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Bacterial RNA Activates PKR-JAK-STAT Signaling and Inflammasome-Associated Apoptosis in Human Cardiac and Stromal Cell Lines: Implications for Sepsis-Induced Cardiac Pathophysiology

Shuhung Kung[1], Farag A. Bleiblo[2*], Madiha W. El-Awamie[2], Nariman A. Elsharif[2], Mohamed M. Khamid[3], Muetaz M. Feetouri[4], Ibtihag S. Alogali[4], Eman M. Almajbry[2], Aseem Kumar[1]

[1]Department of Chemistry and Biochemistry and the Biomolecular Sciences Programme, Laurentian University, Sudbury, Canada
[2]Department of Microbiology, Faculty of Science, University of Benghazi, Benghazi, Libya
[3]Department of Physiology, Faculty of Medicine, University of Benghazi, Benghazi, Libya
[4]Department of Biomedical Sciences, Faculty of Pharmacy, University of Benghazi, Benghazi, Libya
Email: *farag.bleiblo@uob.edu.ly

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Abstract

Background: Sepsis-induced myocardial dysfunction (SIMD) is common and highly predictive of poor outcomes, however the proximal molecular events linking bloodstream infection to myocardial injury remain poorly characterized. Bacterial RNA is an immunostimulatory molecule that could activate the double-stranded RNA-dependent protein kinase (PKR), however, its impact on signaling networks in human cardiac myocytes and non-immune stromal cells has not been explored. **Methods:** Adult human cardiac myocytes and human fibrosarcoma 2fTGH cells, as well as JAK-STAT-IFN pathway-deficient 2fTGH mutants, were treated with purified *Escherichia coli* RNA without transfection reagents. PKR activation was monitored by autophosphorylation and eIF2α phosphorylation. JAK1, JAK2, TYK2, IFNAR2, STAT1 and STAT2 (IRF9-dependent) were investigated using signalling-defective cell lines and *in vitro* kinase assays. STAT1, STAT2, IRF-1 and NF-kB DNA binding were studied by electrophoretic mobility shift assays. Immunoblots for IL-1β induction and caspase activation were performed, and the structural requirements probed using enzymatic or 5′-dephosphorylation of RNA. **Results:** Exogenous bacterial RNA itself was able to induce substantial PKR autophosphorylation and eIF2α phosphorylation in cardiac myocyte and 2fTGH cells. PKR activation was dependent on JAK1, JAK2, TYK2, IFNAR2, STAT1, STAT2 and

IRF9, suggesting their involvement in amplification through an autocrine type I interferon-JAK-STAT signaling. Bacterial RNA induced increased nuclear translocation and DNA binding of STAT1, STAT2, IRF-1, and NF-κB; an up-regulation of IL-1β; as well as activation of caspase-1 and the executioner caspases with mild involvement of casapase-8 consistent with inflammasome-driven apoptosis. These signaling responses were inhibited by RNase digestion RNA dephosphorylation. **Conclusion:** Extracellular bacterial RNA is a potent danger signal for human cardiac myocytes and fibroblast-like cells, which induces PKR-JAK-STAT activation, pro-inflammatory transcription factor recruitment, IL-1β upregulation and caspase-mediated apoptosis. These findings suggest a PKR-mediated mechanism for driving circulating bacterial RNA into pro-inflammatory and pro-apoptotic responses in cardiac myocytes, thereby priming the myocardium for contractile dysfunction and injury in sepsis.

Keywords

Sepsis, PKR, JAK-STAT Signaling, Bacterial RNA, Cardiac Myocytes, Pattern-Recognition Receptors, Apoptosis

1. Introduction

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a major global cause of morbidity. In 2017, the Global Burden of Disease analysis estimated 48.9 million incident cases and 11.0 million sepsis-related deaths, representing about 19.7% of all deaths worldwide [1] [2]. Sepsis-induced myocardial dysfunction (SIMD) occurs in a substantial proportion of patients and is associated with increased intensive-care mortality and prolonged organ failure [3]-[5]. Current management remains purely supportive, and no mechanism-based therapy for SIMD is yet available, underscoring that the key proximal pathways linking infection to myocardial injury remines poorly defined. Pathogen-associated molecular patterns (PAMPs), including microbial nucleic acids, are detected by pattern-recognition receptors (PRRs) such as nucleic acid-sensing Toll-like receptors, RIG-I-like receptors and cytosolic DNA sensors [6]-[8]. These receptors are not only expressed in immune cells, but also in parenchymal cells, including cardiomyocytes and fibroblasts. The immune response to these insults activate NF-κB and interferon signaling pathways and modulate inflammatory signalling, stress responses and cell death [6]-[8]. Experimental data suggested that bacterial DNA induces myocardial cytokine production and reduces cardiomyocyte contractility via Toll-like receptor 9 [9]-[11] and that bacterial RNA binds and activates the double-stranded RNA-dependent protein kinase (PKR), leading to inflammatory signalling and apoptosis in human adult cardiac myocytes [12]-[14].

PKR is an interferon-inducible serine/threonine kinase activated by nonself RNA including viral double-stranded structured RNA (dsRNA), some RNA spe-

cies with secondary structures and other cellular stress signals. It phosphorylates eukaryotic initiation factor 2α (eIF2α) to inhibit protein synthesis to integrated stress response, and can promote inflammatory and apoptotic signalling [15]-[17]. PKR expression and activity are increased in experimental cardiac stress and heart failure, and PKR inhibition reduces myocardial inflammation, cardiomyocyte apoptosis and adverse remodelling [18]-[20]. However, the extent to which bacterial genomic DNA and total RNA from clinically relevant pathogens such as *Escherichia coli* and *Staphylococcus aureus* activate PKR and downstream transcription-factor networks in non-immune human cells remains limited.

