c-Jun N-terminal Kinase-mediated Stabilization of Microsomal Prostaglandin E2 Synthase-1 mRNA Regulates Delayed Microsomal Prostaglandin E2 Synthase-1 Expression and Prostaglandin E2 Biosynthesis by Cardiomyocytes*

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Microsomal prostaglandin (PG) E₂ synthase-1 (mPGES-1) catalyzes the terminal step in the biosynthesis of PGE₂, a key proinflammatory mediator. The purpose of this study was to elucidate the regulation of mPGES-1 mRNA expression in cardiomyocytes, define the role of JNK enzymes in this process, and characterize the role of mPGES-1 in cardiomyocyte PGE₂ biosynthesis. In neonatal cardiomyocytes, interleukin-1β and lipopolysaccharide (LPS) both stimulated mPGES-1 mRNA expression and increased mPGES-1 mRNA stability and protein synthesis but failed to increase mPGES-1 mRNA transcription. Treatment with the JNK1/2 inhibitor, SP600125, abrogated the increases in mPGES-1 mRNA stability, mPGES-1 protein synthesis, and PGE₂ release induced by interleukin-1β or LPS. mPGES-1 protein synthesis was observed in LPS-stimulated neonatal cardiomyocytes from jnk1−/− or jnk2−/− mice. In contrast, infection of jnk1+/− cardiomyocytes with an adenovirus encoding phosphorylation-resistant JNK2 (ad-JNK2-DN), or of jnk2−/− cardiomyocytes with ad-JNK1-DN, significantly decreased LPS-stimulated mPGES-1 protein synthesis. Similarly, co-infection with ad-JNK1-DN and ad-JNK2-DN attenuated LPS-stimulated mPGES-1 protein synthesis in cardiomyocytes from wild type mice. Targeted deletion of the gene encoding mPGES-1 led to a 3.2-fold decrease in LPS-stimulated PGE₂ release by cardiomyocytes in comparison with wild type mice but had no effect on COX-1, COX-2, mPGES-2, or cytosolic PGES mRNA levels. These studies provide direct evidence that mPGES-1 mRNA levels in cardiomyocytes are augmented by stabilization of mPGES-1 mRNA, that JNK1 or JNK2 can participate in the regulation of mPGES-1 protein synthesis in these cells, and that mPGES-1 catalyzes the majority of LPS-induced PGE₂ biosynthesis by cardiomyocytes.

Prostaglandins (PG) are biologically active lipid mediators that regulate protein aspects of cardiac physiology, including coronary vascular permeability and blood flow, myocardial protein synthesis, and myocar-dial hypertrophy (1–3). Atherosclerotic plaque stability also appears to be regulated by PG (4, 5). The biosynthesis of PG is catalyzed by the sequential action of PLA₂, COX, and PG synthase enzymes (6). PLA₂ enzymes release arachidonic acid from phospholipids, and COX enzymes oxidize free arachidonic acid to PGA₂. PG synthases in turn convert PGA₂ to multiple PG, including PGE₂, the principal PG species formed by cardiomyocytes (3, 7). Three PGE₂ synthase (PGES) enzymes have been identified, including the inducible isofom, microsomal PGES-1 (mPGES-1), and the putative constitutively expressed isoforms, mPGES-2 and cytosolic PGES (cPGES) (8–10). Although exposure to cytokines leads to an increase in mPGES-1 protein levels in cardiomyocytes (11), the role individual PGES enzymes play in cardiomyocyte PGE₂ biosynthesis has not been elucidated.

MAPK enzymes, including p38 MAPK, extracellular signal-regulated kinase (ERK), and JNK, have been shown to regulate the expression of PLA₂ and COX enzymes in cardiomyocytes (12, 13). JNK enzymes phosphorylate transcription factors, such as c-Jun, JunD, and ATF2, that participate in the formation and activation of the AP-1 complex (14), and an AP-1-binding site has been tentatively identified in the promoter of the Ptgase gene, which codes for mPGES-1 mRNA. Disruption of the c-Jun-JNK complex attenuates IL-1β-stimulated increases in mPGES-1 mRNA in gingival fibroblasts (15), and treatment with the pharmacologic JNK inhibitor, SP600125, attenuates cytokine-stimulated increases in mPGES-1 levels in rat neonatal cardiomyocytes (11). These observations are consistent with the notion that mPGES-1...
mRNA levels may be regulated through AP-1-stimulated increases in mPGES-1 mRNA transcription (16, 17). In contrast, the expression of some genes that code for prostaglandin biosynthetic enzymes, such as Cox-2, are partially regulated though stabilization of mRNA (13), and JNK enzymes have been shown to stabilize some RNA species (18). JNK family members include JNK1, JNK2, and JNK3, each of which has multiple isoforms that are generated through alternative splicing events (14). JNK1 and JNK2 have been identified in mouse cardiomyocytes (19). The role that individual JNK enzymes play in the regulation of the Ptges gene expression and the role of JNK enzymes in mPGES-1 mRNA transcription and mPGES-1 mRNA stabilization have not been elucidated.

In this study we show the following. 1) mPGES-1 mRNA is constitutively transcribed in neonatal cardiomyocytes. 2) IL-1β and LPS stimulate increases in neonatal cardiomyocyte mPGES-1 mRNA levels by stabilizing mPGES-1 mRNA, in a JNK-dependent fashion, but do not increase mPGES-1 mRNA transcription. 3) JNK1 or JNK2 can participate in the regulation of mPGES-1 protein synthesis in neonatal cardiomyocytes. 4) The majority of PGE2 production by LPS-stimulated neonatal cardiomyocytes is catalyzed by mPGES-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal antiserum specific for phospho-specific c-Jun (sc-7980) and protein G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum against phospho-specific ATF2 (catalog number 9221) and the kinase assay substrates GST-c-Jun and GST-ATF2 were from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal antiserum against JNK1/2 (catalog number 554285) was from Pharmingen. Horseradish peroxidase-linked anti-rabbit secondary antibody was from Pierce. Recombinant human IL-1β was from PeproTech Inc. (Rocky Hill, NJ). Taq polymerase was from MBI Fermentas (Burlington, Ontario, Canada), and mouse GAPDH cDNA was from Ambion (Austin, TX). The PGE2 ELISA kit was from Amersham Biosciences. Rabbit polyclonal primary antibodies raised against mPGES-1 (9) and mPGES-2 (21) were prepared as described previously. Rabbit polyclonal primary antibody raised against cPGES was kindly provided by Dr. M. Murakami and was prepared as described previously (8). The JNK inhibitor SP600125 was obtained from Calbiochem. LPS (from *Escherichia coli* 0111:B4) and all other reagents (analytical or tissue culture grade) were obtained from Sigma.

