Bacterial RNA induces myocyte cellular dysfunction through the activation of PKR

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ABSTRACT

Severe sepsis and the ensuing septic shock are serious life threatening conditions. These diseases are triggered by the host's over exuberant systemic response to the infecting pathogen. Several surveillance mechanisms have evolved to discriminate self from foreign RNA and accordingly trigger effective cellular responses to target the pathogenic threats. The RNA-dependent protein kinase (PKR) is a key component of the cytoplasmic RNA sensors involved in the recognition of viral double-stranded RNA (dsRNA). Here, we identify bacterial RNA as a distinct pathogenic pattern recognized by PKR. Our results indicate that natural RNA derived from bacteria directly binds to and activates PKR. We further show that bacterial RNA induces human cardiac myocyte apoptosis and identify the requirement for PKR in mediating this response. In addition to bacterial immunity, the results presented here may also have implications in cardiac pathophysiology.

KEY WORDS Pattern recognition receptor; bacterial RNA; PKR; apoptosis

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Introduction

Sepsis and septic shock represent the systemic immunologic and pathophysiologic response to overwhelming infection. Approximately 800,000 cases of sepsis are admitted every year to hospitals in the North America and despite aggressive antibiotics and supportive care, over 150,000 patients per year succumb to this disorder (1). Sepsis and sepsis-associated multiple organ failure (MOF) are the leading cause of death in intensive care units (ICUs) of the developed world (2,3). The typical human cardiovascular response to septic shock is characterized by hypotension, decreased systemic vascular resistance and elevated cardiac index. In addition, myocardial depression manifested by reversible biventricular dilation and reduction of ejection fraction has been shown to be common in spontaneous human septic shock (4,5). Septic deaths are typically due to early refractory cardiovascular failure (hypotension or shock) or later multiple organ failure. Bacteria induce mammalian cellular dysfunction by releasing bacterial exotoxins such as Toxic Shock Syndrome Toxin-1 and/or bacterial endotoxin (lipopolysaccharide) (3,6). An emerging area of research indicates that bacterial RNA acts as an antigen. Bacterial RNA presents as an antigen due to nucleic acid sequence, differential secondary and tertiary structure and lack of methylation (7-10).

Discriminating self from nonself is crucial to innate immunity that employs pattern recognition receptors (PRRs) to sense molecular signatures associated with pathogenic moieties including nucleic acids (11). Human PKR is a serine/threonine kinase induced by interferon (IFNs) and characterized by a conserved kinase domain in the C-terminal region of the protein and two dsRNA binding domains (dsRBDs) localized to the N-terminal regulatory region (12-14). Binding of viral dsRNA to the dsRBDs causes PKR to undergo autophosphorylation at multiple kinase residues and subsequent activation (15-17). The alpha subunit of eukaryotic initiation factor 2 (eIF2α) is the best characterized cellular substrate that is phosphorylated by

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PKR. This post-translational modification of eIF2α leads to the inhibition of cellular protein synthesis (18,19). PKR has also been shown to be implicated in regulating physiological and pathological processes such as cellular growth and apoptosis, stress, transcription, and malignancy (20-25).

Recent studies have indicated that other PRRs can recognize distinct subspecies of RNAs released from pathogenic organisms. Of considerable interest are the recent findings that natural bacterial RNA and synthetic oligoribonucleotides can induce antigen specific immunity through the secretion of proinflammatory cytokines and IFNs in a toll like receptor (TLRs)-dependent manner (10,26-29). In addition, the NOD-like receptors (NLRs) have also been identified as an additional quality control mechanism that detects bacterial RNA in a NALP3-dependent manner. However, the NALP3 inflammasome has also been shown to mediate the recognition of different patterns of RNA subtypes such as viral RNA and poly I: C (synthetic dsRNA analogue) (30,31). The retinoic acid-inducible gene I (RIG-I) was first classified as a sensor for dsRNA, however recent reports indicated that single-stranded RNA (ssRNA) containing a 5'-triphosphate end can also function as a ligand for RIG-I (32,33). These observations emphasized the unifying theme in innate immunity whereby the host could use the same immunreceptor to detect various species of foreign RNA. Thus, it is possible that PKR employs a similar strategy to discriminate self from multiple nonself RNA species. For example, it is widely accepted that PKR recognizes viral dsRNA as nonself because it is normally absent in mammalian cells. However, dsRNA and ssRNA ligands with various secondary structures and modifications can also regulate PKR activity (34-36). Although bacterial RNA shares certain structural elements and signaling features with viral RNA, activation of PKR by natural RNA derived from bacteria has not been previously reported.

We reasoned that bacterial RNA could present a distinct pattern to be recognized by PKR as nonself. To address this hypothesis, we examined whether RNAs derived from bacterial or mammalian sources could modulate PKR activity. We determined that PKR recognizes bacterial RNA and we show that bacterial RNA induces apoptosis in a PKR-dependent manner.

Materials and methods

Preparation of bacterial total RNA

Pathogenic isolates of *E. coli* (O18:K1:H7; ATCC #700973) and *S. aureus* [capsular serotype 8, non-toxic shock toxin (TSST-1) producer] were used for this study. A modified hot phenol combined with enzymatic lysis was carried out to isolate bacterial total RNA. Exponentially growing bacteria were harvested in ice cold 5% phenol/ethanol solution and then resuspended in a fresh lysis buffer of TE, 2 mg/mL lysozyme and 5 μg/mL lysostaphin (Sigma Alderish, St.Loius, USA). Samples were brought to 2% SDS and a volume of 850 μL water-saturated phenol was added to each tube and incubated for 5 minutes at 64 ℃. Following centrifugation at 13,000 X g, the aqueous phase was transferred to a fresh tube and equal amounts of chloroform were added and centrifuged for 10 minutes at 4 ℃. The RNA was precipitated by 0.1 vol. 3 M sodium acetate and 2.5 vol. ethanol. The RNA pellets were washed with 70% ethanol and resuspended in DEPC-treated water. The integrity and purity of RNA species were determined by electrophoresis on agarose gels and A_{260}/A_{280} ratios. Extracted crude RNA was treated with RNase-free DNase I (Roche, Indianapolis, USA) to remove contaminant genomic DNA and further cleaned up by the Qiagen method (Qiagen, Valencia, USA) as per the manufacturer's instructions.

