Factor Associated With Neutral Sphingomyelinase Activation and Its Role in Cardiac Cell Death

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Generation of proapoptotic sphingolipids by neutral sphingomyelinase activation is an early response to hypoxia/reoxygenation (HR) in cardiomyocytes. Factor associated with neutral sphingomyelinase activation (FAN) mediates activation of sphingomyelinase and subsequent apoptosis. However, the participation of FAN in HR-induced cardiomyocyte cell death has not been elucidated. We therefore investigated the expression and role of FAN in rat cardiomyocytes. A cDNA was isolated from rat heart encoding putative rat FAN. Reverse transcriptase–polymerase chain reaction, immunoelectron microscopy, and immunofluorescence demonstrated for the first time the expression of FAN specifically in rat cardiomyocytes. FAN expression was confirmed by the finding that expression of a dominant-negative FAN almost completely abrogated HR-induced cell death, whereas overexpression of wild-type FAN led to an increase. Treatment of FAN and dominant-negative FAN–expressing cells with C2-ceramide produced substantial cell death, indicating dominant-negative FAN exerts its protective action by interfering with the activation of the sphingolipid cascade. Taking these results together, we conclude that FAN is a previously undescribed and important HR signaling component in the heart and that inhibition of FAN may provide a novel intervention point for reducing ischemia/reperfusion injury.

The identification of signaling components involved in ischemia/reperfusion (IR) injury is a critical step in designing strategies for mitigating cell death and improving patient outcomes. The sphingolipid-signaling pathway has recently been appreciated as playing a role in apoptotic cell death in cardiomyocytes and cardiovascular disease in general. Activation of neutral sphingomyelinase (nSMase) and generation of ceramide and sphingosine may be one of the earliest events in IR injury. Factor associated with neutral sphingomyelinase activation (FAN) is an adapter protein that is necessary for activation of nSMase by receptors such as tumor necrosis factor receptor type 1 (TNFR1) and CD40. For example, cell death in TNFα-treated fibroblasts from FAN-deficient mice was impaired but could be restored by transfection of FAN.

Considering the central role of FAN in the sphingolipid pathway and the potential role of nSMase in cardiac IR injury, we hypothesized that FAN might also play a role in activating the sphingolipid pathway in the heart. In this study, we report for the first time that FAN is expressed in cardiac cells. We also demonstrate its functional role in HR-induced cell death by providing evidence that cell death is abrogated if FAN is functionally repressed.

Materials and Methods
For Materials and Methods, see the online data supplement, available at http://www.circresaha.org.

Results and Discussion
To determine FAN expression in rat heart, total RNA from adult rat heart was used for reverse transcriptase–polymerase chain reaction (RT-PCR) cloning. A novel 2787-bp clone was found, encoding a putative protein of 920 residues with 95% and 91% identity to human and mouse FAN, respectively (Figure 1A). This putative protein of 104.5 kDa was confirmed by Western analysis in both adult and neonatal cardiomyocytes (data not shown).

In common with human and mouse, rat FAN possesses conserved WD-40 repeat, BEACH, and GRAM domains (Figure 1A). A search of the PROSITE database revealed consensus protein kinase C (PKC), PKA, and tyrosine kinase phosphorylation sites. Their presence implies FAN may be regulated by kinases and that phosphorylation of FAN may play a role in activation nSMase. Identification of consensus myristylation sites indicates that FAN may be membrane-associated.

Figure 1C shows FAN expression in various tissues by RT-PCR and demonstrates that FAN is expressed not only in the rat heart but also in kidney, brain, and skeletal muscle, while not appreciably in lung.

To elucidate FAN expression specifically in cardiomyocytes, RNA from cultured cardiomyocytes was isolated and used for RT-PCR of FAN mRNA. To ensure these cells were free of fibroblasts, RT-PCR of ANF and collagen from cardiomyocytes and COS-7 fibroblast cells was also performed. ANF and collagen are cardiomyocyte- and fibroblast-specific, respectively. cDNAs were cloned and sequenced to verify their identity. A FAN product was obtained from both cell types, whereas only cardiomyocytes expressed ANF (Figure 1C). The cardiomyocytes seemed to be a pure population, determined by a lack of collagen product, whereas collagen was obtained from fibroblasts, as expected.