**Primary Cell Culture and Experimental Protocol**—Rat neonatal cardiomyocytes were isolated from the hearts of 1- to 2-day-old Sprague-Dawley rats (12). Mouse neonatal cardiomyocytes were isolated from 1- to 2-day-old *jnk1*−/−, *jnk1*+/−, *jnk2*−/−, *jnk2*+/−, *Ptges*−/−, or *Ptges*+/− mice using identical methodology. Cells were incubated with adenoviral vectors, as indicated in the figure legends, or with the JNK inhibitor SP600125 (10 µmol/liter) for 30 min, and then treated with vehicle, IL-1β (10 ng/ml), or LPS (5 µg/ml) for up to 32 h. When chemicals were dissolved in Me2SO or ethanol, the identical concentration of these solvents was added to controls. All experiments were done in triplicate and repeated 3–6 times. In all studies, cytotoxicity was assessed by monitoring the extracellular release of lactate dehydrogenase and creatine kinase. Studies in which the release of lactate dehydrogenase or creatine kinase exceeded >5% of total cellular activity were excluded from further analysis. In all studies, isolated cells had characteristic features of cardiomyocytes and beat spontaneously. The methodology used for RNA isolation and Northern and immunoblot analysis has been described (12, 13). All studies were approved by the Animal Care Committee of the Toronto General Hospital.

**Adenoviruses**—The preparation of recombinant adenoviruses (kindly provided by Dr. Jeffery D. Molkentin, University of Cincinnati, OH) encoding murine wild type JNK1 (ad-JNK1-WT), mutated phosphorylation-resistant JNK1 (ad-JNK1-DN), wild type JNK2 (ad-JNK2-WT), and mutated phosphorylation-resistant JNK2 (ad-JNK2-DN) was carried out as described previously (19). ad-JNK1-DN and ad-JNK2-DN express dominant negative proteins, in which the dual phosphorylation motif Thr-Pro-Tyr was converted to Ala-Pro-Phe (19). In all experiments using adenovirally mediated gene transfer, a multiplicity of infection of 25–50 plaque-forming units/cell was used, and cells were infected 24–48 h before stimulation. Viral titers were determined by the agarose gel overlay technique. Cell viability was routinely confirmed by trypan blue exclusion.

**Transgenic Mice**—JNK1 and JNK2 gene-targeted mice were kindly provided by Dr. Michael Karin, University of California, San Diego (23). Construction of the mPGES-1 gene-targeted mouse (*Ptges*+/−) has been reported (24). All adult mice were generated by cross-breeding heterozygous mice (*jnk1*+/−, *jnk2*+/−, or *Ptges*+/−). Homozygous gene-targeted mice and their wild type littermates were identified by tail DNA genotyping.

**Preparation of Total Cell Lysates of Cultured Rat and Mouse Cardiac Myocytes**—In selected studies, cells were lysed in ice-cold lysis buffer A (50 mmol/liter HEPES, pH 7.5, 150 mmol/liter NaCl, 1% Triton X-100, 10% glycerol, 10 mmol/liter MgCl2, 1 mmol/liter phenylmethylsulfonyl fluoride, 1 mmol/liter Na3VO4, 25 mmol/liter NaF, 0.5 mmol/liter nitrophenyl phosphate, 5 mmol/liter glycerophosphate, 2 mmol/liter EDTA, 2 mmol/liter EGTA, 10 µg/ml apronin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin). After 30 min, the cell lysates were passed through a 21-gauge needle and centrifuged at 14,000 × g for 10 min at 4 °C. Supernatants were stored at −20 °C for subsequent immunoblotting studies. The protein content of the supernatant was determined using the BCA protein kit (Pierce).

**Preparation of Microsomal and Cytosolic Fractions of Rat and Mouse Cardiomyocytes**—Cells were washed twice with phosphate-buffered saline and resuspended in 66 mm HEPES buffer, pH 7.5, 1 mm EDTA, 1 mm EGTA, 1 mm Na3VO4, 25 mm NaF, 1 mm diisopropylphosphofluoridate, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Thereafter, cells were sonicated on ice with three bursts (20% maximum power) of 15 s each and then centrifuged for 5 min at 14,000 × g at 4 °C to remove nuclei. The supernatant was then centrifuged at 150,000 × g for 20 min at 4 °C to resolve the cytosolic and microsomal fractions. The cytosolic fraction was decanted into a clean tube; the microsomal pellet was resuspended in 100 µl of ice-cold lysis buffer B. Protein content of the cytosolic and microsomal fractions was determined using the BCA protein kit (Pierce).