In this study, we aimed to investigate PKR-dependent signalling in human fibroblasts and adult cardiac myocytes exposed to purified *E. coli* and *S. aureus* genomic DNA and total RNA. We hypothesized that, even in the absence of other microbial pathogenic signatures, purified bacterial nucleic acids are sufficient to activate PKR, drive NF-κB/STAT/IRF signaling, induce pro-inflammatory cytokine expression, and initiate caspase-dependent apoptosis, thereby providing a mechanistic link between bloodstream infection and sepsis-induced myocardial dysfunction.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Pathogenic *Escherichia coli* O18:K1:H7 (ATCC 700973) and *Staphylococcus aureus* capsular serotype 8, non-TSST-1 producer, were used. Both strains are invasive pathogens in immunocompetent adults. Overnight cultures were grown in Luria-Bertani (LB) medium at 37˚C with aeration. For RNA preparation, 1% (v/v) of the overnight culture was inoculated into fresh LB and grown for 6 - 8 h at 37˚C in an orbital shaker. For genomic DNA preparation, 20 ml overnight cultures were used as starting material, as detailed below.

2.2. Preparation of Bacterial Total RNA

Total RNA from *E. coli* and *S. aureus* was isolated using two approaches. First, RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, with 5 - 7 ml of log-phase culture as input. Second, a modified hot-phenol/enzymatic lysis protocol was employed. Briefly, cultures (75 ml) were harvested by addition of ice-cold 5% phenol/ethanol stop solution, centrifuged, and resuspended in TE buffer containing lysostaphin or lysozyme as appropriate. Cells were lysed in SDS-containing buffer, extracted with water-saturated phenol at 64˚C, and subjected to sequential phenol-chloroform and chloroform extractions. RNA was precipitated with sodium acetate (final 0.25 M) and three volumes of ethanol at −20˚C, collected by centrifugation, washed with 70% ethanol and resuspended in RNase-free water. RNA concentration was determined spectrophotometrically (A260), and integrity was assessed by 1% agarose gel electrophoresis.

2.3. RNA Clean-Up and DNase Treatment

Crude RNA preparations were further purified using RNeasy columns (Qiagen). RNA was bound to the membrane, washed with buffer RW1, and subjected to on-column DNase I digestion to remove contaminating genomic DNA. Columns were washed with buffer RPE and RNA was eluted in RNase-free water. RNA purity and integrity were confirmed by A260/A280 ratios and agarose gel electrophoresis.

2.4. 2FTGH Cell Culture and Stimulation

Human 2FTGH fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37˚C in 5% CO_2. Cells were seeded in 100-mm dishes (10 ml medium) and grown to approximately 70% confluence. Monolayers were then treated for 24 h with bacterial genomic DNA, total RNA, RNase-digested RNA, or polyinosinic-polycytidylic acid (poly I:C), at the indicated concentrations, or left untreated as controls. After treatment, cells were washed with cold PBS and lysed in ice-cold lysis buffer containing non-ionic detergent, protease inhibitors, and phosphatase inhibitors. Lysates were clarified by centrifugation and protein concentrations determined by Bradford assay.

2.5. Cardiac Myocyte Culture and Stimulation

Primary cardiac myocytes were cultured in cardiac myocyte medium (ScienCell Research Laboratories) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% cardiac myocyte growth supplement (CMGS). Cells were maintained at 37˚C in 5% CO_2 in 100-mm dishes (8 ml medium) and grown to approximately 70% confluence. Before stimulation, cultures were switched to cardiac myocyte medium lacking CMGS. Cells were then exposed to bacterial RNA or poly I:C for the indicated time periods. For protein analysis, cells were harvested and lysed as described for 2FTGH cells, with an additional three freeze-thaw cycles before addition of lysis buffer. Culture supernatants were collected for cytokine measurements.

2.6. SDS-PAGE and Immunoblotting

Whole-cell extracts (WCEs) or purified proteins were resolved by SDS-PAGE (typically 7.5% resolving polyacrylamide gels) and transferred to PVDF membranes. Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% non-fat dry milk and incubated with primary antibodies against phospho-PKR, total PKR, phospho-eIF2*α*, or total eIF2*α* (Cell Signaling Technology), followed by appropriate HRP-conjugated secondary antibodies. After washing in TBST, signals were detected by enhanced chemiluminescence and visualised using a ChemiDoc documentation system or X-ray film. Band intensities were quantified using Quantity One software (Bio-Rad) where indicated.

2.7. Expression and Purification of GST-PKR Fusion Proteins

Full-length human PKR and a kinase-defective mutant carrying a Lys296→Arg substitution (mPKR) were expressed as N-terminal GST fusion proteins in pGEX-2T and transformed into *E. coli* BL21. Cultures were grown in LB containing ampicillin and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested, resuspended in PBS with protease inhibitors, treated with lysozyme, and sonicated, followed by incubation with 1% Triton X-100. Lysates were cleared by centrifugation and GST fusion proteins were captured on glutathione-agarose beads (Santa Cruz). After extensive washing with PBS containing protease inhibitors, proteins were eluted with reduced glutathione. Purity and expression levels were assessed by Coomassie Brilliant Blue staining and by immunoblotting with anti-PKR or anti-phospho-PKR antibodies.

2.8. Phosphatase Treatment and PKR Kinase Assays

Purified GST-PKR and GST-mPKR were incubated in dephosphorylation buffer (50 mM Tris-HCl pH 8.5, 0.1 mM EDTA) with calf intestinal alkaline phosphatase (CIP) for 2 h at 37˚C. CIP was removed by washing the beads with PBS and PKR kinase buffer (20 mM Tris-HCl pH 7.6, 100 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 μg/ml aprotinin, 20% glycerol). For autophosphorylation assays, CIP-treated GST-PKR or GST-mPKR bound to glutathione beads was incubated in kinase buffer containing [γ-^{32}P]ATP in the presence or absence of dsRNA (poly I:C), *E. coli* RNA, or *S. aureus* RNA for 20 - 30 min at 30˚C. Reactions were stopped with SDS sample buffer, separated by SDS-PAGE, and analysed by autoradiography. For substrate phosphorylation assays, CIP-treated GST-PKR or GST-mPKR was incubated with [γ-^{32}P] ATP and appropriate substrates (including PRDII-containing oligonucleotides or proteins in WCEs) in kinase buffer. Reactions were processed as above and phosphorylation was detected by autoradiography.