Preparation of mammalian total RNA

Total cellular RNA from human adult cardiac myocytes was prepared with extraction using a silica column-based method, Qiagen RNeasy, according to the manufacturer's instructions (Qiagen, Valencia, USA). 2×10^6 human adult cardiac myocytes cells (ScienCell Research Laboratories) were washed three times with ice-cold PBS and then scraped with 1 mL buffer RLT. Prior to the first RPE wash buffer, DNase l incubation mix was added onto RNeasy silica-gel membrane and allowed to sit for 20 minutes to further eliminate any traces of genomic DNA contamination. RNA was quantified by UV spectrometry and electrophorsed on a 1% agarose gel to verify purity and integrity prior to use.

RNA digestion

Aliquots of RNA samples were incubated in the presence of a heterogeneous mixture of ribonucleases [(1 U per 2 μL of RNA at 1 μg/μL for 1 hr) (Roche, Indianapolis, IN)]. RNA samples were analyzed by denaturing agarose or polyacrylamaide gel electrophoresis for quality assurance. Aliquots of digested total bacterial RNA were added to the media to yield a final concentration of 100 μg/mL or 200 μg/mL.

Cardiac myocytes culture and stimulation

Human adult cardiac myocytes were grown in cardiac myocyte medium supplemented with 10 % fetal bovine serum, growth supplement and 1% of penicillin/streptomycin (ScienCell Research Laboratories). Synthetic dsRNA (polyI: C), and 2-aminopurine (2-AP) were purchased from Sigma Chemical Co. 2×10^6 cardiac cells were treated with or without a final concentration of 10 mM 2-AP for 1 h and then washed 3 times with Hanks solution (Gibco Laboratories) before stimulation.

Cardiac myocytes were stimulated with 100 μg/mL of bacterial total RNA, Poly I:C, digested total RNA, or cardiac myocyte RNA for 24h, or 48h or left untreated as a control.

Immunoprecipitation and immunoblotting

Western blot was performed as previously described (37). Briefly, the cultured cells were washed with cold PBS and resuspended in RIPA lysis buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) plus a protease inhibitor mixture. Cellular lysates were resolved by 10% or 12% SDS-PAGE gels and subjected to immunoblot analysis using antibodies specific for phospho-Ser51 on eIF2α (Cell Signaling), antibodies specific for phospho-Thr451 on PKR (Invitrogen). Caspase-3 antibodies were from Santa Cruz, caspase-8 and caspase-9 antibodies were from Cell Signaling. Membranes were stripped and probed for total eIF2α protein or total human PKR protein (Cell Signaling). For Immunoprecipitation, 500 μg of the cellular lysates were first precleared with the addition of 50 μL of 50% protein G-Sepharose beads (Santa Cruz) for 1 h at 4 °C and then incubated with 5 μL of anti-PKR polyclonal antibody overnight with rotation. The beads were washed twice with RIPA, collected, and immunoprecipitates were subjected to either electrophoresis or *in vitro* PKR binding assay.

In vitro PKR binding assays

The ability of tested RNAs to bind to and activate PKR was carried out as previously described (21,22). Briefly, immunoprecipitated PKR was incubated in kinase buffer containing 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 50 mM KCl, 0.1 mM EDTA, 1 μg/mL Aprotinin, 1mM DTT, 20% glycerol, 10 μCi [γ-^{32}P] and 1 μg/mL RNA for 20 min at 30 °C. The beads were washed 6X with the salt buffer and the reaction was terminated by the addition of SDS sample buffer, heated at 95 °C for 5 min, and loaded on a 10% SDS-PAGE gel.

Analysis of genomic DNA fragmentation

Isolation of genomic DNA fragments was performed as previously described (37). Briefly, treated cells were collected and resuspended in lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). The supernatants contained fragmented DNA were brought to 1% SDS and treated with RNase A (0.5 mg/mL) and proteinase K (1 mg/mL). The DNA was precipitated and genomic DNA was fractionated by 1% agarose electrophoresis.

Results

Bacterial RNA can form pathogen associated molecular patterns (PAMPs) and serve as a danger signal to cells (10,26). To assess the ability of bacterial RNA to modulate PKR activation, cardiac cells were incubated in the presence of 100 μg/mL of RNA derived from bacterial or mammalian origins for 24 h. Total RNA from *E. coli* and *S. aureus* were shown to be potent activators of PKR (Figure 1A, lane 5 and 6). Self RNA derived from cardiac cells lacks the ability to activate PKR (Figure 1A, lane 2). Excluding the possibility that the observed effects on PKR activation were induced by potential contaminants in the RNA preparations, bacterial RNA was subjected to digestion with RNase. Digested bacterial RNA did not activate PKR suggesting that intact bacterial RNA is required for PKR phosphorylation (Figure 1A, lanes 3 and 4). As a positive control, poly I:C treatment of cardiac myocytes activated PKR to an extent comparable to that generated by bacterial RNA (Figure 1A, lane 7 *vs.* lanes 5 and 6).

To investigate the biological significance of PKR activation by bacterial RNA, we tested whether bacterial RNA could induce the phosphorylation of eIF2α. Immunolotting experiments revealed enhanced eIF2α on Ser 51 phosphorylation induced by bacterial RNA (Figure 1B, lanes 5 and 6). RNase treatment of bacterial RNA samples resulted in a reduction of the eIF2α phosphorylation (Figure 1B, lanes 3 and 4). In response to bacterial RNA, the levels of eIF2α phosphorylation were correlated to the PKR activation levels (Figure 1A and Figure 1B, lanes 5 and 6).