**JNK Immunoprecipitation and JNK Kinase Assay**—Cells were infected with ad-JNK1-WT, ad-JNK1-DN, ad-JNK2-WT, or ad-JNK2-DN and lysed as described above, and the JNK isoforms were immunoprecipitated by incubating with anti-JNK1/2 antibody for 16 h. After addition of protein G-agarose beads and incubation for 3 h at 4 °C, immunoprecipitated proteins were washed three times with buffer A and twice with kinase buffer (25 mmol/liter HEPES, pH 7.4, 25 mmol/liter glycerophosphate, 25 mmol/liter MgCl2, 0.5 mmol/liter dithiothreitol, and 0.1 mmol/liter Na3VO4). Kinase reactions were initiated by addition of 1 µg of GST-ATF2 or 1 µg of GST-c-Jun and 200 µmol/liter ATP in 40 µl of kinase buffer. After 30 min at 30 °C, reactions were terminated by addition of Laemmli buffer and boiling. Phosphorylation of ATF2 or c-Jun was evaluated by immunoblot analysis with an anti-phospho-ATF2 or an anti-phospho-c-Jun antibody.

**RNA Isolation and Northern Blot Analysis**—Total cellular RNA was extracted from rat or mouse cardiac myocytes or from mouse left ven-
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mPGES-1 mRNA Expression and Stability—To assess the role of mRNA stabilization in the expression of mPGES-1 mRNA transcripts, confluent rat neonatal cardiac myocytes were treated for 16 h with vehicle, LPS (5 μg/ml), or IL-1β (10 ng/ml). Cells were then treated with actinomycin D (5 μg/ml), which arrests transcription. In other experiments, cells were additionally treated with 0.1% MeSO or 10 μM SP600125, forward primer 5′-TGG TGG GTG TGA ACC ATG GAG AAG G-3′ and reverse primer 5′-TCC GCC ACC GTG TGT-3′, PCR product expected size of 115 bp; for rat GAPDH, forward primer 5′-CTG AAG GTG GAA GA-3′ and reverse primer 5′-GTC CCT AT-3′, PCR product expected size of 375 bp; for mouse mPGES-1 (GenBank™ accession number NM_022415), forward primer 5′-TGA TGG GTG TGA ACC AC-3′ and reverse primer 5′-GAT GT-3′, PCR product expected size of 111 bp. PCR products were sized by 10 min at 72 °C. The following primers were used in this study: for mouse mPGES-1 (GenBank™ accession number NM_022415), forward primer 5′-CTT TCT GCT CTG CAG CAC ACT-3′ and reverse primer 5′-GCG ATG GAG AAA CAG GAG AAC-3′, PCR product expected size of 378 bp; for mouse COX-2 (GenBank™ accession number NM_021198), forward primer 5′-CCA GAG CAG AGA GAT GAA AT-3′ and reverse primer 5′-TAC TGT AGG GTT AAT GTC AT-3′, PCR product expected size of 469 bp; for mouse cPGES (GenBank™ accession number NM_019766), forward primer 5′-CGA AGG GAC TAT GTA TTC ATT G-3′ and reverse primer 5′-ATC CTC ATC ACC CAT GT-3′, PCR product expected size of 375 bp; for mouse mPGES-2 (NM_133783), forward primer 5′-ACT TCC ACT CCC TGC CCT AT-3′ and reverse primer 5′-CAG GTA CCA AGG CTT GT-3′, PCR product expected size of 731 bp; for mouse GAPDH (GenBank™ accession number XM_214281), forward primer 5′-TTC TG(TGA TGG GTG TGA ACC ATG GAG AAG G-3′ and reverse primer 5′-TCC GCC ACC GTG TGT-3′, giving an expected size for the PCR product of 502 bp. All product levels were normalized to GAPDH levels. Reverse transcription-PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis, and specificities were confirmed by DNA sequence analysis with an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). The absence of genomic DNA in the RNA preparations was confirmed by performing PCR analysis of “minus-RT controls.”

Assessment of mPGES-1 mRNA Expression and Stability—To assess the role of mRNA stabilization in the expression of mPGES-1 mRNA transcripts, confluent rat neonatal cardiac myocytes were treated for 16 h with vehicle, LPS (5 μg/ml), or IL-1β (10 ng/ml). Cells were then treated with actinomycin D (5 μg/ml), which arrests transcription. In other experiments, cells were additionally treated with 0.1% MeSO or 10 μM SP600125, forward primer 5′-TGG TGG GTG TGA ACC ATG GAG AAG G-3′ and reverse primer 5′-TCC GCC ACC GTG TGT-3′, PCR product expected size of 115 bp; for rat GAPDH, forward primer 5′-CTG AAG GTG GAA GA-3′ and reverse primer 5′-GTC CCT AT-3′, PCR product expected size of 375 bp; for mouse mPGES-1 (GenBank™ accession number NM_022415), forward primer 5′-CTT TCT GCT CTG CAG CAC ACT-3′ and reverse primer 5′-GCG ATG GAG AAA CAG GAG AAC-3′, PCR product expected size of 378 bp; for mouse COX-2 (GenBank™ accession number NM_021198), forward primer 5′-CCA GAG CAG AGA GAT GAA AT-3′ and reverse primer 5′-TAC TGT AGG GTT AAT GTC AT-3′, PCR product expected size of 469 bp; for mouse cPGES (GenBank™ accession number NM_019766), forward primer 5′-CGA AGG GAC TAT GTA TTC ATT G-3′ and reverse primer 5′-ATC CTC ATC ACC CAT GT-3′, PCR product expected size of 375 bp; for mouse mPGES-2 (NM_133783), forward primer 5′-ACT TCC ACT CCC TGC CCT AT-3′ and reverse primer 5′-CAG GTA CCA AGG CTT GT-3′, PCR product expected size of 731 bp; for mouse GAPDH (GenBank™ accession number XM_214281), forward primer 5′-TTC TG(TGA TGG GTG TGA ACC ATG GAG AAG G-3′ and reverse primer 5′-TCC GCC ACC GTG TGT-3′, giving an expected size for the PCR product of 502 bp. All product levels were normalized to GAPDH levels. Reverse transcription-PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis, and specificities were confirmed by DNA sequence analysis with an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). The absence of genomic DNA in the RNA preparations was confirmed by performing PCR analysis of “minus-RT controls.”