2.9. Co-Immunoprecipitation of PKR and eIF2α

To examine PKR-eIF2α complexes in cells, 2FTGH WCEs (2 mg total protein) were incubated with polyclonal anti-PKR antibody on ice, followed by addition of glutathione-agarose beads and overnight rotation at 4˚C. Beads were washed with lysis buffer, and bound proteins were eluted in SDS sample buffer and analysed by SDS-PAGE and immunoblotting with anti-eIF2α antibody.

2.10. Electrophoretic Mobility Shift Assay (EMSA)

NF-κB DNA-binding activity was assessed by EMSA using nuclear extracts or WCEs. Protein samples were incubated with a [γ-^{32}P] ATP end-labelled double-stranded oligonucleotide containing the PRDII element of the IFN-β promoter. Binding reactions were resolved on non-denaturing polyacrylamide gels in Tris-glycine buffer, dried, and exposed to X-ray film at −80˚C. In some experiments,

GST-PKR or GST-mPKR pre-treated with dsRNA or bacterial RNA was added to the binding reactions. Supershift assays were performed with antibodies against NF-κB p65 or p50 where indicated.

2.11. Enzyme-Linked Immunosorbent Assay (ELISA)

Culture supernatants from cardiac myocytes were collected after stimulation with bacterial RNA or poly I:C, clarified by centrifugation, and stored at −70˚C. Concentrations of pro-inflammatory cytokines were quantified using commercially available ELISA kits according to the manufacturers' instructions. Absorbance was measured with a microplate reader and cytokine concentrations were calculated from standard curves.

2.12. Statistical Analysis

Quantitative data were analysed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. A two-sided Pvalue < 0.05 was considered statistically significant. Specific information on the number of experiments and technical replicates is provided in the figure legends.

3. Results

3.1. Optimisation and Quality Control of Bacterial RNA Preparations

Total RNA was isolated from *E. coli* and *S. aureus* using either a commercial silica-membrane kit or a hot phenol-enzymatic lysis method. Agarose gel electrophoresis showed that the hot phenol-enzymatic protocol yielded intact, high-molecular weight RNA with clearly resolved 23S and 16S rRNA bands for both organisms, whereas the commercial kit produced weaker bands with partial degradation, especially for *S. aureus* (**Figure 1**). Quantitative spectrophotometry confirmed that hot phenol extraction gave substantially higher yields (approximately 3.52 mg RNA from *E. coli* and 3.60 mg from *S. aureus*) with A260/280 ratios of 2.0, compared with 114.6 µg and 10.2 µg, respectively, obtained using the kit, which also showed A260/280 ratios of 2.0.

3.2. Bacterial RNA Activate PKR-eIF2α Signalling in 2FTGH Fibroblasts

The ability of bacterial total RNA to activate PKR was next examined in 2FTGH cells treated with 100 µg/ml RNA from *E. coli* or *S. aureus* for 24 h. Both RNAs induced phosphorylation of PKR and eIF2α, with *E. coli* RNA eliciting a stronger increase in phospho-PKR than *S. aureus* RNA. Poly(I:C) again served as a positive control and produced the highest level of PKR and eIF2α phosphorylation. RNase-digested bacterial RNA induced weaker PKR activation than intact RNA, indicating that higher-order RNA structure contributes to PKR activation (**Figure 2**).

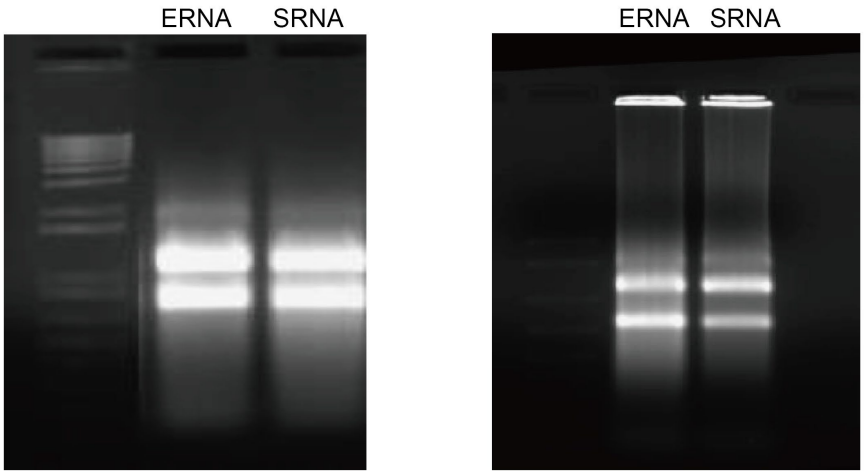

Figure 1. Comparison of RNA isolation methods. (a) The combination of hot-phenol and enzymatic methods is left. Lane 1 is marker. Lane 2 is *E. coli* RNA (5 μg) and Lane 3 is *S. aureus* RNA (5 μg). (b), this is Qiagen method (right). Lane 1 is marker. Lane 2 is *E. coli* RNA (1.28 μg) and Lane 3 is *S. aureus* RNA (0.8 μg).

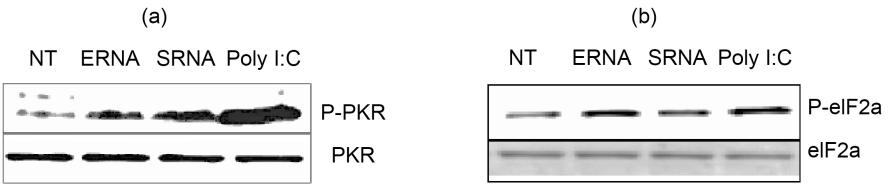

Figure 2. Phosphorylation of PKR and eIF2α observed in bacterial RNAs stimulation in 2FTGH cells. 2FTGH cells were treated with 100 μg/ml of *E. coli* RNA (lane 2), *S. aureus* RNA (lane 3) and poly I:C for 18 h in serum free condition. Western blot was performed using phospho-PKR (a) and phospho- eIF2α (b) NT (No treatment) = 2FTGH cells were incubated with medium alone.

