Original Article

Nitric Oxide Induces Gene Expression of Jumonji and Retinoblastoma 2 Protein while Reducing Expression of Atrial Natriuretic Peptide Precursor Type B in Cardiomyocytes

(jumonji / nitric oxide / cardiomyocyte / heart / atrial natriuretic peptides / retinoblastoma)

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Abstract. Jumonji (JMJ, Jarid2), a prototypical member of the jumonji domain-containing protein family, plays a major role in embryonic cardiac development, but its role in the developed heart is unclear. Cardiomyocytes from neonatal mouse heart were treated in culture with NO donor SIN-1, 500 µM, for 2, 4, and 20 h. SIN-1 treatment was associated with a significant and 6.9 ± 2.5 fold increase in *jmj* gene expression over all time points. The expression of *jmj* increased markedly and significantly 4.2 ± 1.1 fold, 16.6 ± 4.1 fold, and 2.7 ± 0.3 fold, respectively, at time points 2 h, 4 h, and 20 h after treatment. The ability of the increase in gene expression to translate into an increase in cellular protein expression was ascertained by Western blotting, which showed an increase in the JMJ protein in whole-cell lysates. Because of the relationship of JMJ to Rb and ANP in the heart, gene expression of these proteins was also examined. SIN-1 produced a small but significant increase in Rb2, but not Rb1 or Rb-binding proteins 4, 6, or 7. In contrast, SIN-1 produced a marked and significant reduction in natriuretic peptide precursor type B but not type C to 0.24 ± 0.09 fold of the control. These data suggest that JMJ may be a critical, previously unrecognized factor that mediates some of the cellular effects of NO, that NO may be able to increase JMJ in diseases associated with reduced JMJ expression.

Introduction

The protein Jumonji (JMJ, Jarid2), first discovered in a developmental mouse gene trap experiment (Takeuchi et al., 1995), is the prototypical member of the since-identified jumonji domain-containing protein family (Takahashi et al., 2007). Proteins in this family regulate gene expression at both the level of chromatin regulation, by modifying chromatin (de)methylation and accessibility, and more discretely as transcription factors by acting as gene repressors (Takahashi et al., 2007). The ability of JMJ to regulate gene expression established JMJ as a factor that controls organ development (Kim et al., 2003). A major role for JMJ was identified in cardiac development as *jmj*-mutant mice have a range of cardiac defects including ventricular septal defects, non-compaction of the ventricular wall and double-outlet right ventricle (Lee et al., 2000). JMJ interacts with the retinoblastoma protein (Rb), suggesting that JMJ-induced down-regulation of cardiac cell growth via interaction with Rb is a mechanism underlying the development of cardiac defects (Jung et al., 2005). In the heart, JMJ represses atrial natriuretic peptide (*ANP*) gene expression by inhibiting transcriptional activities of Nkx2.5 and GATA4 (Kim et al., 2004). The function of JMJ is not limited only to the heart as *jmj* mutation manifests CNS abnormalities, hepatic cell death, and haematopoietic cell accumulation in the thymus and spleen (Motoyama et al., 1997). The function of JMJ in the developed heart, however, is unclear.

Nitric oxide (NO) is a major regulator of cardiac function and cardiomyocyte cell viability. NO can alter mitochondrial function (Davidson and Duchen, 2006) and regulate cardiac contractility (Massion et al., 2005). NO can play a role in the development of heart failure (Hare and Stamler, 2005). NO plays a major role in cardiomyocyte cell viability as it can protect cardiomyocytes against cell injury or death (Cohen et al., 2006) while under some condition it can induce cardiomyocyte cell death (Rabkin and Kong, 2000; Andreka et al., 2004). Thus, NO is an important factor in myocardial reperfusion injury (Jones and Bolli, 2006). The effect of NO on JMJ has not previously been examined. We asked the question - what is the effect of NO on jumonji and genes related to jumonji in the heart? This study provides the first evidence that NO upregulates *jmj* gene expression, links NO to *Rb* and *ANP* gene expression

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Abbreviations: ANP – atrial natriuretic peptides, JMJ – jumonji, NO – nitric oxide, Rb – retinoblastoma, SIN-1 – 3-morpholino-sydnonimine hydrochloride.

and suggests that JMJ may be part of a novel pathway mediating some of the effects of NO.