Commununofluorescence was performed to additionally evaluate expression of FAN in cardiomyocytes and its subcellular location. A polyclonal antibody was produced against a peptide (amino acids 537 to 550 of rat FAN). Neonatal cardiomyocytes
were coincubated with FAN and caveolin-3 (cav3) antibodies. Cav3 is a sarcolemmal muscle-specific isoform of caveolin used as a marker to identify cardiomyocytes. To verify the specificity of the FAN antibody, antibodies were preincubated with increasing amounts of FAN peptide. FAN, but not cav3 staining, was substantially reduced when antibodies were preincubated with FAN peptide (Figure 2A), indicating FAN antibodies were specific.

Importantly, FAN staining was seen at the edge of neonatal cardiomyocytes (Figures 2A and 2B, arrows), which was partially colocalized with cav3 at the sarcolemma (yellow in Figure 2B overlay). FAN staining also appeared to be intracellular, possibly vesicular. These data suggest that FAN is expressed at the plasma membrane, consistent with previous reports on human FAN. Immunoelectron microscopy was used to additionally elucidate the localization of FAN in adult cardiomyocytes. Gold particles were localized principally to the cytoplasmic surfaces of the sarcolemma (Figure 2C, arrows), which was not seen using preimmune serum, additionally supporting the novel finding that FAN is expressed in cardiomyocytes at the sarcolemma.

FAN couples various receptors to nSMase activation in other cell types to mediate apoptosis. Considering that nSMase activation is one of the first responses to HR in cardiomyocytes, we determined if FAN is necessary for HR-induced cell death using neonatal rat cardiomyocytes and a well-characterized model simulating IR injury. Cells were cotransfected with GFP and empty-vector, wild-type, or dominant-negative FAN (DN-FAN) V5-tagged constructs. The DN-FAN was designed to encode only the WD-40 repeats. Others have shown that, although still capable of interacting with upstream proteins, the DN-FAN renders wild-type FAN incapable of nSMase activation. The action of this construct was confirmed by demonstration that the DN-FAN abrogated TNFα-induced nSMase activation in COS-7 cells (online Figure 1). Gene transfer and V5 expression were confirmed by Western analysis (Figure 3A).

HR elicited cell death in 13.5 ± 3.2% of control GFP-expressing cells, corresponding to a 7.5-fold activation of cell death over nontreatment (Figure 3B). HR-treated cells overexpressing wild-type FAN exhibited a 45% increase in cell death compared with HR control cells, with levels reaching 19.7 ± 1.2%. This increase was not seen in untreated cells overexpressing FAN, indicating FAN may be playing a role in cell death in response to HR, but not basally. Moreover, HR-treated cells expressing DN-FAN exhibited 3.1 ± 2.7%
death, corresponding to a 5-fold decrease in cell death from empty vector HR-treated cells, indicating interruption of FAN signaling can reduce cell death induced by HR.

To determine whether a downstream effector of the nSMase pathway could overcome the inhibitory effects of DN-FAN on cell death, FAN or DN-FAN expressing cells were treated with cell-permeable C2-ceramide and analyzed by TUNEL. Treatment resulted in 19.4 ± 0.5% cell death, a 7.3-fold increase over nontreated cells (Figure 3C). This level of cell death was not significantly affected by expression of FAN-V5 or DN-FAN-V5, which resulted in 28.5 ± 0.1% and 20.1 ± 6.5% cell death, respectively. Together, these data indicate that ceramide acts downstream of FAN and confirms that DN-FAN inhibits cell death by blocking activation of nSMase.

The presented data suggest FAN may be a critical gatekeeper in the sphingolipid pathway, leading to cell death in the cardiomyocyte. Results implying nSMase involvement in the heart’s response to IR are consistent with previous work demonstrating a central role of this signaling cascade in IR injury.2–4 The novel discovery that FAN may be playing a crucial role in the molecular mechanism of HR-induced cell death suggests TNFR1 or another receptor may be involved in the FAN/nSMase signaling system in the heart. TNFα is released by cardiomyocytes during hypoxia5 and may play a central role in heart failure.13 However, although TNF-α can induce cardiomyocyte apoptosis,14 it can also be protective.15 Additional studies are needed to evaluate whether the role of FAN in HR-induced cell death is through TNFR1 or a novel mechanism. Furthermore, identifying the specific nSMase isoform activated by FAN during IR signaling will be crucial to understanding the molecular pathways underlying IR injury.