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RT-PCR Analysis—After isolation, total RNA was treated with DNase I (RNase-free) and converted to cDNA mixtures exactly as described (13). Semi-quantitative PCR was performed in a 50-μl reaction containing 75 mmol/liter Tris-HCl, pH 8.8, 20 mmol/liter (NH4)2 SO4, 1.5 mmol/liter MgCl2, 0.01% Tween 20, dNTP mixture (0.2 mmol/liter each), 2 units of Taq polymerase (MBI Fermentas), and 0.4 μmol/liter of the specific primers. After 4 min at 95 °C, 28 cycles of amplification with a PTC-100 Thermal Cycler (MJ Research, Waltham, MA) was carried out as follows: 30 s at 95 °C, 1 min at 60 °C, and 45 s at 72 °C followed by 10 min at 72 °C. The following primers were used in this study: for mouse mPGES-1 (GenBank™ accession number NM_022415), forward primer 5′-CTT TCT GCT CTG CAG CAC ACT-3′ and reverse primer 5′-GCG ATG GAG AAA CAG GAG AAC-3′, PCR product expected size of 378 bp; for mouse COX-2 (GenBank™ accession number NM_021198), forward primer 5′-CCA GAG CAG AGA GAT GAA AT-3′ and reverse primer 5′-TAC TGT AGG GTT AAT GTC AT-3′, PCR product expected size of 469 bp; for mouse cPGES (GenBank™ accession number NM_019766), forward primer 5′-CGA AGG GAC TAT GTA TTC ATT G-3′ and reverse primer 5′-ATC CTC ATC ACC CAT GT-3′, PCR product expected size of 375 bp; for mouse mPGES-2 (NM_133783), forward primer 5′-ACT TCC ACT CCC TGC CCT AT-3′ and reverse primer 5′-CAG GTA CCA AGG CTT GT-3′, PCR product expected size of 731 bp; for mouse GAPDH (GenBank™ accession number XM_214281), forward primer 5′-TTC TG(TGA TGG GTG TGA ACC ATG GAG AAG G-3′ and reverse primer 5′-TCC GCC ACC GTG TGT-3′, giving an expected size for the PCR product of 502 bp. All product levels were normalized to GAPDH levels. Reverse transcription-PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis, and specificities were confirmed by DNA sequence analysis with an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). The absence of genomic DNA in the RNA preparations was confirmed by performing PCR analysis of “minus-RT controls.”
levels were determined by real time quantitative PCR and normalized to GAPDH cDNA levels, as described above.

Statistical Analysis—All results are expressed as the mean ± S.E. of 3–6 experiments, carried out in triplicate. Comparisons between groups were made by repeated measures analysis of variance, followed by post-hoc analysis with paired t tests, two-tailed, unequal variance. When multiple comparisons between groups were made, a Bonferroni correction was applied. A p value <0.05 was considered to be significant.

RESULTS

IL-1β and LPS Both Increase PGE₂ Release, PGES Activity, and Ptges Gene Expression in Cardiomyocytes—Treatment with IL-1β or LPS increased PGE₂ release by primary cultures of rat neonatal cardiomyocytes in comparison with vehicle-treated cells (Fig. 1A). PGE₂ release by cardiomyocytes was delayed, as no PGE₂ was detected after 8 h of exposure to IL-1β or LPS, whereas significant PGE₂ release was identified after exposure to IL-1β or LPS for 24 or 32 h. Treatment of cultured cardiomyocytes with IL-1β or LPS significantly increased the rate of conversion of PGH₂ to PGE₂ in vitro by microsomes prepared from these cells in comparison with vehicle-treated cells (Fig. 1B), thereby documenting an IL-1β- or LPS-stimulated increase in microsomal PGES synthase activity. In contrast, PGE₂ synthase activity was not detected in cytosolic fractions of cardiomyocytes, despite the presence of cPGES, verified by Western blot analysis (data not shown).

The effect of IL-1β or LPS on mPGES-1 mRNA expression, as well as mPGES-1, mPGES-2, and cPGES protein levels was then assessed. Quantitative PCR analysis documented the presence of mPGES-1 mRNA in preparations of unstimulated rat neonatal cardiomyocytes, with ~4 × 10⁴ copies of mPGES-1 mRNA/10⁶-plated cells. Treatment with IL-1β or LPS for 2 h stimulated an increase in mPGES-1 mRNA expression in cardiomyocytes, and continuous exposure to IL-1β or LPS for 24 h led to ~9-fold increases in mPGES-1 mRNA levels in these cells (Fig. 1C). mPGES-1 protein was not identified in untreated cardiomyocytes (Fig. 1D). Exposure to IL-1β or LPS led to mPGES-1 protein expression in cardiomyocytes after a lag time of 8 h (Fig. 1D). Therefore, the increase in mPGES-1 protein expression correlated with the increase in microsomal PGES synthase activity in IL-1β- or LPS-treated cardiomyocytes. mPGES-2 and cPGES proteins were identified in untreated cells. In contrast to mPGES-1, exposure to IL-1β or LPS had minimal effect on mPGES-2 or cPGES protein levels in cardiomyocytes (Fig. 1, E and F). The low levels of PGE₂ synthase activity detected in control microsomes may originate from mPGES-2 or the nonenzymatic degradation of PGH₂. Taken together, these data show that exposure to IL-1β or LPS led to a selective but delayed increase in mPGES-1 protein synthesis and PGE₂ release by neonatal cardiomyocytes.