Material and Methods

Cell culture

Neonatal mouse cardiomyocytes were cultured from CD1 mice as described (Kong and Rabkin, 2000) using a protocol approved by the University committee on animal care. Briefly, hearts from 1-day-old mice, sacrificed by decapitation, were transferred to a petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum and 1% antibiotic/antimycotic (10 mg/ml streptomycin sulphate, 10 U/ml penicillin G sodium, and 25 μg/ml amphotericin B). The ventricles were manually minced in collagenase solution (1 mg/ml collagenase type I, 0.6 mg/ml bovine serum albumin, 3 μM $CaCl_2$ in DMEM). The initial digest was discarded, and the digestion was repeated two more times with collagenase solution. Following that, the remaining tissue was subjected to periods of agitation in digestion medium (0.005% trypsin, 0.1% bovine serum albumin (BSA), 1.1 x 10^5 Dornase units DNAse in DMS8 salt solution (116 mM NaCl, 5.4 mM KCl, 0.4 mM NaH_2PO_4-H_2O, 0.75 mM Na_2PO_4-$7H_2O$, 5.5 mM glucose in water)), with the digest being added to tubes containing DMEM. The digest was then centrifuged at 1000 *g* for 5 min to pellet the cells. The cell pellet was resuspended in DMEM with 10% FCS and 1% antibiotic/antimycotic, and incubated at 37°C and 5% CO_2 in 60 mm culture dishes at a density of 3×10^6 cells per dish.

Gene expression

Gene expression studies were performed as previously outlined (Rabkin and Klassen, 2007). Briefly, cells were cultured in dishes for 72 h. At 20, 4, and 2 h prior to cell collection, 3-morpholinosydnonimine hydrochloride (SIN-1), 0.5 mM, or diluent was added to the medium. Following the incubation time, cells were collected and RNA isolated with Qiagen RNeasy Mini kit (Qiagen, Mississauga, Canada). A 3DNA Array 350™ Expression Array Detection Kit (Genisphere, Hatfield, PA) was used for labelling and hybridizing RNA to microarray slides. Microarray procedures were followed as outlined in the manufacturer's protocols. cDNA microarrays were printed at the Gene Array Facility at Vancouver Hospital, and contained the mouse gene for Jumonji (Jarid2). Procedures were followed as outlined in the manufacturer's protocols. cDNA was combined with reverse transcriptase (RT) primer (Cy5 for controls, Cy3 for treatments) and heated at 80° C to facilitate annealing. Superase-In RNase inhibitor was added, and then SuperScript II First Strand Buffer (Gibco, Burlington, Canada), dNTP mix, DTT, and Superscript II enzyme (Gibco) were added. Following incubation allowed the reverse transcription of cDNA to occur, and NaOH/EDTA was added to stop the reaction. Incubation denatured the DNA/RNA hybrids and degraded the RNA. The reaction was neutralized by the addition of Tris-HCl, pH 7.5. The cDNA controls and treatments from each time point were combined and concentrated using Millipore Microcon® YM-30 Centrifugal Filter Devices. cDNA was resuspended in a formamide-based hybridization buffer and added to a microarray beneath a Sigmacote-treated LifterSlip. Hybridization of the cDNA to the array proceeded overnight in a dark humidified chamber at 42° C. The LifterSlip was then removed and the array washed and centrifuged to dry. Fluorophores Cy3 and Cy5 3DNA capture reagents were then mixed with formamide-based hybridization buffer and anti-fade reagent, warmed, and added to the array beneath the LifterSlip. Following the hybridization, the LifterSlip was removed and the arrays washed with a series of SSC/SDS buffers and centrifuged to dry. They were stored in a light-protective box until scanned. Microarrays were scanned in a Perkin Elmer Microarray Analysis System utilizing ScanArray Express version 2.2 software (Perkin Elmer, Fremont, CA) to obtain the array image files. Array image files were then analysed using Imagene 6.0 (BioDiscovery, El Segundo, CA) and Genespring 7.0 (Silicon Genetics, Palo Alto, CA) software to quantitate the fluorophores' intensity.