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References


Key Words: FAN • sphingomyelinase • apoptosis • hypoxia
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Materials and Methods

RT-PCR, Cloning and Expression of rat FAN

Total RNA was extracted from rat cardiac tissue according to manufacturer’s protocol (RNeasy, Qiagen). Rat FAN was isolated by RT-PCR (Qiagen One Step) using primer sets 5’-ggg cct cca tgg cgt t-3’/5’-ccg ttg cac cat cca aac-3’ and 5’-gtt tgg atg caa cg-3’/5’-gga gag gag aag gca ctt aat-3’, designed from mouse FAN mRNA (accession number NM010945). The two RT-PCR fragments were subsequently extended in a second PCR reaction to produce a partial cDNA encoding the full-length FAN open reading frame (ORF), which was cloned into pCR2.1-TOPO (Invitrogen), and sequenced in both directions (San Diego State University DNA Core Facility). Sequence analysis was performed using CLUSTAL W alignment program and by searching the PROSITE database.

For expression of FAN, the entire FAN ORF was subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen). A truncated, dominant-negative form of FAN (DN-FAN, amino acids 706-920) was also subcloned into pcDNA3.1/V5-His-TOPO using an upstream primer containing a Kozak sequence at its 5’ end: 5’-cca cca tgt ttg gaa gac gcc aag aca-3’.

For RT-PCR analysis a partial rat collagen alpha 1 type 1 (coll1a1) cDNA (GenBank accession no. Z78279) was amplified by RT-PCR for analysis using primers 5’-tgg tct tgg agg aaa ctt-3’ and 5’-cca tct tca cca cga gca-3’. A partial rat ANF cDNA (GenBank accession no. M27498) was amplified by RT-PCR for analysis using primers 5’-cgt gcc ceg acc cac gcc-3’ and 5’-ggc tcc gag gcc cag cga g-3’.

Cell Culture

Neonatal and adult rat ventricular myocytes
Rat neonatal cardiomyocytes were isolated from 1-4 day old Harlan Sprague-Dawley rats as previously described. Adult Harlan Sprague-Dawley rat cardiomyocytes were prepared as previously described. All procedures involving experimental animals were conducted following institutional and NIH guidelines for the ethical treatment of animals.

COS-7 cells

COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) until transfections.

**Antibody Development and Western Analysis**

A putative antigenic FAN sequence, GGVDLNSIEDPDDK (amino acids 537 – 550) synthesized and used for the generation of a rabbit polyclonal antibody (Sigma-Genosys). Preimmune and anti-serum from the fourth bleed were titered by ELISA and IgG column purified by Protein A column chromatography (Bio-RAD).

For Western analysis, cultured cardiomyocytes were rinsed twice in ice-cold PBS, lysed in 1% (v/v) Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris, pH 8, and 5 µl/mL protease inhibitor cocktail (Sigma). Resulting lysates were centrifuged at 12,000 x g for 5 min and protein concentration was determined on supernatants using Bradford reagent (Sigma). 40 µg were fractionated by SDS-PAGE, and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories Inc.). Protein labeling was performed by blocking in 10% normal goat serum (NGS) in Tris-buffered saline with .1% Tween 20 (TBST), followed by incubation for one hour each with .1 ng/mL mouse anti-V5 (Invitrogen) and 1/10,000 goat anti-mouse-peroxidase (Jackson ImmunoResearch) antibodies in 10% NGS/TBST. Detection was performed using enhanced chemiluminescence detection reagents (NEN).
Immunocytochemistry, Confocal Microscopy and Immunoelectron Microscopy

Cultured cardiomyocytes were rinsed twice in ice-cold PBS, fixed in –10°C methanol for 5 min and washed in phosphate buffered saline (PBS) before blocking with 10% normal goat serum (NGS) in PBS for an hour. Cells were incubated for an hour with anti-cav3 (BD Transduction Laboratories, 1/500) or anti-FAN (1/100) in 10% NGS/PBS. After several washes in PBS, cells were incubated with anti-mouse-FITC and anti-rabbit-RRX (1/500, Jackson ImmunoResearch Laboratories, Inc.) in 10% NGS/PBS. Confocal microscopy analysis was performed using a Leica TCS SP2 inverted microscope and images acquired using Leica Confocal Image Analysis Software. Immunoelectron microscopy was performed using a pre-embedding technique on whole cardiac ventricular fibers incubated overnight with anti-FAN (1/50) and goat anti-rabbit-nanogold (1.4nm, Aurion), followed by silver enhancement (Nanoprobes), as originally described by Ralston and Ploug and modified by us for heart tissue.

