not affect that of active STAT3. Unexpectedly, wortmannin markedly reduced STAT3 protein level by 54%, as well as AKT protein level though to a less extent (by 27%). Consistently, real-time RT-PCR showed significant diminishment of STAT3 and AKT mRNA levels (by 46% and by 23%, respectively) by wortmannin (Fig. 4A). On the other hand, treatment of cells with AG490 (100 μ M), a JAK2 inhibitor, caused some 49% and 45% decreases of AKT mRNA and protein levels, respectively,

and 22% and 25% decreases in STAT3 mRNA and proteins (Fig. 4B). By comparison, ZM449829 (100 μ M), a putative JAK3 inhibitor, only produced minor effects on STAT3 and AKT expressions (data not shown).

Transfection of cells with constitutively active form of AKT (caAKT) substantially enhanced STAT3 expressions by 1.2 folds at protein level and by 1.5 folds at mRNA level (Fig. 4C). In sharp contrast, transfection of dominant negative construct of AKT (dnAKT) considerably reduced STAT3 protein and mRNA levels by 48% and 42%, respectively. Neither caAKT nor dnAKT affected significantly the level of active STAT3 under basal conditions (Fig. 4C).

The small interference RNA targeting STAT3 transcripts (STAT3-siRNA) diminished both STAT3 and AKT mRNA levels (STAT3-m and AKT-m, respectively), but did not affect ERK1 transcripts (Fig. 4D). Effects of the decoy oligodeoxynucleotides targeting STAT3 proteins (STAT3-dODN) on STAT3-m and AKT-m were also investigated. Decoy ODN acts to trap its target

transcription factor by binding specifically to the transcription factor to prevent their access to the endogenous *cis*-elements and to knockdown transcription of genes regulated by this transcription factor, say STAT3 [9, 21, 22]. If AKT transcription is regulated by STAT3 in cardiomyocytes, then STAT3-dODN should be able to alter AKT expression. This was indeed verified by our experiments. As illustrated in Figure 4D, STAT3-dODN produced significant downregulation on both STAT3-m and AKT-m. Negative controls for STAT3-siRNA using mismatched siRNA and for STAT3-dODN using mutated dODN did not alter STAT3-m and AKT-m, confirming the specificity of the molecules. The ability of the STAT3-dODN used in this study to bind to STAT3 *cis*-acting elements has been confirmed by our previous study [9].

AKT kinase and STAT3 transcription factors can both induce antiapoptotic gene expression including Bcl-XL that binds to proapoptotic Bax molecules and prevent formation of proapoptotic Bax homodimers. Besides acting on Bcl family of survival genes, AKT also promotes cell survival through activates transcription of antiapoptotic genes through phosphorylation of IKK and activation of NF- κ B [23-27]. In our study, caAKT increased the level of active NF- κ B whereas dnAKT slightly decreased it. Incubation of cells with NF- κ B activator heregulin (1 μ M) markedly enhanced STAT3 expressions at both protein and transcript levels (Fig. 4E). This effect was seen even in the cells transfected with dnAKT. Co-application of heregulin with NF- κ B inhibitor ligand (NIL) abolished the promoting effects of the former on STAT3 expression.

Noticeably, PI3K and JAK2, the upstream molecules of PI3K/AKT and JAK/STAT pathways, were not significantly different in their protein levels and activities between H₂O₂ and control groups. This fact suggested that the interactions between the two pathways occur downstream to PI3K and JAK.

Evidence for a Positive Transactivation Loop Formed by AKT/NF- κ B and JAK2/STAT3 Pathways

The above results indicate that there exists a mutual transactivation loop between AKT/NF- κ B and JAK2/STAT3 in neonatal rat ventricular myocytes and this crosstalk maintains higher levels of AKT and STAT3 expressions. In order to investigate whether this crosstalk accounts, at least partially, for the intrinsic apoptosis-resistance, we performed the following experiments.

In the experiments illustrated in Figure 5A, caAKT enhanced the apoptosis-resistance of rat ventricular myocytes; in cells transfected with caAKT, the dose-

response curve of H₂O₂-induced reduction of cell viability was remarkably shifted to right with an *IC*₅₀ of ~130 μ M, compared with the *IC*₅₀ of ~70 μ M in caAKT-non-transfected cells. This enhancement of apoptosis-resistance was abolished by co-transfection with STAT3-siRNA or incubation with NF- κ B inhibitor ligand, as indicated by an abolishment of shifts of dose-response curves or of changes of *IC*₅₀ values. STAT3-siRNA alone promoted apoptotic cell death, as indicated by a marked leftward shift of the dose-response curve (Fig. 5A). In sharp contrast, cells transfected with dnAKT lost their apoptosis-tolerance, as indicated by a leftward shift of dose-response curve and the reduced *IC*₅₀ of H₂O₂ to 22.0 $\pm$ 1.7 μ M (Fig. 5A). However, application of heregulin rescued the anti-apoptotic ability in the presence of dnAKT by increasing *IC*₅₀ of H₂O₂ to ~90 μ M. Co-transfection of cells with dnAKT and STAT3-siRNA together eradicated the apoptosis-tolerance, resulting in a reduction of *IC*₅₀ to a concentration of H₂O₂ as low as 10 μ M.