3.3. Bacterial RNAs Modulate Recombinant PKR Activity and NF-κB *in Vitro*

3.3.1. Activation of Recombinant GST-PKR by Bacterial RNAs

To test whether bacterial RNAs could directly activate PKR, an *in vitro* kinase assay was performed using GST-PKR fusion proteins pretreated with calf intestinal phosphatase (GST-PKR + CIP) and incubated with dsRNA, *E. coli* RNA, or *S. aureus* RNA in the presence of [γ-32P] ATP. Autoradiographic analysis of SDS-PAGE-resolved GST-PKR showed that synthetic dsRNA poly(dI:dC) potently induced PKR autophosphorylation. Remarkably, total RNA isolated from *E. coli* and *S. aureus* also triggered robust PKR activation, with phospho-PKR band intensities comparable to those obtained with poly(dI:dC) and clearly exceeding the GST-PKR + CIP background (**Figure 3**), indicating that bacterial RNA can function as an efficient PKR agonist. Substrate phosphorylation assays were then carried out by adding recombinant eIF2α to the kinase reactions. A ~40 kDa band corresponding to phosphorylated eIF2α was detected in reactions containing dsRNA as well as bacterial RNAs, but not in reactions containing mutant GST-

mPKR + CIP with dsRNA. These results indicate that GST-PKR + CIP retained dsRNA-dependent kinase activity and could phosphorylate eIF2*α*, whereas the mutant PKR was inactive (Figure 3(b)). The modest effect of bacterial RNA in this assay suggests that, under these conditions, bacterial RNA is a relatively weak direct activator of recombinant PKR.

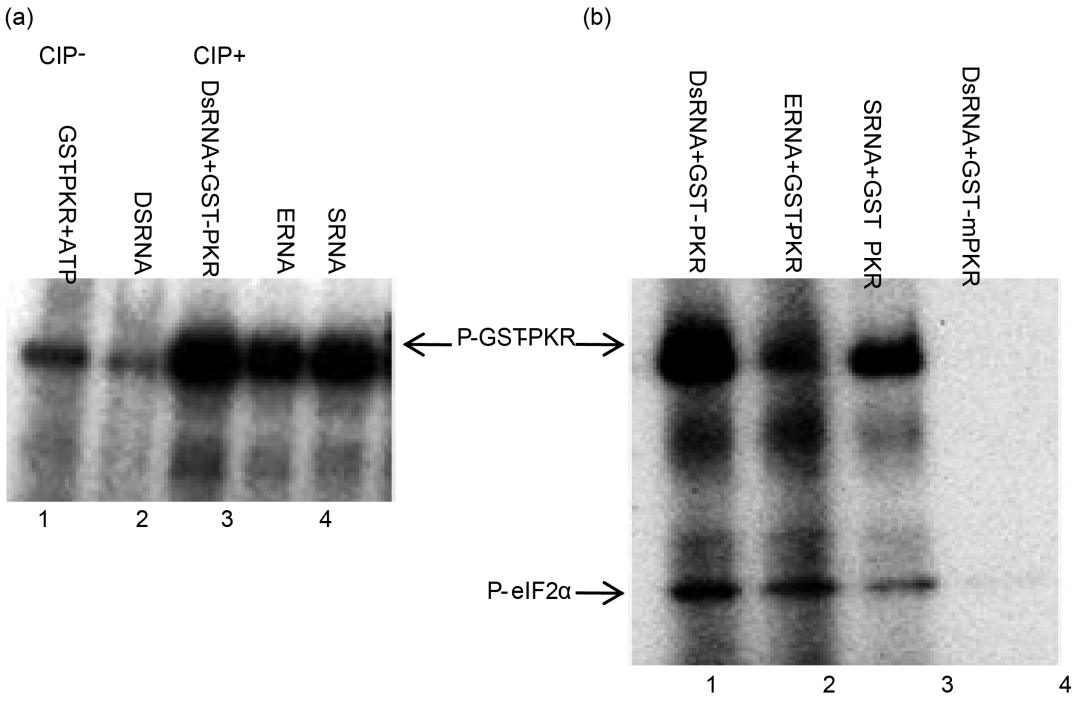

Figure 3. Effects of various RNAs on autophosphorylation of GST-PKR. GST-PKR was incubated with 14.8 KBq [γ-^{32}P] ATP in the presence of the indicated concentration of dsRNA, *E. coli* RNA or *S. aureus* RNA for 30 min at room temperature. After reaction, the samples were separated by 7.5% SDS-PAGE and exposed to an x-ray film. Lanes 1 - 2 GST-PKR untreated with CIP (Calf intestinal phosphatase). Lanes 3 - 5 GST-PKR treated with CIP. (b) Substrate specificity of GST-PKR. GST-PKR and GST-mPKR were pre-treated with CIP and then subjected into kinase buffer in the presence of dsRNA, *E. coli* RNA or *S. aureus* RNA. After 30 min, Recombinant human eIF2-*α* (Biosource) was added into each reaction mixture with additional 10 min. After that, the reaction was terminated and the samples were subjected to 7.5% SDS-PAGE. The gel was dried, exposed to X-ray film, and analyzed.