Western blotting

Neonatal mouse cardiomyocytes in culture dishes were treated with SIN-1, 0.5 mM, or diluent and maintained at 37°C and 5% CO_2 for 24 h. Cells were harvested and Western blotting was performed as previously outlined (Kong et al., 2005). Anti-jumonji rabbit antibodies were a kind gift from Dr. Youngsook Lee of the University of Wisconsin. They are specific for the Jumonji (Jarid2) peptide fragment between residues 100–116. Western blots were scanned and subjected to densitometry using Scion Image 4.0.2 computer software (Scion Corp, Frederick, MD).

Materials, drugs and chemicals

Culture media, foetal calf serum, antibiotic-antimycotic was obtained from Gibco. SIN-1 was from Sigma Chemical Co. (St. Louis, MO). All chemicals, unless otherwise stated, were purchased from Fischer Scientific (Ottawa, Ontario, Canada).

Data Analysis

The data are presented as the mean ± SEM Hypothesis testing used one-way ANOVA.

Results

Microarray gene expression data indicated that following treatment with the NO donor, SIN-1, *jmj* expression increased markedly. The magnitude of the increase was the largest amongst the changes in gene expression on the microarray in response to SIN-1 (Rabkin and Klassen, 2007). Cells were treated with SIN-1, 500 μM, for 2, 4, and 20 h and the intensity value at each time point was divided by the intensity value of the control at

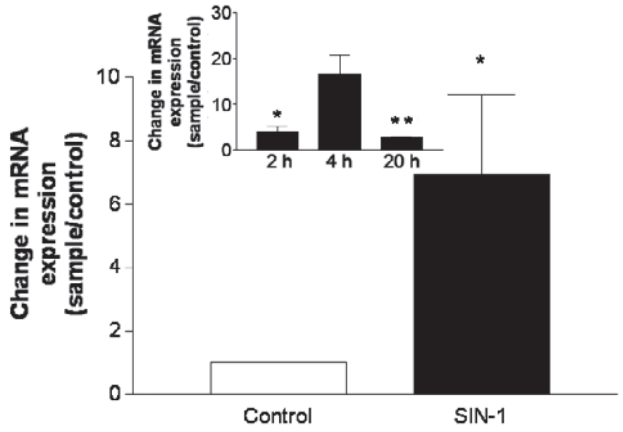

Fig. 1. The nitric oxide donor SIN-1 increases jmj gene expression in cardiomyocytes
Cardiomyocytes were maintained in culture for 72 h. At 20, 4, and 2 h prior to cell collection, SIN-1, 500 μM, or diluent was added to the medium and returned to the incubator. RNA was isolated and microarray procedures were followed as described in Material and Methods. The intensity value for *jmj* at each treatment time point was divided by the intensity value of the control (diluent treatment) at the same time point to yield the fold change in RNA expression relative to the control. The main figure shows the average change in RNA expression for *jmj* over the 20-h period, which is the average for all time points. The inset shows the mean ± SEM (N = 3) for each time point (* $P < 0.05$;** $P < 0.01$).