To test whether the activation of PKR by bacterial RNA is dose-dependent, we challenged the cardiac myocytes with bacterial RNA at 100 or 200 μg/mL. The data from these experiments indicated that the extent of PKR activation was dependent on the amount of RNA that was added (Figure 2A, lanes 2 and 3 *vs.* lanes 4 and 5). However, the phosphorylation levels of eIF2α indicated that cardiac cells treated with 200 μg/mL bacterial RNA were not different from those treated with 100 μg/mL (Figure 2B, lanes 2 and 3 *vs.* lanes 4 and 5).

To further determine the role of PKR signaling in bacterial RNA recognition, we used 2-AP which is widely used as a selective inhibitor for PKR (38-40). Cardiac cells left untreated or treated with 10 mM 2-AP for 1 h and then stimulated without or with bacterial RNA (Figure 3A and 3B). Bacterial RNA-induced phosphorylation of PKR was significantly reduced when PKR was inhibited with 2-AP (Figure 3Aa, lanes 2 and 3 *vs.* lanes 6 and 7). These results suggested that recognition of bacterial RNA is mediated by PKR. Inhibition of PKR activity by 2-AP also resulted in a significant reduction of eIF2α phosphorylation by bacterial RNA (Figure 3Ba, lanes 2 and 3 *vs.* 6 and 7). This observation further suggested that PKR is the kinase responsible for induction of eIF2α phosphorylation.

Although the above results demonstrated that bacterial RNA is a potential activator for PKR, the mechanism of this activation remains undetermined. Therefore, we immunoprecipitated PKR and subsequently performed *in vitro* PKR binding assay

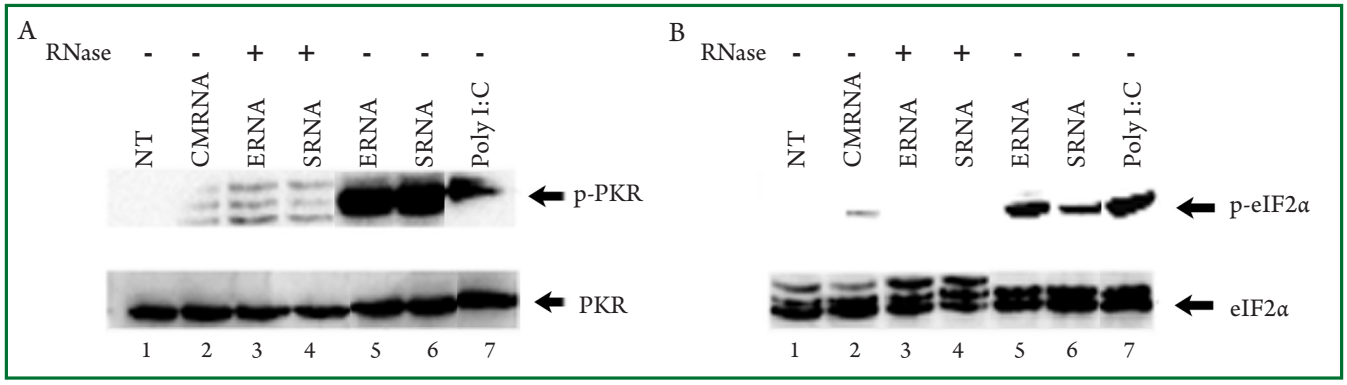

Figure 1. Bacterial RNAs activate PKR and induces eIF2α phosphorylation. Cardiac myocytes were stimulated for 24 h with 100 μg/mL RNAs from various origins (lanes 2-7) or unstimulated (lane1). Western blots were probed with antibodies to (A) pPKR (Thr446), (B) phosphor-eIF2α (Ser51) then stripped, and reprobed with antibodies to total PKR (A, bottom panel) and total eIF2α (B, bottom panel). NT: nontreated cells, CMRNA: cardiac myocytes RNA, ERNA: total RNA isolated from E. coli, SRNA: total RNA isolated from S. aureus, Poly I:C: synthetic RNA mimics viral dsRNA. RNA was digested with RNase (+) or left undigested (-) as indicated.

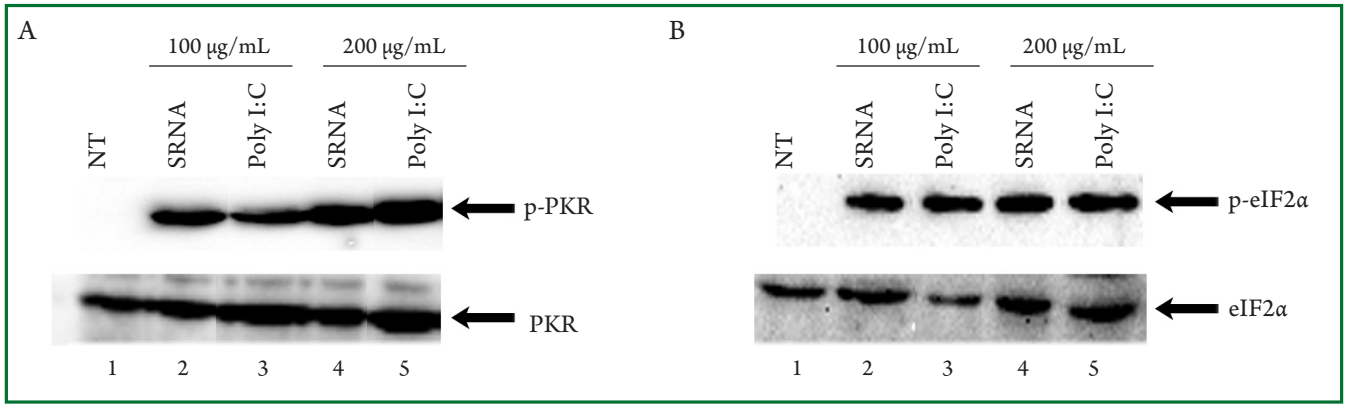

Figure 2. Activation of PKR is RNA dose- dependent. Cardiac myocytes were stimulated for 24 h with either 100 μg/mL RNAs (lanes 2 and 3) or 200 μg/mL RNAs (lanes 4 and 5) or left unstimulated (lane1). Cell lysates were separated by 10% SDS-PAGE, and Western blots were probed with antibodies to (A, top panel) pPKR (Thr446) or stripped, and reprobed with antibodies to total PKR (A, bottom panel) phospho-eIF2α (Ser51) (B, top panel) stripped, and reprobed with antibodies to total eIF2α (B, bottom panel). Positions are indicated. NT: nontreated cells, SRNA: total RNA isolated from S. aureus, PolyIC as a positive control for PKR activation.