3.3.2. NF-κB Activation *in Vitro* via GST-PKR

To examine whether bacterial RNAs could induce NF-*κ*B activation through PKR *in vitro*, electrophoretic mobility shift assays were performed using whole-cell extracts from 2FTGH cells combined with GST-PKR + CIP or GST-mPKR + CIP pretreated with dsRNA or bacterial RNAs. Poly(I:C) clearly induced NF-*κ*B DNA-binding activity, and supershift assays with anti-p65 and anti-p50 antibodies confirmed the identity of the complex as NF-*κ*B. Treatment with *E. coli* or *S. aureus* RNA also produced detectable NF-*κ*B-DNA complexes in the presence of GST-PKR + CIP, whereas no complexes were observed when GST-mPKR + CIP was used with dsRNA. These findings support a role for PKR in bacterial RNA-induced NF-*κ*B activation *in vitro* (Figure 4).

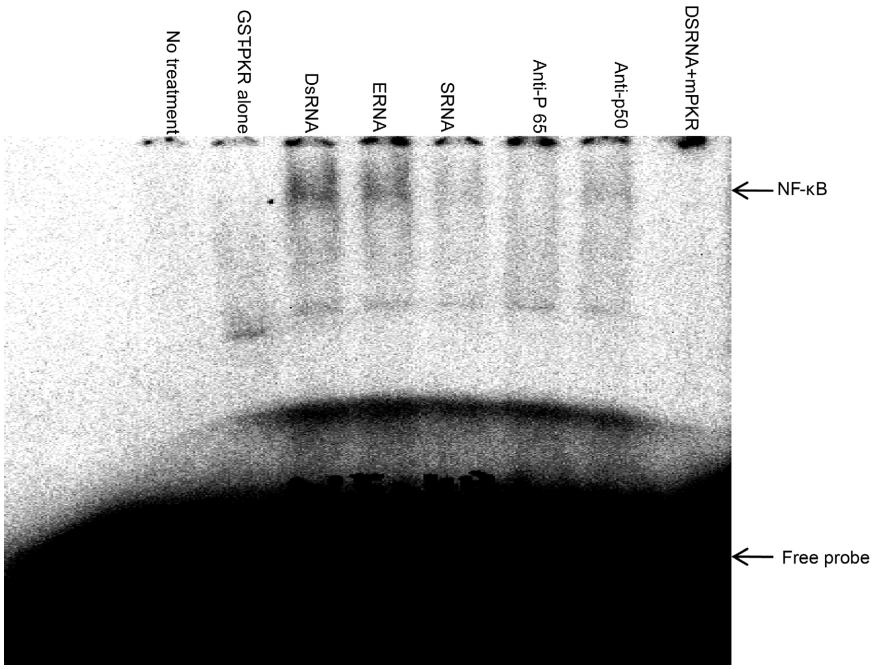

Figure 4. Identification of the GST-PKR with various RNAs-induced complexes as NF-*κ*B. The GST-PKR proteins were dephosphorylated by CIPs, and then incubated with various RNA as indicated in material and method section. The NF-*κ*B complex was activated in 2FTGH WCE extracts with the addition of GST-PKR or GST-mPKR and the labeled DNA oligonucleotide competitors used was the PRDII elements. Lanes: 1, negative control (2FTGH WCE extract with the end-labeled PRDII probe without the addition of PKR-GST); 2, positive control (same as lane 1 with PKR-GST); 3 - 5, the same as lane 2 except GST-PKR was pre-treated with dsRNA, ERNA, or SRNA. 6 - 7, Supershift assay were performed with the antibody P65 and P50 against GST-PKR with dsRNA. 8, GST-mPKR + dsRNA.

3.4. Bacterial RNAs Activate PKR, Transcription Factors, and IL-1*β* Production in Adult Human Cardiac Myocytes

3.4.1. PKR Activation in Adult Cardiac Myocytes

Adult human cardiac myocytes were treated with 100 μg/ml total RNA from *E. coli* or *S. aureus* or with poly(I:C), and PKR activation was assessed at multiple time points. Phospho-PKR levels increased after stimulation with both bacterial RNAs and poly(I:C), with an early rise detectable at 10 - 15 min and peak activation between 30 and 60 min, depending on the stimulus. *E. coli* RNA generally induced stronger PKR phosphorylation than *S. aureus* RNA, while poly(I:C) produced the most robust and sustained activation. PKR phosphorylation declined toward baseline by 120 min (**Figure 5**).

3.4.2. Activation of NF-*κ*B, STAT1 and IRF1 in Adult Cardiac Myocytes

Nuclear extracts from RNA-stimulated adult cardiac myocytes were analysed by EMSA for NF-*κ*B, STAT1 (GAS), and IRF1 DNA-binding activity. *E. coli* and *S. aureus* RNAs both increased NF-*κ*B binding compared with untreated cells, with maximal activation typically observed around 30 - 60 min. Poly(I:C) induced potent NF-*κ*B activation similar to that of bacterial RNA (**Figure 6(a)**) Bacterial

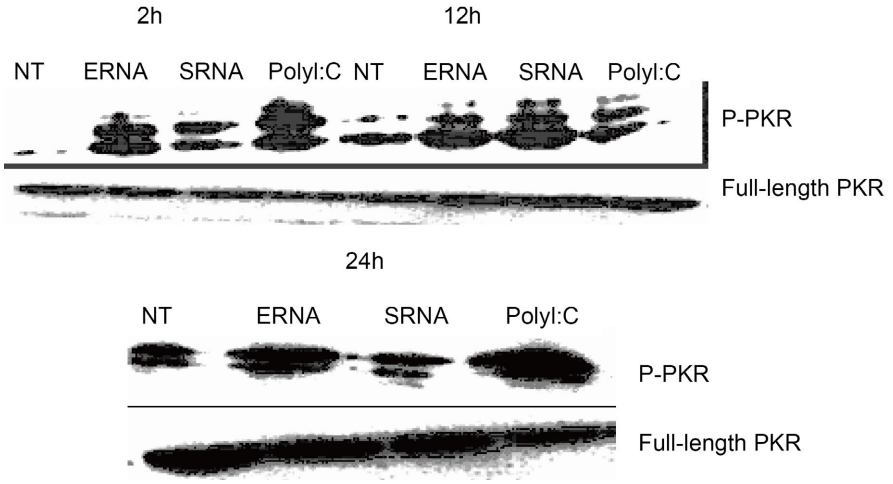

Figure 5. Time course study for PKR activation in adult cardiac myocytes stimulated with bacterial RNAs. Cardiac myocytes were incubated in medium alone (NT), 100 μg/ml of *E. coli* RNA, *S. aureus* RNA or Poly I:C (dsRNA) for 2 h, 12 h and 24 h. At each time period, protein extracts were prepared from each sample and used for western blot experiments. The PVDF membrane was probed with anti phosphor-PKR antibody and then re-probed with anti full length PKR antibody.