Transfection by Electroporation

Cardiomyocytes were co-transfected essentially as previously described. Cells were suspended in serum-free DMEM:F-12 (1:1) following the final dissociation step. Approximately 4 x 10^6 cells were placed with 25 µg pGreen Lantern (Clontech) and 80 µg pcDNA3.1, FAN-V5 or DN-FAN-V5 plasmid DNA in 300 µl total volume DMEM:F-12 (1:1). Electroporations were performed using a Gene Pulser (Bio-Rad) at 550 V, 25 µF and 100 Ω in a .2 cm gap electroporation cuvette (Bio-Rad). Cells were subsequently culured at a density of .56 x 10^6 cells per well in 1.7 cm² chamber slides for 16 – 24 hours in 10% FCS in DMEM:F-12 (1:1). Previous observations determined that, under these conditions, approximately 5 – 10%
transfection efficiency was obtained and approximately 85% of transfected cells expressed both plasmids, consistent with previous studies.  

_Transfection by Lipofectamine_

COS-7 cells were transferred to serum-free DMEM and transfected with 2 µg of either pcDNA3.1 or DN-FAN-V5 using Lipofectamine 2000 reagent according to manufacturer’s protocol (Invitrogen). Cells were subsequently cultured for 24 hours in serum-free DMEM prior to experiment.

_Neutral Sphingomyelinase Assay_

Transfected COS-7 cells cultured to confluency in 3.8 cm² wells were treated with 100 ng/ml rat TNF-α for 1.5 min, previously shown to maximally activate nSMase. Treatment was stopped by floating the plate in a methanol-dry ice bath for approximately 10 seconds. Cells were washed twice in ice-cold PBS and lysed in 35 µl/well 100 mM Tris, pH 7.5, 10 mM MgCl₂, .2% Triton-X100, and 5 µl/ml protease inhibitor cocktail (Sigma). Protein concentrations were determined using Bradford reagent (Sigma). Lysates from two wells were combined and centrifuged at 15,000 x g to pellet cell debris. 50 µl lysates were combined with an equal volume sphingomyelin (SM) substrate solution consisting of 100 mM Tris, pH 7.5, 10 mM MgCl₂, 0.2 mM ovine SM (Sigma), 0.2% Triton, and 0.3 mCi/ml [N-methyl-¹⁴C]SM (specific activity 55 mCi/mmol, Amersham). Reactions were incubated for one hour at 37°C, under conditions where the reaction rate was linear and the amount of SM hydrolyzed did not exceed 10% of the total amount of SM added. Phosphorylcholine was then extracted with 400 µl chloroform:methanol and measured by scintillation counting. SMase activity was normalized for protein content.
Hypoxia Reoxygenation and C2-ceramide Treatments

Transfected cardiomyocytes were washed with DMEM/F-12 (1:1) after initial culturing and cultured an additional 48 hours in serum free DMEM/F-12 (1:1) supplemented with 1 mg/ml BSA (minimal media). Cells were then treated with 12 h hypoxia and 30 h reoxygenation, as previously described 3. Cells treated with C2-ceramide were placed into minimal media containing 20 µM cell permeable D-erythro-C2-ceramide (Matreya) for eight hours, changing the media every 2 hours.

TUNEL Analysis

HR or ceramide treated cells were washed twice in ice-cold PBS and fixed in 4% freshly depolymerized paraformaldehyde in PBS for 20 min. The slides were then processed immediately or stored at 4°C in .4% paraformaldehyde in PBS. TUNEL analysis was subsequently performed according to manufacturer’s protocol (Roche).

References


Supplemental Figure 1: DN-FAN-V5 expression decreases TNF-\(\alpha\) induced nSMase activity. COS-7 cells were transfected with pcDNA3.1 or FAN-V5 and treated with 100 ng/ml TNF-\(\alpha\) for 1.5 min. nSMase activities were determined and plotted as the mean fold activity over control \(\pm\) S.E. (n = 3 individual cultures). *, \(p < 0.05\) different from empty vector non-treated control; †, \(p < 0.05\) different from empty vector TNF-\(\alpha\)-treated control, as determined by one-way ANOVA followed by Neuman-Keuls post hoc analysis of variance.
nSMase activity (fold of control)

control    TNF

pcDNA3.1    DN-FAN-V5

*    †