IL-1β and LPS Both Increase mPGES-1 mRNA Stability but Do Not Increase mPGES-1 mRNA Transcription—To determine whether treatment of cardiomyocytes with IL-1β or LPS increases RNA pol II recruitment to the mPGES-1 proximal promoter, ChIP was performed using an RNA pol II-specific antibody. Loading of RNA pol II onto the mPGES-1 proximal promoter was similar in vehicle-, IL-1β-, and LPS-treated cells (Fig. 2A). mPGES-1 mRNA transcription, assessed by nuclear run-off assay (13), was also detected at base line and was similar in vehicle-, IL-1β-, and LPS-treated cardiomyocytes (Fig. 2B). In contrast, IL-1β and LPS both increased COX-2 mRNA transcription (Fig. 2B), as described previously (13). We take the results of the RNA pol II binding and nuclear run-off assays to indicate that, at base line, the Ptges gene is transcriptionally active. Basal mPGES-1 mRNA transcription does not lead to accumulation of mPGES-1 mRNA in cardiomyocytes at steady state. Furthermore, treatment with IL-1β or LPS does not increase mPGES-1 mRNA transcription in these cells.

To assess the stability of mPGES-1 mRNA in vehicle-, IL-1β-, or LPS-treated cardiomyocytes, cells were incubated with vehicle, IL-1β,
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mPGES-1 mRNA Stability and PGE2 Release by Cardiomyocytes—Treatment with IL-1β or LPS increases mPGES-1 mRNA stability in cardiomyocytes (11, 27). To define the potential role of JNK activation in the regulation of Ptges gene expression, cardiomyocytes were treated with vehicle or the JNK inhibitor SP600125 (28) prior to exposure to IL-1β or LPS. Treatment with SP600125 attenuated IL-1β- or LPS-induced increases in mPGES-1 mRNA expression, mPGES-1 protein levels, and PGE_2 release by neonatal cardiomyocytes, a finding consistent with the results of a previous study (11), but had no effect on mPGES-2 protein levels in these cells (Fig. 3, A–E).

Preincubation with SP600125 had no effect on in vitro mPGES-1 mRNA transcription by nuclei isolated from vehicle-, IL-1β-, or LPS-treated cardiomyocytes, as assessed by nuclear run-off assay (Fig. 3F). In contrast, preincubation with SP600125 decreased the stability of mPGES-1 mRNA in both IL-1β- (Fig. 3G) and LPS (Fig. 3H)-treated cardiomyocytes, such that the half-life of mPGES-1 mRNA in cardiomyocytes treated with vehicle alone, SP600125 alone, or SP600125 plus IL-1β or LPS was similar (~3.5–4 h). These results are consistent with the notion that JNK activity is necessary for IL-1β- or LPS-induced increases in mPGES-1 mRNA stability and PGE_2 release by rat neonatal cardiomyocytes. The failure of treatment with SP600125 to attenuate mPGES-1 mRNA transcription (Fig. 3F) suggests that JNK-mediated AP-1 activation does not play an important role in the regulation of Ptges gene expression in neonatal cardiomyocytes.

JNK1 or JNK2 Activity Is Necessary for LPS-induced mPGES-1 Protein Synthesis in Mouse Neonatal Cardiomyocytes in Vitro—To determine which JNK isoform(s) regulate expression of the gene that encodes mPGES-1 mRNA expression in cardiomyocytes in vitro, primary cultures of neonatal cardiomyocytes from jnk1−/− mice were infected with a control adenovirus (ad-GFP) or adenoviruses that encode wild type JNK2 or phosphorylation-resistant (dominant negative) JNK2 (ad-JNK2-WT and ad-JNK2-DN, respectively). As shown in Fig. 4A, mPGES-1 protein was not identified in cardiomyocytes from jnk1−/− mice after infection with ad-GFP, ad-JNK2-WT, or ad-JNK2-DN. Treatment with ad-JNK2-WT increased cellular c-Jun and ATF2 phosphorylation, indices of JNK activity, but had no effect on mPGES-1 protein levels in these cells. Thus JNK2 activation, in the absence of LPS, is not sufficient to induce mPGES-1 protein synthesis in jnk1−/− cardiomyocytes. Treatment with LPS increased mPGES-1 protein levels in cardiomyocytes from jnk1−/− mice, thereby demonstrating that JNK1 is not necessary for LPS-induced mPGES-1 protein synthesis in these cells. The increase in mPGES-1 protein induced by LPS in cardiomyocytes from jnk1−/− mice was attenuated by infection with ad-JNK2-DN, but not ad-JNK2-WT, thereby suggesting a role for JNK2 in LPS-induced mPGES-1 protein synthesis in cells from jnk1−/− mice.

mPGES-1 protein was not identified in cardiomyocytes from jnk2−/− mice after infection with ad-GFP, ad-JNK1-WT, or ad-JNK1-DN (Fig. 4B). Treatment with ad-JNK1-WT increased c-Jun and ATF2 phosphorylation in these cells but had no effect on mPGES-1 protein levels, so JNK1 activation in the absence of LPS is not sufficient to induce mPGES-1 protein synthesis in jnk2−/− cardiomyocytes. mPGES-1 protein levels increased in cardiomyocytes from jnk2−/− mice after treatment with LPS. Therefore, JNK2 is not necessary for LPS-induced mPGES-1 protein synthesis in these cells (Fig. 4B). The LPS-induced increase in mPGES-1 protein levels in cardiomyocytes from jnk2−/−

or LPS for 16 h and then exposed to actinomycin D, which arrests transcription (13). The half-life of mPGES-1 mRNA in untreated cardiomyocytes was ~3 h (Fig. 2C). In contrast, treatment with IL-1β or LPS increased the half-life of mPGES-1 mRNA in cardiomyocytes to >6 h (Fig. 2C). Importantly, exposure to IL-1β or LPS for 4 h increased mPGES-1 mRNA levels 4- and 6.3-fold, respectively (Fig. 1C), but had no effect on mPGES-1 mRNA transcription, which remained at baseline levels (Fig. 2, A and B). Taken together, these results demonstrate that, in rat neonatal cardiomyocytes, IL-1β or LPS induced increases in mPGES-1 mRNA levels by increasing the stability of mPGES-1 mRNA.

mPGES-1 mRNA levels 4- and 6.3-fold, respectively (Fig. 1).