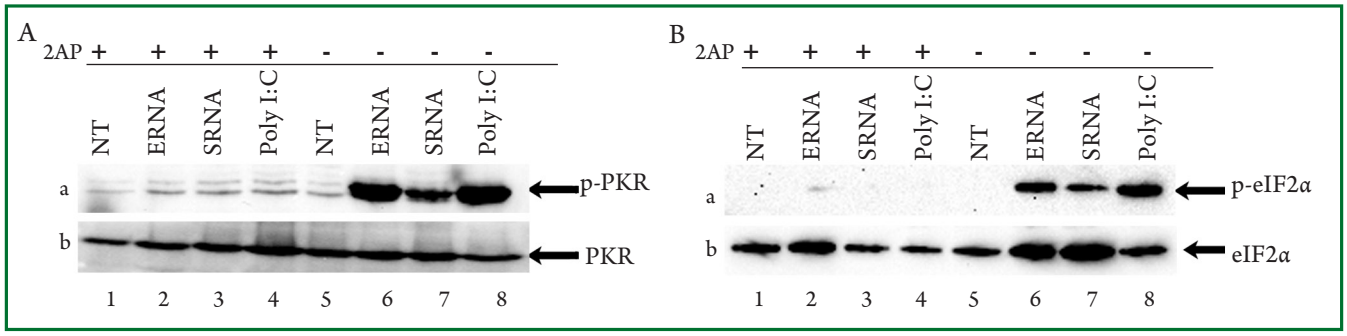

Figure 3. Inhibition of PKR suppresses its RNA-mediated activation and reduces eIF2α phosphorylation. Cardiac cells were pretreated with 10 mM 2-aminopurine (2-AP) for 1h (+) or left untreated (-) and then cells were stimulated with or without RNAs for 24 h. Western blots were probed with antibodies to (Aa) pPKR (Thr446), or stripped, and reprobed with antibodies to total PKR (Ab) phospho-eIF2α (Ser51) (Ba) stripped, and reprobed with antibodies to total eIF2α (Bb). Positions are indicated. NT: nonstimulated cells, ERNA: total RNA isolated from E. coli, SRNA: total RNA isolated from S. aureus, Poly I:C: synthetic RNA mimics viral dsRNA used as a positive control for PKR activation.

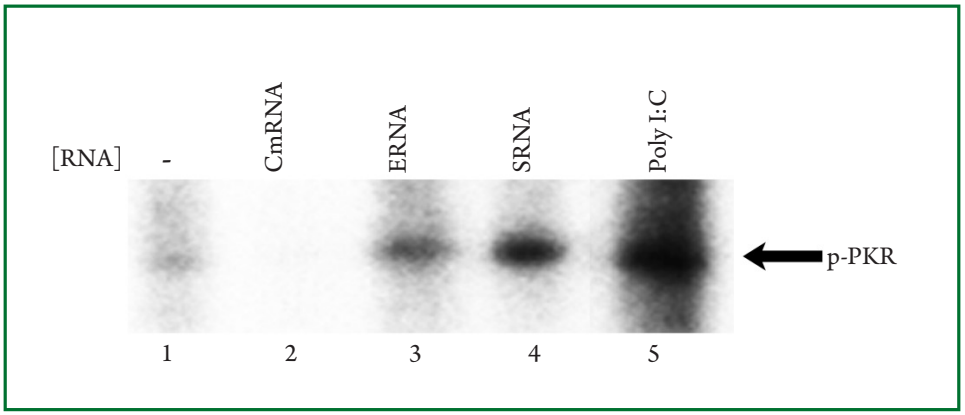

Figure 4. Bacterial RNAs directly binds to and activates PKR. PKR was immunoprecipitated from cardiac myocytes and subjected to in vitro binding assay. Purified PKR was incubated without (lane 1) or 1 μg/mL of total RNA from cardiac (lane 2), E. coli (lane 3), S. aureus (lane 4) or PolyI:C (lane 5). After 20 minutes, the reaction was terminated and the samples were subjected to 10% SDS-PAGE. The gel was dried, exposed and analyzed.

to examine the ability of various RNAs to bind to the purified PKR. The results from this assay revealed that the purified PKR was efficiently activated by total bacterial RNA (Figure 4, lanes 3 and 4). The efficiency of PKR activation by bacterial RNA was comparable to that of poly I: C (Figure 4, lane 5). Cardiac RNA failed to activate PKR (Figure 4, lane 2). These results suggested that bacterial RNA contains structural features that directly bind to PKR. Therefore, PKR is a direct receptor responsible for the recognition of bacterial RNA.

Viral and bacterial RNAs share many immunostimulatory potentials such as production of inflammatory cytokines which is considered as a hallmark of the cellular response to nonself RNA. These inflammatory mediators may exert pathological effects and harm the host. It is well established that innate immunity-mediated detection of viral dsRNA can trigger an apoptotic response. However, there have been no reports which describe the role of bacterial RNA as an inducer of apoptosis. Therefore, we tested whether bacterial RNA could provoke an apoptotic response. We observed that cardiac myocytes challenged with total bacterial RNAs for 48 h exhibited a number of morphological changes which are characteristic of apoptosis which were cell shrinkage, membrane blebbing, and apoptotic bodies. (Figure 5, G and H). Digested bacterial RNA failed to trigger these apoptotic responses (Figure 5, B and C). This confirms that intact bacterial RNA is the active inducer of cardiac cell death. We examined next the involvement of activated PKR signaling in bacterial RNA-induced cardiac apoptosis. Resistance to apoptosis was observed when cardiac PKR was inhibited with 2-AP (Figure 5, E and F *vs.* control D). To assess cardiac cell viability, we performed similar experiments using trypan blue exclusion assay. Bacterial RNA induced an average of 17% apoptosis in the cardiac myocytes (Figure 6). In contrast, only 2% and 3% cardiac apoptosis was detected when the cells were treated with digested bacterial RNA and 2-AP respectively.