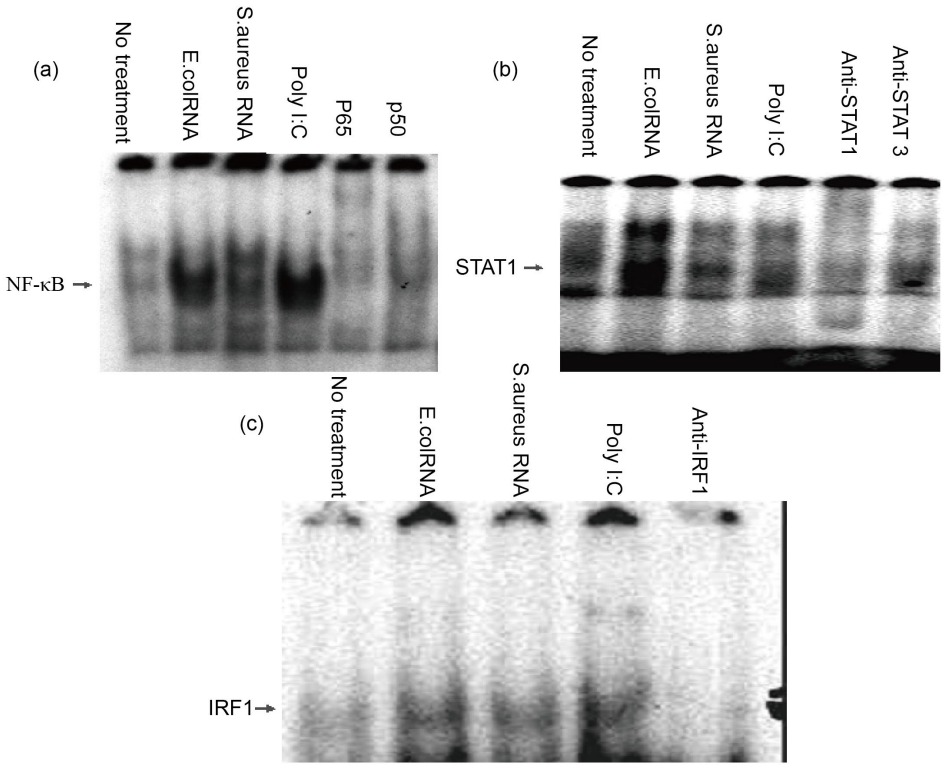

Figure 6. Bacterial RNAs treatments of human adult myocytes activate transcription factors NF-κB, STAT1 and IRF1. (a) human adult myocytes were treated with 100 μg/ml of *E. coli* RNA, *S. aureus* RNA or Poly I:C (dsRNA) for 12h. (a) EMSA was performed on 10 μg of protein extracts with the radiolabeled NF-κB DNA regulatory element. (b) was prepared similarly to A, except that the STAT regulatory element was used as the radiolabeled probe. (c) was prepared similarly to *A*, except that the IRF hexamer regulatory element was used as the radiolabeled probe. p50 = anti-p50 antibody, p 65 = anti-p65 antibody.

RNAs also modulated STAT1 and IRF1. GAS-binding activity attributable to STAT1 was modestly enhanced by bacterial RNAs and more strongly by poly(I:C), consistent with type I interferon signalling (Figure 6(b)). IRF1 DNA-binding activity was induced by both *E. coli* and *S. aureus* RNAs, again with poly(I:C) producing the most pronounced response (Figure 6(c)). These data indicate that bacterial RNA can activate PKR and downstream transcription factors in adult human cardiac myocytes.

3.4.3. Induction of IL-1β Secretion by Bacterial RNA

To determine whether bacterial RNA-induced signalling translates into pro-inflammatory cytokine production, IL-1β release from adult cardiac myocytes was measured after 24 h of stimulation. Both *E. coli* and *S. aureus* RNAs significantly increased IL-1β secretion compared with untreated cells, with *S. aureus* RNA generally eliciting higher levels than *E. coli* RNA. Combinations of bacterial RNA with TNF-α and IFN-γ further augmented IL-1β production, indicating synergy between RNA- and cytokine-mediated signalling. Statistical analysis (one-way ANOVA with Tukey's post hoc test) showed that IL-1β levels in RNA-treated and RNA-plus-cytokine-treated cells were significantly higher than in controls ($p < 0.001$; Table 1).

Table 1. *E. coli* or *S. aureus* RNAs stimulate the adult myocyte to produce IL-1β. Cardiac myocytes were incubated in medium alone (NT), 100 μg/ml of *E. coli* RNA, *S. aureus* RNA or Poly I:C (dsRNA) for 2 h, 12 h and 24 h. At each time point, the cell medium was collected and used for ELISA assay. Stars represent the samples were statistically significant ($p < 0.001$) when compared to their respective control (NT).