FIGURE 2. IL-1β and LPS both increase the stability of mPGES-1 mRNA but have no effect on mPGES-1 mRNA transcription. A, cells were treated with 0.1% Me2SO (control), 10 ng/ml IL-1β, or 5 ng/ml LPS for 4 h. The number of copies of the mPGES-1 promoter immunoprecipitated by the RNA pol II antibody was determined by real time quantitative PCR. Immunoprecipitated DNA was determined by subtracting the number of copies of mPGES-1 promoter bound in a no antibody control immunoprecipitation and dividing by a diluted input sample to normalize for starting chromatin between samples. Results from one of three representative experiments, performed in triplicate, are shown. B, cells were treated with 0.1% Me2SO, 10 ng/ml IL-1β or 5 ng/ml LPS for 4 h, and the transcription of mPGES-1, COX-2 and GAPDH mRNA was assessed by nuclear run-off assay. Representative blots from three independent experiments are shown. C, cells were incubated with 0.1% Me2SO (filled squares), 10 ng/ml IL-1β (open squares), or 5 ng/ml LPS (filled triangles) for 16 h and then incubated with 5 μg/ml actinomycin D for up to 8 h, followed by quantitative real-time PCR analysis for mPGES-1 and GAPDH mRNA levels. The mPGES-1 mRNA/GAPDH mRNA ratio is reported. ANOVA, p < 10−10 for the effect of group (vehicle, IL-1β or LPS) on mPGES-1 mRNA/GAPDH mRNA levels. *, p < 0.01, IL-1β versus vehicle; **, p < 0.01, LPS versus vehicle at each time point. The means ± S.E. of four independent experiments are shown.
FIGURE 3. JNK enzymes regulate mPGES-1 mRNA stability, mPGES-1 mRNA and protein levels, and PGE₂ release in neonatal cardiomyocytes. Cells were pretreated with 0.1% Me₂SO or 10 μM SP600125 and then incubated with 0.1% Me₂SO, 10 ng/ml IL-1β, or 5 ng/ml LPS for 24 h, as indicated. A, mPGES-1 mRNA; B, GAPDH mRNA levels, Northern blot analysis. C, mPGES-1 and D, mPGES-2 protein levels, immunoblot analysis. Representative blots from five independent experiments are shown, and the corresponding densitometric analysis. E, PGE₂ (pg/ml) levels, nuclear run-off assay. F, pcDNA, mPGES-1, GAPDH, COX-2 expression levels, immunoblot analysis. G, mPGES-1 mRNA levels (percent of baseline), H, mPGES-1 mRNA levels (percent of baseline), Western blot analysis.
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A, neonatal cardiomyocytes from jnk1−/− mice were infected with adenoviruses encoding GFP, wild type, or phosphorylation-resistant JNK2 (ad-GFP, ad-JNK2-WT, and ad-JNK2-DN, respectively). B, neonatal cardiomyocytes from jnk2−/− mice were infected with ad-GFP, ad-JNK1-WT, or ad-JNK1-DN. After 24 h, cells were treated with vehicle or 5 ng/ml LPS for 16 h. Cellular mPGES-1 and mPGES-2 proteins (immunoblot), as well as c-Jun and ATF2 phosphorylation (immunoprecipitation followed by immunoblot with antibodies directed against phospho-c-Jun or phospho-ATF2), were assessed. C, neonatal cardiomyocytes from wild type mice were infected with ad-GFP, ad-JNK1-WT, and ad-JNK2-WT, or ad-JNK1-DN and ad-JNK2-DN, and then treated with vehicle or 5 ng/ml LPS for 16 h. mPGES-1 and mPGES-2 levels were assessed by densitometric analysis of mPGES-1 and mPGES-2 protein levels in these independent experiments is presented below A and B and adjacent to C.* p < 0.01, LPS versus vehicle; ** p < 0.01, ad-GFP + LPS versus ad-JNK2-DN + LPS (A), ad-GFP + LPS versus ad-JNK1-DN + LPS (B), ad-GFP + LPS versus ad-JNK1-DN + ad-JNK2-DN + LPS (C).

Together, these results provide direct evidence that either JNK1 or JNK2 can participate in the signaling cascade that regulates LPS-induced mPGES-1 protein synthesis in mouse neonatal cardiomyocytes.

To further assess the roles of JNK1 and JNK2 in mPGES-1 protein synthesis in cardiomyocytes, cells from wild type mice were simultaneously infected with either ad-JNK1-WT and ad-JNK2-WT, with ad-JNK1-DN and ad-JNK2-DN, or with ad-GFP and were then treated with vehicle or LPS. mPGES-1 protein was not identified in vehicle-treated cells infected with ad-GFP, ad-JNK1-WT, and ad-JNK2-WT or ad-JNK1-DN and ad-JNK2-DN (Fig. 4C). After exposure to LPS, mPGES-1 protein levels were similar in cardiomyocytes infected with ad-GFP or with ad-JNK1-DN and ad-JNK2-DN significantly decreased LPS-stimulated mPGES-1 protein synthesis in these cells. Simultaneous infection with adenoviruses encoding wild type or dominant negative JNK1 or JNK2 had no effect on mPGES-2 protein levels in LPS-treated cells (Fig. 4C). Taken together, these results provide direct evidence that either JNK1 or JNK2 can participate in the signaling cascade that regulates LPS-induced mPGES-1 protein synthesis in mouse neonatal cardiomyocytes.