Because DNA fragmentation is considered as a hallmark of apoptosis, we next repeated these experiments and evaluated the potency of bacterial RNA to trigger genomic DNA fragmentation. Consistent with the above assays, we found that stimulation of the cells with *E. coli* and *S. aureus* RNA induced cardiac DNA fragmentation (Figure 7, lanes 8 and 9). However, genomic DNA laddering was not apparent in cardiac cells treated with either digested bacterial RNA (Figure 7, lanes 3 and 4) or 2-AP (Figure 7, lanes 6 and 7). Taken together, the above observations revealed that bacterial RNA is an inducer of cardiac myocyte apoptosis and implicates PKR in mediating the apoptotic process.

To understand how bacterial RNA triggers cardiac apoptosis, we tested the cleavage of caspases as key regulators of apoptosis. While bacterial RNA treatment induced the cleavage of caspase 8, caspase 9, and caspase 3 (Figure 8 A-C, lanes 7 and 8), digested RNA suppressed the production of active caspase fragments (Figure 8, lanes 2 and 3). PKR inhibition by 2-AP also prevented production of the caspases fragments (Figure 8, lanes 5 and 6). These results suggested the requirement of PKR for bacterial RNA-induced caspase activation.

Discussion

We show that bacterial but not mammalian RNA is a novel activator of PKR. This observation suggested that cardiac myocytes are fully able to distinguish between self and foreign RNA. Although the primary function of cardiac myocytes is contraction, accumulating evidence suggested that cardiac

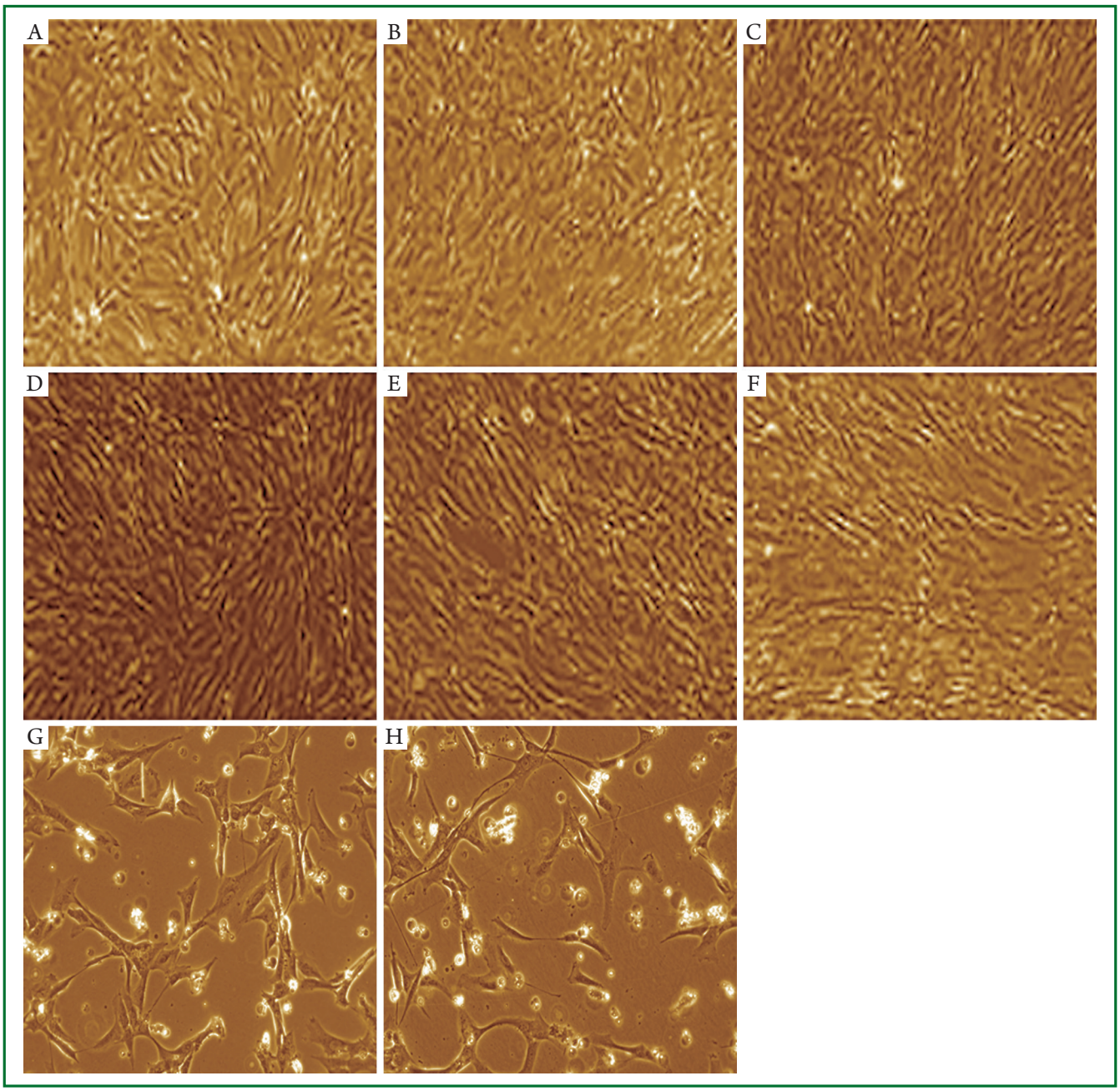

Figure 5. Bacterial RNAs induces morphological features of cardiac apoptosis. Cardiac myocytes were treated with 200 μg/mL for 48 h and evaluated by phase contrast microscopy. Cells were left untreated (A), or incubated with digested RNA from E. coli or S. aureus RNA (images B and C) respectively. Cardiac cells were pretreated with 10 mM 2AP (images D-F) and then stimulated without (image D) or with E. coli RNA (E) or S. aureus RNA (F). Cardiac myocytes were challenged with E. coli RNA or S. aureus RNA (G, H) respectively. Following treatment, micrographs were taken at (100 ×) magnification.