Hour	NT	ERNA	SRNA	dsRNA
2 h	16.4 pg/ml	15 pg/ml	9 pg/ml	26.6* pg/ml
12 h	7.2 pg/ml	35.6* pg/ml	35.4 *pg/ml	15.6* pg/ml
24 h	14 pg/ml	49.2* pg/ml	37* pg/ml	39.6* pg/ml

3.5. Bacterial RNA Induces Apoptosis and Caspase Activation in 2FTGH Fibroblasts and Adult Cardiac Myocytes

3.5.1. Bacterial RNA-Induced Apoptosis in 2FTGH Cells

Cell viability and morphology were assessed in 2FTGH fibroblasts treated for up to 72 h with 100 μg/ml *S. aureus* RNA, poly(I:C), or a cytokine mixture (TNF-α + IFN-γ). Trypan blue exclusion showed that spontaneous cell death remained low in untreated controls (approximately 2% - 4% over 72 h). In contrast, *S. aureus* RNA increased cell death progressively, reaching ~13% at 48 h and remaining elevated at 72 h. Poly(I:C) and the cytokine mixture induced even higher levels of cell death (up to ~20% and ~14% at 48 h, respectively). Bright-field microscopy confirmed characteristic features of apoptosis, including cell shrinkage, membrane blebbing, and nuclear condensation, particularly in cells treated with *S. aureus* RNA or poly(I:C) (Figure 7).

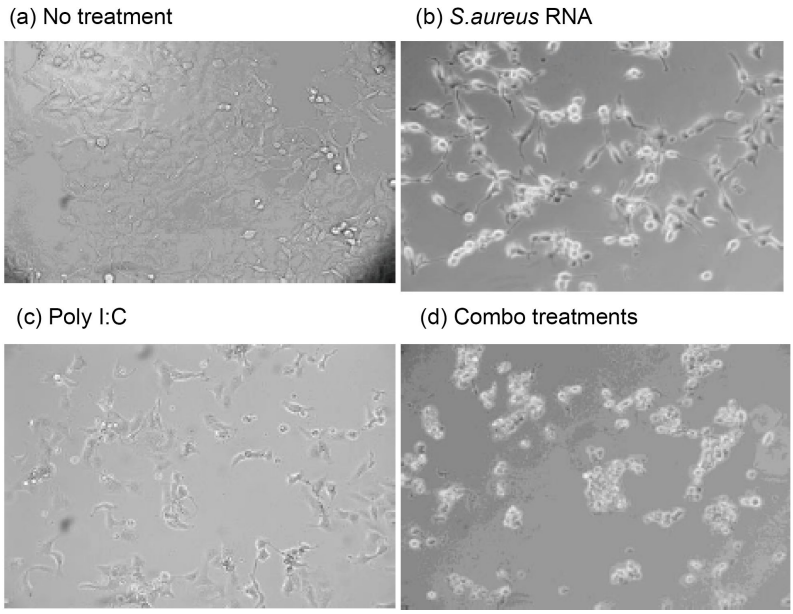

Figure 7. Bacterial RNA-Induced apoptosis. After 48 h, apoptosis was observed in *S. aureus* RNA stimulation of 2FTGH cells 1) Under Bright light microscopy, membrane blebbing, enlarged nucleus and apoptotic bodies (indicated apoptosis occurring) in 2FTGH cell treated with 100 μg/ml *S. aureus* RNA (b), 100 μg/ml Poly I:C (c) and cytokines combo treatments (Poly I:C, TNF-α, IFN-γ IL-1β 100 ug, 20 ng, 500 ug and 20 ng per ml) (d) at 48h post treatment in serum condition. 2) The cell viability was determined by tyrpan blue exclusion assay. Star represent the samples were statistically significant ($p < 0.001$) when compared to their respective control (NT).

3.5.2. Caspase-8 Activation in 2FTGH Cells Treated with Bacterial RNA

To investigate whether caspase-8 participates in bacterial RNA-induced apoptosis, 2FTGH cells were treated with *S. aureus* RNA, poly(I:C), or cytokines, and caspase-8 processing was analysed by immunoblotting. As expected, TNF-α plus cycloheximide (positive control) induced robust cleavage of pro-caspase-8 into its active intermediate forms. In contrast, *S. aureus* RNA alone did not produce a clear caspase-8 cleavage pattern, and poly(I:C) generated only weak intermediate bands. The cytokine mixture also yielded limited caspase-8 activation under these conditions. Overall, these data indicate that bacterial RNA-induced cell death in 2FTGH cells occurs with minimal caspase-8 processing (**Figure 8**).

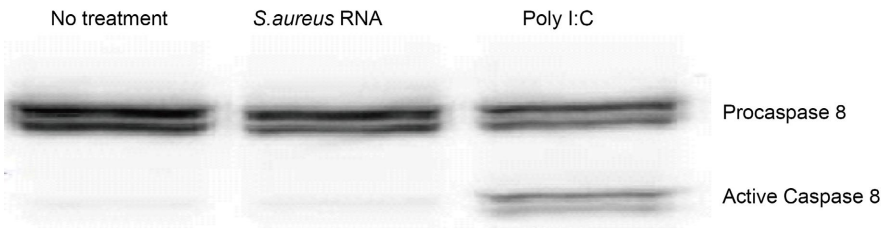

Figure 8. Bacterial RNA treatment of 2FTGH cells induces the formation of a caspase 8 intermediate as determined by immunoblot assay. The 2FTGH cells were treated with 100 μg/ml *S. aureus* RNA or 100 μg/ml Poly I:C as indicated for 12 h in serum free condition, and Western blot was performed using polyclonal caspase 8 antibody.

3.5.3. Caspase-1 Activation in Cardiac Myocytes Treated with Bacterial RNA

Finally, caspase-1 processing was examined in adult cardiac myocytes exposed to *E. coli* RNA, *S. aureus* RNA, poly(I:C), or cytokines. Immunoblotting revealed the appearance of a caspase-1 intermediate (~p20 subunit) in cells treated with both bacterial RNAs and with poly(I:C), while little or no processing was detected in untreated controls. Cytokine-treated cells also showed evidence of caspase-1 activation. These findings indicate that bacterial RNAs can trigger caspase-1 processing in human myocardial cells, consistent with engagement of inflammatory caspase pathways (**Figure 9**).