Analysis of mPGES-1 mRNA and mPGES-1 protein levels in these independent experiments is presented adjacent to the immunoblots in A and C. E. PGE2 release (ELISA) after exposure to vehicle (open bars), 10 ng/ml IL-1β (closed bars, left panel), or 5 ng/ml LPS (closed bars, right panel); pretreatment with 10 μM SP600125. The means ± S.E. of four independent experiments are shown, * p < 0.01, IL-1β or LPS versus vehicle; ** p < 0.01, IL-1β or LPS versus IL-1β or LPS + SP600125. F. Nuclear run-off analysis of mPGES-1, COX-2, and GAPDH mRNA with pretreatment with 10 μM SP600125 and incubation with 1% Me2SO, 10 ng/ml IL-1β, or 5 ng/ml LPS. Representative results from three independent experiments are shown. Cardiomyocytes were incubated with 1% Me2SO (open circles), 10 μM SP600125 (closed circles), 10 ng/ml IL-1β (open squares), or 10 ng/ml IL-1β + 10 μM SP600125 (closed squares) (G), or 0.1% Me2SO (open circles), 10 μM SP600125 (closed circles), 5 ng/ml LPS (open squares), or 5 ng/ml LPS + 10 μM SP600125 (closed squares) (H) for 16 h and then incubated with 5 μg/ml actinomycin D for up to 8 h, followed by quantitative real-time PCR analysis for mPGES-1 and GAPDH mRNA levels. ANOVA, F = 10−15 for the effect of group (vehicle, SP600125, IL-1β, or vehicle + SP600125 + IL-1β); or vehicle, SP600125, LPS, or SP600125 + LPS on mPGES-1 mRNA/GAPDH mRNA levels. * p < 0.01, IL-1β versus vehicle, SP600125, or IL-1β + SP600125 (G); ** p < 0.01, LPS versus vehicle, SP600125, or LPS + SP600125 (H) at each time point. The means ± S.E. of four independent experiments are shown.
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Figure 5. JNK1 or JNK2 can participate in the signaling cascade that regulates LPS-induced mPGES-1 protein synthesis in mouse myocardium in vivo. Adult jnk1+/-, jnk1+/−/jnk2+/-, and jnk2−/− mice were injected intraperitoneally with saline (open bars) or LPS (closed bars). After 24 h, the ratio of mPGES-1 mRNA to GAPDH mRNA in left ventricular tissue was assessed by quantitative RT-PCR. ANOVA, p < 0.0005 for the effect of group (jnk1−/+ + vehicle, jnk1−/+ + LPS, jnk1−/+ + vehicle, and jnk1−/+ + LPS) on the ratio of mPGES-1 mRNA to GAPDH mRNA. ANOVA, p < 0.02 for the effect of group (jnk2−/- + vehicle, jnk2−/- + LPS, jnk2−/- + vehicle, and jnk2−/- + LPS) on the ratio of mPGES-1 mRNA to GAPDH mRNA. *p < 0.05, vehicle versus LPS. The means ± S.E. of five independent experiments are shown.

Effect of Ptges Gene Disruption on PG Biosynthetic Enzyme Gene Expression and PGE2 Release by Neonatal Cardiomyocytes.—To begin to explore the role of mPGES-1 in cardiomyocyte eicosanoid biosynthesis, myocytes from neonatal Ptges+/+ and Ptges−/− mice were isolated and exposed to vehicle or LPS for 24 h. mPGES-1 mRNA was identified in vehicle-treated mouse neonatal cardiomyocytes (Fig. 6A). In comparison with vehicle-treated cells, LPS stimulated an increase in mPGES-1 mRNA levels in cardiomyocytes from Ptges−/− mice, measured by semi-quantitative RT-PCR (Fig. 6A). As expected, mPGES-1 mRNA was not identified in cardiomyocytes from Ptges−/− mice. COX-2 mRNA was not identified in vehicle-treated cardiomyocytes from Ptges−/− or Ptges−/− mice. Exposure to LPS led to increased mPGES-2 mRNA levels in cardiomyocytes from Ptges+/+ and Ptges−/− mice. COX-1, mPGES-2, cPGES, and GAPDH mRNA levels were similar in both vehicle- and LPS-treated cardiomyocytes from Ptges+/+ mice (Fig. 6A).

The biosynthesis of PGE2 by vehicle-treated cardiomyocytes from Ptges+/+ and Ptges−/− mice was similar (Fig. 6B). Exposure to LPS led to a significant increase in PGE2 release by cardiomyocytes from both Ptges+/+ and Ptges−/− mice (Fig. 6B) in comparison with vehicle-treated cells. However, the PGE2 release induced by LPS was significantly greater in cardiomyocytes from Ptges+/+ mice than from Ptges−/− mice (54.3 ± 85 versus 169 ± 90 pg/ml, p = 0.04, unpaired t test, two-tailed, unequal variance). These experiments provide direct evidence that mPGES-1 catalyzes the majority of the PGE2 release induced by LPS in neonatal cardiomyocytes. The increase in PGE2 formation by LPS-treated cardiomyocytes from Ptges−/− mice, in comparison with PGE2 formation by vehicle-treated cells from Ptges−/− mice, may be the result of the nonenzymatic breakdown of PGH2 produced by COX-2. Alternatively, in Ptges−/− cardiomyocytes, mPGES-2 may catalyze PGE2 formation from PGH2 generated by COX-2.

Discussion

PGE2 regulates multiple biologically relevant functions in the normal and diseased heart (1–3). mPGES-1 has been shown to regulate PGE2 biosynthesis in many tissues and cells, and increased mPGES-1 mRNA and protein levels have been documented in cytokine-treated cardiomyocytes (11). The molecular mechanisms that regulate mPGES-1 mRNA expression, mPGES-1 protein synthesis, and PGE2 production in cardiomyocytes have not been completely defined.