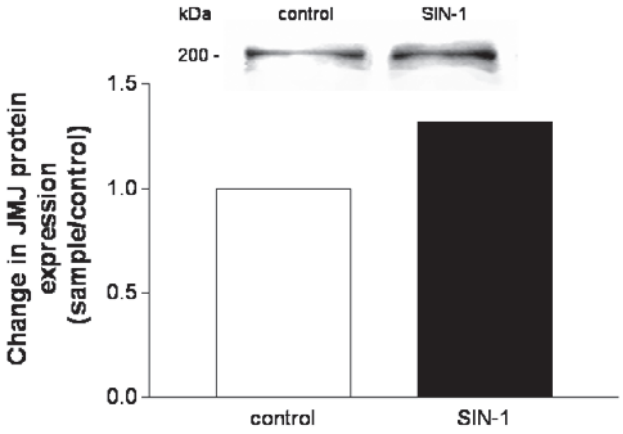

Fig. 2. The nitric oxide donor SIN-1 increases JMJ protein expression in cardiomyocytes
Cardiomyocytes in culture dishes were treated with SIN-1 and maintained at 37°C and 5% CO_2 for 24 h. Cells were then harvested and Western blotting was performed. Anti-jumonji rabbit antibodies were a kind gift from Dr. Youngsook Lee of the University of Wisconsin. They are specific for the Jumonji (Jarid2) peptide fragment between residues 100–116. Western blots were scanned and subjected to densitometry.

the same time point to yield the fold change relative to the control. This concentration of SIN-1, 500 μM, produced an increase in nitrite in the medium, as measured by the Greiss reaction (Rabkin and Klassen, 2007), that

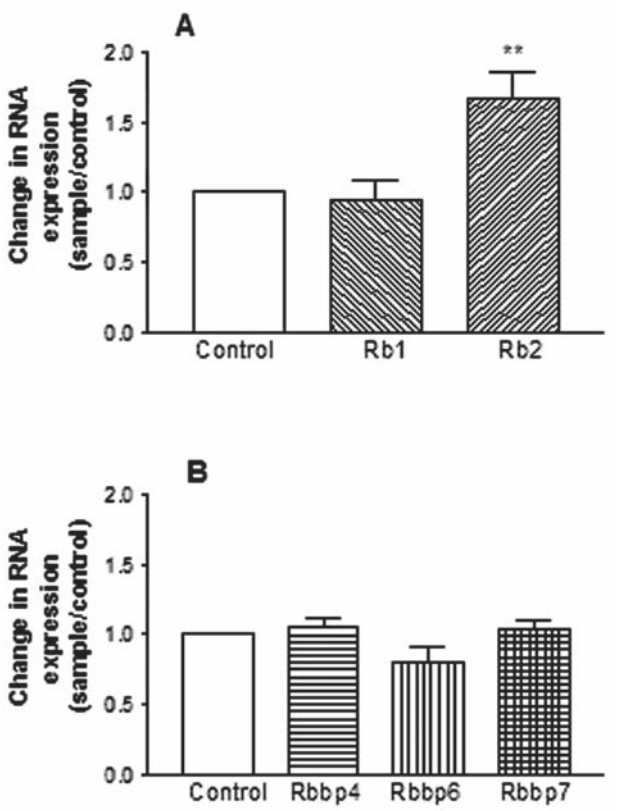

Fig. 3. The nitric oxide donor SIN-1 increases Rb2 but not Rb1 or Rbp 4, 6 or 7 gene expression in cardiomyocytes
Cardiomyocytes were maintained in culture for 72 h. At 20, 4, and 2 h prior to cell collection, SIN-1, 500 μM, or diluent was added to the medium. RNA intensity values at each time point were divided by the intensity value of the control at the same time point to yield the fold change relative to control. The mean ± SEM for the entire observation periods is shown for Rb1 and Rb2 in panel A (** $P < 0.01$) and for Rbp 4, 6, and 7 in Panel B.

was similar to the mean plasma nitrite concentration in the mouse heart 30 days after acute coronary artery ligation (Feng et al., 2001). Considering the entire 20-h period, SIN-1 treatment was associated with significant ($P < 0.01$) and 6.9 ± 2.5 fold increase in *jmj* expression. The expression of *jmj* increased markedly and significantly ($P < 0.01$) 4.2 ± 1.1 fold, 16.6 ± 4.1 fold, and 2.7 ± 0.3 fold, respectively, at time points 2 h, 4 h, and 20 h after treatment (Fig. 1). The ability of the increase in gene expression to translate into an increase in cellular protein expression was ascertained by Western blotting. Densitometric analysis of Western blots indicated an increase of the JMJ protein in whole-cell lysates (Fig. 2).