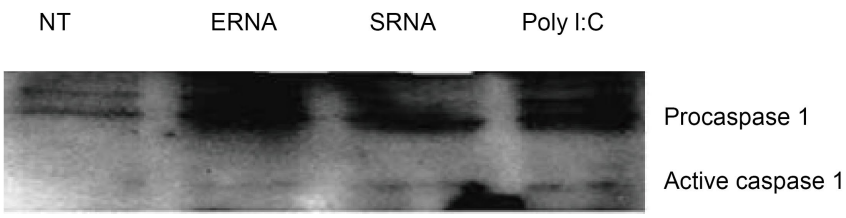

Figure 9. Bacterial RNAs treatment of human myocardial cells induces the formation of a caspase 1 intermediate as determined by immunoblot assay. Human myocardial cells cells were treated with 100 μg/ml *E.col* RNA, 100 μg/ml *S. aureus* RNA or 100 μg/ml poly I:C as indicated for 12 h in serum condition, and Western blot was performed using polyclonal caspase 1 antibody.

4. Discussion

Our experiments show that extracellular bacterial nucleic acids are sufficient to activate PKR and its downstream signalling network in non-immune human cells. Both bacterial RNA and DNA induced PKR autophosphorylation, eIF2α phosphorylation and a stress-response signature, with bacterial RNA generally more potent than DNA. This was accompanied by nuclear translocation and DNA-binding activity of STAT1, STAT2, IRF-1 and NF-κB, induction of IL-1β and caspase-dependent cell death. Together, these findings support a model in which bacterial nucleic acids behave as danger signals that couple PKR activation to JAK-STAT and inflammasome-related pathways in stromal cells, not only in professional immune cells [6]-[8] [12] [15]-[17].

These data extend previous work demonstrating that bacterial DNA and CpG motifs impair cardiomyocyte contractility and promote myocardial inflammation through TLR9-dependent mechanisms [9]-[11] and that bacterial RNA directly activates PKR in human adult cardiac myocytes, leading to eIF2α phosphorylation, contractile dysfunction and apoptosis [12]. Our current results in 2fTGH cells and signalling-deficient derivatives confirm that bacterial RNA is a strong PKR agonist in non-immune human cells as well and support the concept that bacterial RNA is a bona fide pathogen-associated molecular pattern for PKR in multiple tissues [12] [15] [16].

The requirement for JAK kinases and type I interferon components in bacterial RNA-triggered PKR activation provides a mechanistic link between nucleic-acid

sensing and canonical cytokine signalling. Using JAK- and STAT-deficient 2fTGH variants, we found that full PKR activation by bacterial RNA depends on JAK1, JAK2, TYK2, IFNAR2, STAT1, STAT2 and IRF9. This refines earlier work showing that JAK kinases are required for bacterial RNA- and poly(I:C)-induced PKR tyrosine phosphorylation [13] and is consistent with models in which an initial nucleic-acid trigger is amplified by autocrine type I interferon and JAK-STAT signalling [15]-[17]. The partial preservation of early PKR phosphorylation in some knock-out lines suggests that direct interaction of PKR with bacterial RNA, or with upstream RNA sensors, initiates signalling, whereas JAK-STAT pathways consolidate a second wave of PKR engagement.

We also observed rapid nuclear accumulation and enhanced DNA binding of STAT1, STAT2, IRF-1 and NF-κB in bacterial RNA-treated cells in parallel with PKR activation. This transcription-factor profile is typical of type I interferon and inflammatory responses downstream of nucleic-acid-sensing receptors in cardiac and stromal cells [6]-[8]. It also agrees with our previous microarray study in human adult cardiomyocytes, where exposure to non-self RNA induced broad transcriptional reprogramming affecting apoptosis, metabolism and innate immunity [14]. Our current results indicate that PKR and JAK-STAT signalling contribute to this transcriptional landscape and position non-immune structural cells within the wider network of sepsis-related signalling circuits [3]-[5] [8].

The induction of IL-1β and caspase-1 activation by bacterial RNA and DNA in our system connects PKR activity to inflammasome-associated effector functions. PKR has been implicated as a regulator of inflammasome activation and cytokine release in several models of infection and stress [15] [16] and our data extend this concept to fibroblast-like cells by showing that bacterial nucleic acids can drive PKR-dependent caspase-1 activation and IL-1β processing. Along with caspase-dependent apoptosis and limited caspase-8 activation, these findings support a model in which PKR acts as a nodal kinase that couples nucleic-acid sensing to both inflammatory cytokine production and programmed cell death [15]-[17].

From a translational perspective, our observations reinforce the idea that PKR is a potential therapeutic target in septic cardiomyopathy and related forms of organ dysfunction driven by nucleic-acid-mediated innate immune activation [3]-[5] [12] [16]. Experimental models show that PKR deficiency or pharmacological inhibition protects the heart from systolic overload, oxidant stress and isoproterenol-induced injury by reducing inflammation, apoptosis and adverse remodelling [18]-[20]. Together with our data, this suggests that PKR activation by bacterial nucleic acids may lower the threshold for apoptosis in parenchymal cells exposed to sepsis-related injury, thereby contributing to myocardial dysfunction and multi-organ failure. Any therapeutic approach, however, must balance attenuation of harmful PKR signalling with preservation of essential antiviral and antibacterial defence [15]-[17].

Several important limitations should be acknowledged. First, we relied mainly on immortalised human cell lines and purified bacterial nucleic acids at relatively

high concentrations, which may not fully recapitulate nucleic-acid delivery, compartmentalisation or receptor usage in vivo. Second, we did not identify the upstream pattern-recognition receptors that connect extracellular bacterial RNA to JAK-PKR activation in these cells; candidates include TLR3, TLR7/8, RIG-I-like receptors and cytosolic DNA sensors, which warrant direct testing [6]-[8]. Third, we focused on PKR, JAK-STAT and a limited set of downstream readouts; integration with other sepsis-relevant pathways, such as the integrated stress response and mitochondrial danger signalling, remains to be explored [17]. Future studies in primary human cardiomyocytes and in vivo models of sepsis-induced myocardial dysfunction should address these questions.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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