myocytes respond to pathogenic insults with a complex inflammatory response. Cardiac myocytes express PRRs and can detect a diverse range of pathogenic patterns including nucleic acids (41-46). Several lines of investigation support the idea that immuoreceptors sense bacterial RNA while ignoring the endogenous RNA. For example, bacterial but not mammalian RNA exerts immunostimulatory potential in TLR-dependent and NALP3-dependent fashion (31,47). These observations raised the question of how the immune sensors discriminate self from foreign RNA. Recent reports have indicated that specific nucleoside modifications and structural elements are differentially represented in either microbial or mammalian RNA

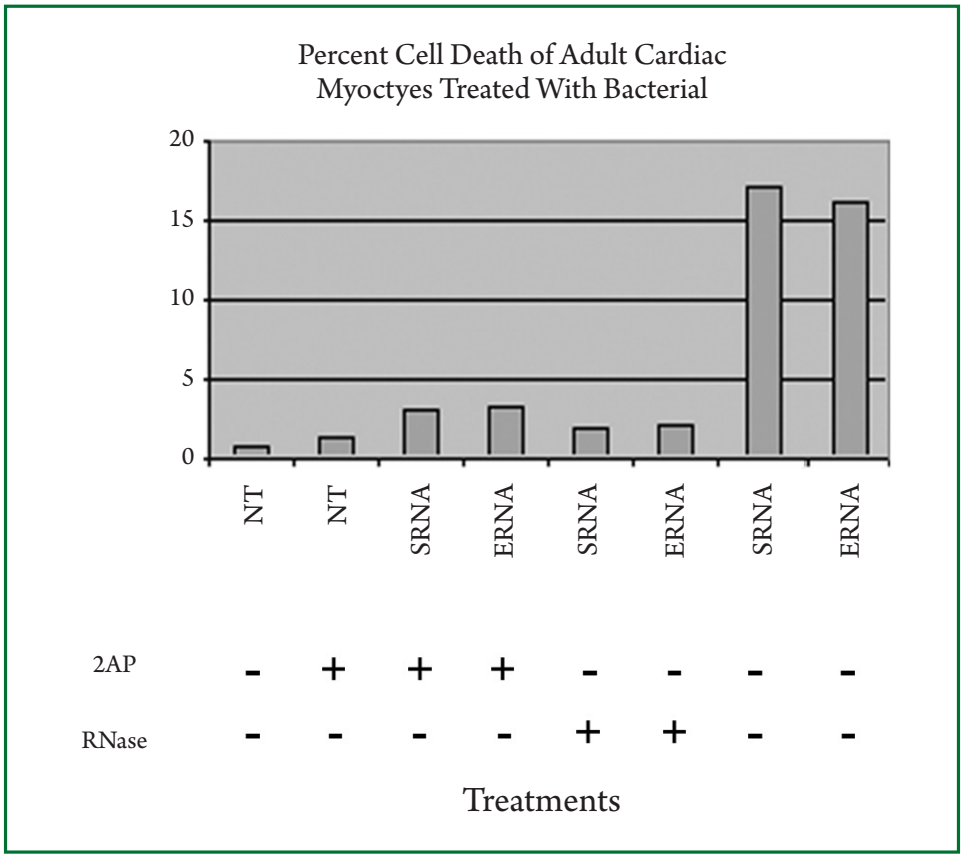

Figure 6. Percentage of cardiac myocytes apoptosis. Following treatment with (+) or without (–) digested bacterial RNAs, 10 mM 2-AP, or intact bacterial RNAs as indicated, Trypan Blue was used for viable cell count.

and therefore provide a molecular mechanism to discriminate pathogenic from self RNA (9,10,48,49). Importantly, the type and extension of these modifications depends on the evolutionary level of the organism from which the RNA subspecies was derived. In contrast to most cellular species of RNA that are heavily modified, unmodified motifs or low degree of nucleoside modifications are abundant in bacterial and viral RNAs. For example, cellular primary transcripts undergo various modifications to acquire a 7-methylguanosine (7 mG) cap structure at its 5'-end while bacterial and viral mRNAs possess a 5'-triphosphate at their terminal end (50-52). It has been recently reported that *in vitro* transcribed RNA with limited secondary structures activate PKR in a 5′-triphosphate–dependent manner (53). The investigators suggested that PKR uses this feature, which is primarily present in foreign transcripts such as viral and bacterial mRNA, as a part of the RNA-quality mechanism to distinguish between endogenous and pathogenic RNAs. Furthermore, internal nucleosides modifications including nucleobase, sugar, and phosphodiester which mimic their natural modifications modulates PKR activation in RNA structure-specific context (54). In addition to capping, posttranscriptional modifications such as pseudouridine and methylated nucleosides exist unequally in either mammalian or microbial RNA. Unmethylated CpG motifs are found more frequently in bacterial RNA and certain DNA viruses than that of eukaryotic rRNA and mRNA. It has been demonstrated that RNA with unmethylated CpG motifs is immunostimulatory. Methylation of the 5' position of the C in this RNA, mimicks the viral CpG DNA and abrogates its immune activity (55). Internal regions of select RNA viruses such as adenoviruses, influenza, and herpes simplex contain the modification N6-methyladenosine (m6A) to a greater extent than the cellular and bacterial RNA. This modification, which destabilizes RNA duplexes, suppresses the immune activation (10). Pseudouridin is a more frequent modification in human RNA as compared to RNAs of bacterial and viral origins. It has been recently demonstrated that the incorporation of uridine in mRNA activates PKR and enhances translation. In contrast, pseudouridin reduced PKR activation (10,56). Importantly, we show that the potential of bacterial RNA to activate PKR was sensitive to RNase that removes the base-paired secondary structures indicating the dependence of PKR on this feature as a key for the molecular recognition