Because JMJ interacts with Rb (Jung et al., 2005), the effect of SIN-1 on Rb1, Rb2 as well as several Rb-binding proteins (Rbp) 4, 6, and 7 were assessed. SIN-1 produced a small but highly significant ($P < 0.01$) increase in Rb2 but not Rb1 or Rbp4, 6, or 7 (Fig 3).

Because JMJ represses *ANP* gene expression in the heart, we sought to determine whether NO also represses

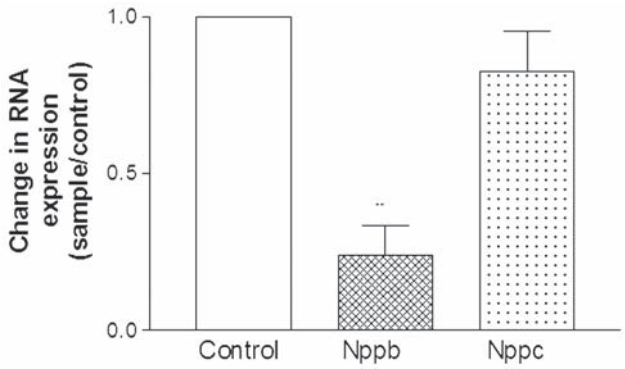

Fig. 4. The nitric oxide donor SIN-1 decreases natriuretic peptide precursor type B (Nppb) but not type C (Nppc) gene expression in cardiomyocytes
Cardiomyocytes were maintained in culture for 72 h. At 20, 4, and 2 h prior to cell collection, SIN-1, 500 μM, or diluent was added to the medium. RNA intensity values at each time point were divided by the intensity value of the control at the same time point to yield the fold change relative to control. The mean ± SEM for the entire observation period is shown (**P < 0.010).

ANP gene expression, which would be consistent with an effect potentially operating though JMJ. SIN-1 induced a marked and significant (P < 0.01) reduction in expression of natriuretic peptide precursor type B to 0.24 ± 0.09 fold of the control (Fig 4). Natriuretic peptide precursor type C was not altered.

Discussion

This study is the first to identify a potential role for JMJ in the developed heart as well as identifying the ability of NO to induce an increase in the expression of *jmj* (*Jarid2*). Our findings are consistent with the exciting emerging concept of NO as a regulator of the expression of certain genes. NO modulates gene expression of certain signal transduction pathways in lung endothelial cells (Li et al., 2004). NO stimulates the activity of the insulin gene with the resultant increase in endogenous insulin mRNA levels in isolated islets of Langerhans (Campbell et al., 2007). In cells from the islets of Langerhans, NO stimulates the nuclear localization and DNA-binding activity of the β-cell transcription factor PDX-1 (Campbell et al., 2007). NO regulates gene transcription in other cell types through various processes. NO up-regulates tumour necrosis factor α by decreasing Sp1 binding to a proximal GC-box element (Zhang et al., 2003). Phorbol ester response element-regulated genes are activated by NO-releasing agents and appear to be mediated by the AP-1 (Jun/Fos) transcription factor complex because there is an increased DNA binding of AP-1 and increased *junB* and c-*fos* mRNA in cells treated with these agents (Pilz et al., 1995). The mechanism of gene activation by NO is distinct from that used by phorbol esters and cAMP (Pilz et al., 1995). NO inhibits *IL-2* gene expression in murine T-lymphoma cells, perhaps through NO-induced abrogation of the DNA-binding activities of the regulating transcription factors Sp1 and EGR-1 (Berendji et al., 1999). NO donors up-regulated the activity of the human VEGF promoter in normoxic human glioblastoma and hepatoma cells through a cGMP-independent process utilizing the hypoxia-response element (Kimura et al., 2000). NO may also trigger gene regulation that stabilizes mRNA, through a p38 mitogen-activated protein kinase (MAPK) mechanism (Wang et al., 2006). Many of these genes, such as those related to insulin production, are not relevant to the heart, which does not produce insulin, but are nevertheless discussed because of their importance within the construct of NO modulation of gene expression.