To further clarify that the levels of AKT-T or STAT3-T correlate with the apoptosis-tolerance of cardiac cells, we carried out additional experiments. The cells survived from the initial oxidative challenge were treated with H₂O₂ at concentrations ranging from 10 to 200 μ M for 30 min before measurements for cell viability using MTT assay. The dead cells were removed and the cells that survived from this secondary oxidative insult were used for immunoblotting for quantifying AKT-T and STAT3-T for each single concentration of H₂O₂. Then the cell viability was plotted as a function of double normalized AKT-T or STAT3-T levels (first normalized to GAPDH and then normalized to control H₂O₂-non-treated samples, determined at various H₂O₂ concentrations). The data were used to perform correlation analysis and the results are summarized in Figure 5B, which indeed indicate good correlation between the levels of AKT-T/STAT3-T and the apoptosis resistance in rat ventricular myocytes.

To further verify that the siRNA used in our study is specific to STAT3, we studied the effects of this siRNA on STAT3 and STAT1 expression at both mRNA and protein levels. The results clearly indicate the specificity of the STAT3-siRNA for it knocked down STAT3 expression but did not affect STAT1 expression (Fig. 5C).

Discussion

We showed here rat ventricular myocytes possess unique antiapoptotic property or apoptosis-resistance and

this trait can last for at least four days in culture. The apoptosis-resistance is likely afforded by high levels of basal AKT and STAT3 proteins and transcripts. The crosstalk or the positive transactivation loop between AKT/NF- κ B and STAT3 may be one of the mechanisms for keeping higher levels of AKT and STAT3 expressions in apoptosis-resistant cells.

Apoptosis, besides its well-known characteristic alterations of cell morphology and DNA fragmentation, possesses several important features in terms of the patterns of cell death. First, cells can be protected against apoptosis by a transient insult, a preconditioning property of apoptosis [8]. Second, cells can also demonstrate self-sustained apoptosis following withdrawal from a transient apoptotic insult, a remodeling process of apoptosis [28]. And third, apoptosis changes with different cell densities with low density more susceptible to apoptotic inducers, the cell density-dependence of apoptosis [29]. Here, we revealed another distinct characteristic of cells: the resistance or tolerance to apoptosis.

Several lines of evidence from this work and from previous studies as well support the existence of the positive transactivation loop between AKT/NF- κ B and STAT3. First, the cells possessing apoptosis-resistance had higher levels of AKT and STAT3 expressions. Second, inhibition of AKT activation by PI3K inhibitor and dnAKT downregulated STAT3 level, and vice versa inhibition of STAT3 activation by JAK2 inhibitor also downregulated AKT level. Third, activation of AKT using caAKT upregulated STAT3 expression, and similarly, activation of NF- κ B by its activator also increased STAT3 expression.

Interestingly, it has been shown that PI3K/AKT pathway plays a role in tyrosine phosphorylation of STATs [30, 31], possibly via members of the Src tyrosine kinase family that link cytokine receptors to PI3K through JAK [32], resulting in inhibition of STAT-transcriptional activities via their effects on JAK2 [33]. Our data in this study indeed support this notion. Either inhibition of PI3K with wortmannin or inhibition of AKT with dnAKT increased both JAK2 and STAT3 activities and on the contrary, artificial activation of AKT with caAKT diminished JAK2 and STAT3 activities despite that it increased the expression of STAT3. This negative feedback regulation of activities may counteract with the positive regulation of expressions.

Based on these findings, we proposed dual crosstalks between PI3K/AKT/NF- κ B and JAK2/STAT3 pathways: the positive transactivation loop that maintains higher levels of AKT and STAT3 and the negative

feedback mechanism that keeps a balanced AKT and STAT3 activities, as schematically depicted in Figure 5D. Activation of PI3K activates AKT that in turn activates NF- κ B. NF- κ B protects cells from apoptosis on one hand and promotes STAT3 expression on the other hand. Similarly, STAT3, which can be activated by JAK2 and promoted by NF- κ B, produces protective effects against apoptosis and also promotes AKT expression as well. These interactions form a positive feedback mechanism against apoptosis. On the other hand, an increase in AKT can also give rise to a downregulation to JAK2, which then reduces STAT3 and AKT expression. These latter interactions may form a negative feedback loop to balance AKT and STAT3 activities.

In conclusion, our study demonstrated the apoptosis-resistance, a novel property of apoptosis, in NRVMs and the interactions between AKT/NF- κ B and JAK2/STAT3 at the transcription level, a novel crosstalk between the two signaling pathways. These findings shed new light on the networking governing the fate of cells: death or survival, and may lead us to better understanding of many pathological processes related to apoptosis and over proliferation of cells.

Abbreviations

AKT (protein kinase B); caAKT (constitutively activated AKT); dnAKT (dominant negative AKT); ERK1/ERK2 (extracellular signal-regulated kinases); HR (heregulin (NF- κ B activator)); JAK1 and JAK2 (the janus activated kinases); NC (negative control (scrambled siRNA)); NIL (NF- κ B inhibitor ligand); NRVM (neonatal rat ventricular myocyte); STAT1 and STAT3 (the signal transducers and activators of transcription); STAT3-siRNA (siRNA specifically targeting STAT3).

Acknowledgements

The authors thank XiaoFan Yang for excellent technical support. This work was supported in part by the National Sciences and Engineering Research Council of Canada and Fonds de la Recherche de l'Institut de Cardiologie de Montreal, awarded to Dr. Z Wang and by the National Nature Science Foundation of China (30430780), the Foundation of National Department of Science and Technology of China (2004CCA06700), awarded to Dr. B Yang. Dr. Z. Wang is a senior research scholar of the Fonds de Recherche en Sante de Quebec.

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