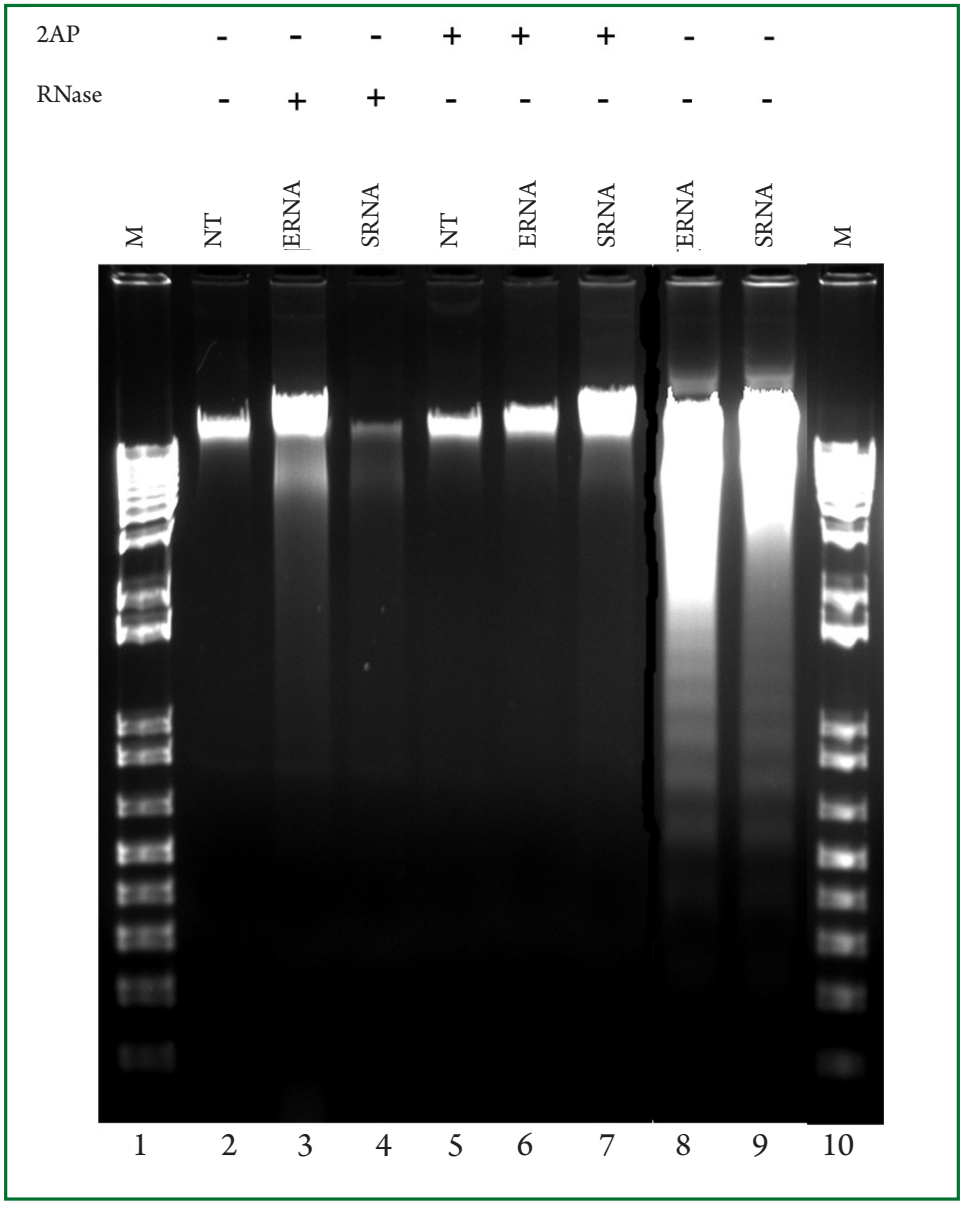

Figure 7. Bacterial RNAs induce genomic DNA laddering in cardiac myocytes and inhibition of PKR protects from DNA fragmentation. Cardiac myocytes post treated with 200 μg/mL bacterial RNAs for 48 hr or pretreated first with 2-AP for 1hr and then genomic DNA was fractionated by 1% agarose gel electrophoresis. Lanes 1 and 10 DNA marker (M); lane 2 (NT) untreated cells; lanes 3 and 4 cells treated with digested E. coli RNA (ERNA), S. aureus RNA (SRNA) respectively; lanes 5, 6, 7 cells were pretreated with 10 mM 2AP and then stimulated without (lane 5) or with E. coli RNA (ERNA) or S. aureus RNA (SRNA) (lanes 6 and 7 respectively); lanes 8 and 9, cells challenged with E. coli RNA (ERNA) or S. aureus RNA (SRNA).

and signaling. Several RNA ligands with various modifications activate PKR to a different extent; however, secondary structure elements are the common theme for these ligands to regulate PKR signaling (34-36,57). We next determined that bacterial RNA binds to and activates PKR which suggests that bacterial RNA has structural features involved in the direct activation of PKR and thus PKR is a molecular sensor for bacterial RNA. Although dsRNA was first proposed as a ligand and activator for PKR, a diverse range of biological RNA patterns with various structural elements such as dsRNA, bulges, stem- loops, and internal loops can also regulate PKR activity in a sequence-independent manner (57). These secondary structures are also