The magnitude of the increase in JMJ protein expression is less than the magnitude of the increase in *jmj* gene expression. This may be due to the time lag between the increase in gene expression and the maximum amount of protein synthesis or the multiple factors influencing protein synthesis and degradation that determine the amount of a cellular protein.

Our finding that SIN-1 increases gene expression of *jmj* and *Rb2* raises questions for further research on the commonality of factors that would lead to the increased expression of these two genes in response to NO and/or its products. JMJ contains an AT-rich interaction domain that is also conserved in Rb-binding protein 1 (Rbp1) and Rbp2 (Fattaey et al., 1993; Balciunas and Ronne, 2000; Jung et al., 2005). The products of the *Rbp1* and *Rbp2* genes are ubiquitously expressed nuclear phosphoproteins with structural motifs that suggest a role in transcriptional regulation (Fattaey et al., 1993). Data suggest that there is a JMJ/Rb interaction in the heart consistent with JMJ down-regulation of cell growth via interaction with Rb (Jung et al., 2005). Our data suggest two sites of action for NO namely to increase expression of both *jmj* and *Rb2*; and in so doing magnify the effect of JMJ on cell physiology. Our data are also consistent with the effect of JMJ on ANP. JMJ induces repression of *ANP* gene expression, which is thought to occur through a protein-protein interaction via specific JMJ domains (Kim et al., 2004). We found that NO produced a marked reduction in natriuretic peptide precursor type B gene expression. This finding suggests a novel potential site of interaction of JMJ to reduce *ANP* expression, as we propose a conceptual framework whereby NO increases *jmj* expression, which in turn leads to an increase in cellular JMJ levels resulting in a reduction in ANP by reducing gene expression of natriuretic peptide precursor type B.

Several limitations of this study should be borne in mind. First, we used microarrays and mRNA expression profiling, which have some inherent uncertainty. The use of small amounts of RNA to perform an experiment leads to the risk that minor discrepancies in specificity or binding between experiments will produce seemingly large fold changes when comparing treatments to stand-

ards. We minimized these problems by examining different time points during NO treatment. Distinguishing changes in fluorophore intensity from background intensity can at times be challenging and has the potential to produce false-positive reports (Kothapalli et al., 2002). However, we obtained further verification of changes in JMJ with the use of Western blotting. A second consideration is that we examined only one of the jumonji family genes and proteins; it remains to be determined whether NO similarly affects other jumonji genes. Thirdly, we examined only the association of SIN-1, which releases NO and produces NO by-products, on the potential downstream effectors - Rb2 and ANP.

While it is speculative to consider the potential implications of NO in up-regulation of the *jmj* gene and protein products and potential downstream effectors, several areas of further research are suggested. Developmental defects of the heart and central nervous system are subject to JMJ regulation (Lee et al., 2000; Jensen et al., 2005) so it is intriguing to speculate whether NO could alter these conditions. Considering the important roles that NO plays in the heart, from the physiologic regulation of cardiac contractility to disease states such as heart failure and myocardial infarction (Davidson and Duchen, 2006), the present study opens new avenues for exploration of a central role for jumonji to mediate the cellular effects of NO in normal cardiac function and clinical disease.

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