In this study, we present four independent lines of evidence that suggest a role for JNK enzymes in the regulation of mPGES-1 mRNA expression and protein synthesis in cardiomyocytes. First, treatment with the JNK inhibitor SP600125 attenuated IL-1β- and LPS-induced mPGES-1 mRNA and protein synthesis in cardiomyocytes. These results must be interpreted with caution, as SP600125 has been shown to inhibit multiple kinases at concentrations approximating the IC50 for inhibition of JNK activity (30). Second, infection of cardiomyocytes from jnk1−/− mice with an adenovirus encoding phosphorylation-resistant JNK2 (ad-JNK2-DN) abolished cellular JNK activity, as demonstrated by the lack of c-Jun and ATF2 phosphorylation, and decreased LPS-induced mPGES-1 protein synthesis by 78% (Fig. 4A). Third, infection of cardiomyocytes from jnk2−/− mice with ad-JNK1-DN also abolished cellular JNK activity and was associated with a 65% decrease in mPGES-1 protein synthesis induced by LPS (Fig. 4B). Fourth, infection of cardiomyocytes from wild type mice with ad-JNK1-DN and ad-JNK2-DN decreased LPS-induced mPGES-1 protein synthesis in these cells by 73% (Fig. 4C). Taken together, these results provide direct evidence that JNK1 or JNK2 can participate in the regulation of mPGES-1 protein synthesis in neonatal cardiomyocytes. Importantly, mPGES-1 protein synthesis was reduced, but not eliminated, under experimental conditions where JNK activity was attenuated (Fig. 3A and Fig. 4, A–C). Multiple MAPK enzymes (11) and transcription factors (17, 31) have been implicated in the regulation of mPGES-1 mRNA expression and mPGES-1 protein synthesis. Therefore, some or all of these pathways may have been functioning under conditions where JNK
activity was inhibited and may have enabled LPS-stimulated mPGES-1 protein synthesis in the absence of JNK activity.

In murine fibroblasts, most of the cellular JNK activity is attributable to JNK1. In contrast, JNK2 regulates the stability of its downstream target, c-Jun, and only makes a small contribution to overall fibroblast JNK activity (32). Therefore, the molecular mechanisms through which JNK1 and JNK2 participate in LPS-stimulated Ptges gene expression in cardiomyocytes may differ, despite the fact that either JNK1 or JNK2 can support Ptges gene expression in these cells. Redundant JNK1 and JNK2 signaling has also been observed in T cell activation and apoptosis (23).

Exposure to IL-1β or LPS increases the luciferase activity of mPGES-1 promoter-luciferase reporter constructs (16, 17), a finding consistent with the hypothesis that pro-inflammatory mediators stimulate mPGES-1 mRNA transcription. In contrast, we found that two independent indices of mPGES-1 mRNA transcription, RNA pol II binding to the proximal mPGES-1 promoter (measured by ChIP and independent indices of mPGES-1 mRNA transcription, RNA pol II and LPS-treated cells. The explanation for the apparent discrepancy between our results and studies reporting transcriptional regulation of mPGES-1 mRNA levels is unclear but may be due to the fact that a segment of the promoter region, rather than the full gene encoding mPGES-1 mRNA, was evaluated in the latter studies. In addition, our studies and those identifying transcriptional regulation of mPGES-1 mRNA levels (16, 17) were carried out in different cell types. The biological relevance of our studies is confirmed by the fact that we used assays to study mPGES-1 mRNA expression that assessed the function of the endogenous locus (i.e. chromosome based) rather than binding to transfected plasmids (16, 17).

The experiments presented herein demonstrate that IL-1β and LPS both increase mPGES-1 mRNA levels in cardiomyocytes through stabilization of constitutively transcribed mPGES-1 mRNA. To our knowledge, these data constitute the first demonstration of a role for mRNA stabilization in the regulation of Ptges gene expression in any cell type. The time required to increase mPGES-1 mRNA levels through mRNA stabilization may explain the relatively long lag phase (at least 8 h) between exposure to pro-inflammatory stimuli and increases in mPGES-1 protein synthesis and PGE₂ release by cardiomyocytes (Fig. 1) and other cells (33, 34). The purpose of the relatively slow induction of mPGES-1 mRNA expression, in contrast to the rapid induction of COX-2 mRNA expression and protein synthesis in cytokine-treated cardiomyocytes (13), may be to delay the onset of PGE₂ biosynthesis in comparison with the biosynthesis of other PG.

The stability of mRNA is regulated by cis-acting elements that are present in the 5'-untranslated, coding, and 3'-untranslated regions of mRNAs (35). Adenosine- or uridine-rich elements, especially in the 3'-untranslated region of mRNAs, have been implicated in the destabilization of mRNAs and are frequently characterized by the pentanucleotide AUUUA (36). Mouse mPGES-1 mRNA does not contain AUUUA motifs but does contain two AUUUUA motifs (positions 1015–1021 and 2353–2359) and one AUUUA motif (positions 2106–2111) in the 3'-untranslated region of the mRNA. Identical AUUUUA and AUUUA motifs have been shown to destabilize IL-6 mRNA (37) and may therefore decrease the stability of mPGES-1 mRNA. Our demonstration that the increase in mPGES-1 mRNA stability induced by IL-1β or LPS is decreased to base-line levels in cardiomyocytes pre-treated with SP600125 is consistent with the notion that JNK enzymes act directly on mPGES-1 mRNA or act on intermediate mediators such as nucleolin and YB-1, which bind to the JNK-response element in the 5'-untranslated region of IL-2 transcripts and stabilize this mRNA (18). The trans-acting factors and mechanism through which JNK enzymes stabilize mPGES-1 mRNA in cardiomyocytes remains to be defined. In addition to regulating mPGES-1 mRNA stability, JNK enzymes may also participate in the regulation of mPGES-1 mRNA translation and/or the regulation of mPGES-1 protein stability, as JNK enzymes have been shown to stabilize many proteins (20, 22, 38, 39).

Recently, deletion of the PGE₂ receptor EP4 was shown to augment myocardial ischemia/reperfusion injury in a murine model of left anterior descending coronary artery occlusion and reperfusion (29). We show herein that targeted deletion of the gene encoding mPGES-1 results in a 69% decrease in PGE₂ biosynthesis by LPS-treated cardiomyocytes (cf. Fig. 6B). Therefore, evaluation of new drugs targeting mPGES-1-mediated PGE₂ biosynthesis should include consideration of putative deleterious effects on the heart after cardiac ischemic events.

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