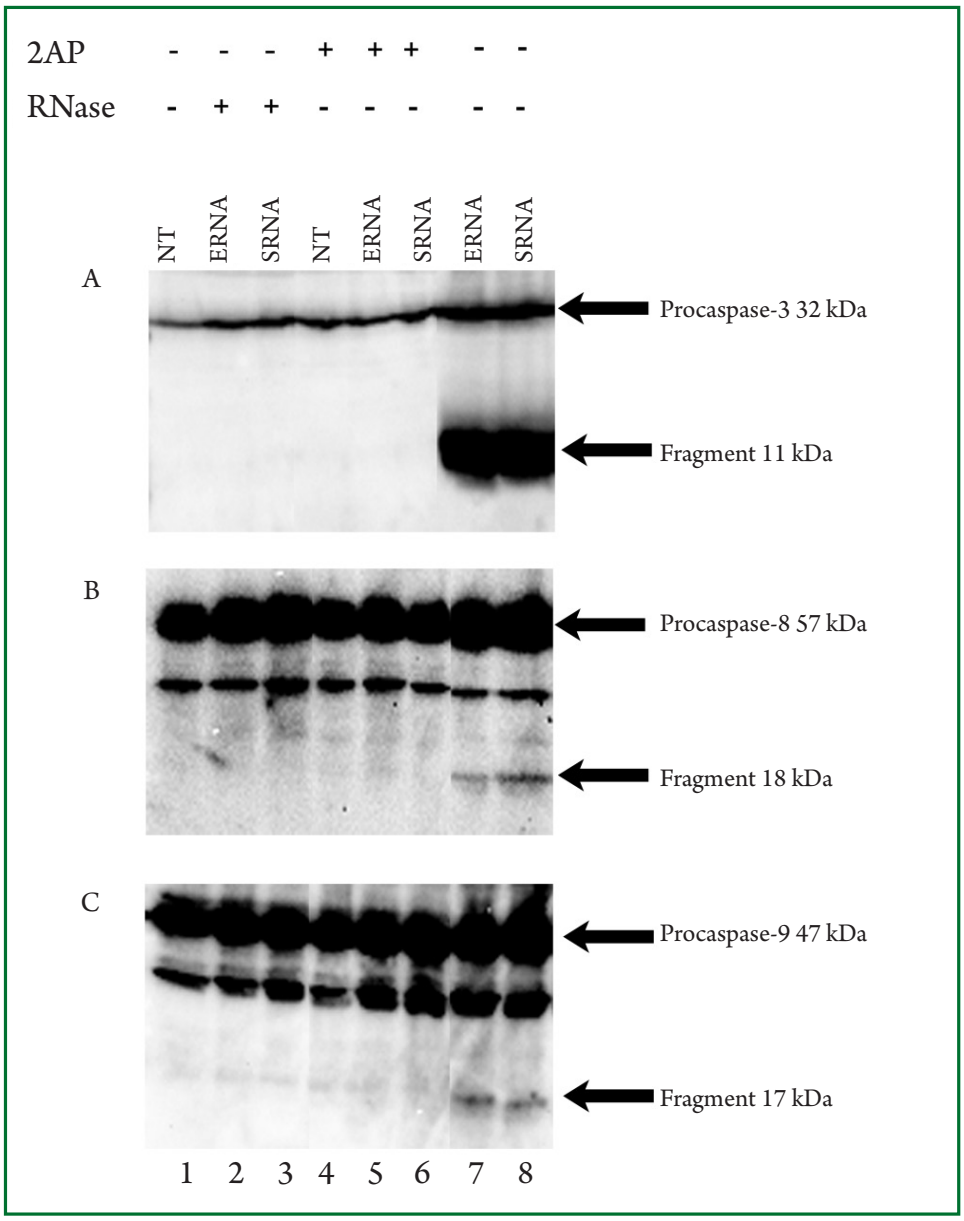

Figure 8. Bacterial RNAs induces cleavage of caspase-3, -8 and -9 mediated by PKR. Extracts from cardiac myocytes challenged with 200 μg/mL bacterial RNA for 48 h were analyzed by Western blotting using antibodies to caspase-3 (A), caspase-8 (B), or caspase-9 (C). Cardiac Cells untreated (lane 1), or incubated with digested E. coli RNA or S. aureus RNA (lanes 2 and 3) respectively; or pretreated with 10 mM 2AP (lanes 4-6) and stimulated without (lane 4) or with E. coli RNA or S. aureus RNA (lanes 5 and 6) respectively; or challenged with E. coli RNA or S. aureus RNA (lanes 7,8).

a common characteristic to bacterial RNA. Therefore, it seems likely that these modifications affect the RNA structure which impact on PKR activation.

We also provided evidence that bacterial RNA itself can elicit a lethal response and identified the requirements of PKR for the induction of cardiac apoptosis. Although much progress has been made to understand the PKR signaling in apoptosis induced by different stimuli, the role of bacterial RNA in this response remains unexplored (58-64). Herein, we linked the caspases-3, -8 and -9 activation to bacterial RNA-induced apoptosis and identified PKR-dependent signaling for activation of these caspases and induction of apoptosis. Our findings are in agreement with previous reports which established the PKR-dependent activation of caspases 1, -3, -7, -8, and -9 and PKR is essential for different forms of stress-induced apoptosis (62-68). Additionally, it has been shown that PKR is essential for

macrophage apoptosis which is induced by bacterial pathogens including *Bacillus, Yersinia and Salmonella* (69). Collectively, we identified bacterial RNA as a novel inducer of apoptosis in PKR-dependent manner.

Bacterial RNA and modified ssRNA are potent immunostimulatory agents characterized by the induction of proinflammatory cytokines and interferons (10,26,28,29). Although triggering the immune response is generally considered as protective, the interplay between the pathways that are modulated may harm the host. For example, cytokines can modulate cardiac function and induce apoptosis and thus serve as prognostic markers for morbidity and mortality in patients with microbial infections, septic shock, and cardiac failure (37,70-72). We previously demonstrated that bacterial and viral nucleic acids induce cardiac dysfunction (73). Loss of cardiac myocytes by apoptosis is a serious and frequent complication from microbial infections, septic shock and myocardial infarction (37,71,74,75). Several bacterial pathogens are epidemiologically linked to human heart diseases such as endocarditis and myocarditis. Although it has been established that bacterial infections of cardiac myocytes induces apoptosis and caspases activation, the role that PKR plays in regulating these responses has not been previously described (76,77). We propose that bacterial RNA may contribute to myocardial dysfunction during bacterial sepsis.

Although PKR is considered as a principal player in antiviral protection, its contribution to cellular defenses against bacterial infections has not been previously described. Our studies suggested that activation of PKR by bacterial RNA may play a pivotal role in the innate response to bacterial infections. The results described above also show that the bacterial RNA-driven immune response can provoke an apoptotic response and suggested the potential role of these immunostimulatory agents in cardiac pathophysiology.

Acknowledgements

The first two authors contributed equally to this paper.

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