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RESEARCH ARTICLE | *Metabolism, Cell Signaling and Disease*

FoxO1 regulates myocardial glucose oxidation rates via transcriptional control of pyruvate dehydrogenase kinase 4 expression

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Gopal K, Saleme B, Al Batran R, Aburasayn H, Eshreif A, Ho KL, Ma WK, Almutairi M, Eaton F, Gandhi M, Park EA, Sutendra G, Ussher JR. FoxO1 regulates myocardial glucose oxidation rates via transcriptional control of pyruvate dehydrogenase kinase 4 expression. *Am J Physiol Heart Circ Physiol* 313: H479–H490, 2017. First published July 7, 2017; doi:10.1152/ajpheart.00191.2017.—Pyruvate dehydrogenase (PDH) is the rate-limiting enzyme for glucose oxidation and a critical regulator of metabolic flexibility during the fasting to feeding transition. PDH is regulated via both PDH kinases (PDHK) and PDH phosphatases, which phosphorylate/inactivate and dephosphorylate/activate PDH, respectively. Our goal was to determine whether the transcription factor forkhead box O1 (FoxO1) regulates PDH activity and glucose oxidation in the heart via increasing the expression of *Pdk4*, the gene encoding PDHK4. To address this question, we differentiated H9c2 myoblasts into cardiac myocytes and modulated FoxO1 activity, after which *Pdk4*/PDHK4 expression and PDH phosphorylation/activity were assessed. We assessed binding of FoxO1 to the *Pdk4* promoter in cardiac myocytes in conjunction with measuring the role of FoxO1 on glucose oxidation in the isolated working heart. Both pharmacological (1 μM AS1842856) and genetic (siRNA mediated) inhibition of FoxO1 decreased *Pdk4*/PDHK4 expression and subsequent PDH phosphorylation in H9c2 cardiac myocytes, whereas 10 μM dexamethasone-induced *Pdk4*/PDHK4 expression was abolished via pretreatment with 1 μM AS1842856. Furthermore, transfection of H9c2 cardiac myocytes with a vector expressing FoxO1 increased luciferase activity driven by a *Pdk4* promoter construct containing the FoxO1 DNA-binding element region, but not in a *Pdk4* promoter construct lacking this region. Finally, AS1842856 treatment in fasted mice enhanced glucose oxidation rates during aerobic isolated working heart perfusions. Taken together, FoxO1 directly regulates *Pdk4* transcription in the heart, thereby controlling PDH activity and subsequent glucose oxidation rates.

NEW & NOTEWORTHY Although studies have shown an association between FoxO1 activity and pyruvate dehydrogenase kinase 4 expression, our study demonstrated that pyruvate dehydrogenase kinase 4 is a direct transcriptional target of FoxO1 (but not FoxO3/FoxO4) in the heart. Furthermore, we report here, for the first time, that FoxO1 inhibition increases glucose oxidation in the isolated working mouse heart.

forkhead box protein O1; pyruvate dehydrogenase; pyruvate dehydrogenase kinase; glucose oxidation; heart

TO ENSURE SUSTENANCE, the heart must continually pump blood throughout the body to support the functions of numerous bodily organs. Thus, the heart must constantly use and generate ATP at a tremendous rate. In the healthy adult heart, virtually all ATP production is derived from mitochondrial oxidative metabolism (~90%), with the remainder arising primarily from substrate-level phosphorylation during glycolysis (11, 21). The majority of myocardial mitochondrial oxidative metabolism is achieved via oxidation of fatty acids and carbohydrates (i.e., glucose and lactate), although other energy sources, including amino acids and ketone bodies, can also be oxidized by the heart, depending on nutritional and hormonal status, as well as their circulating concentrations (16, 21).

Glucose oxidation is a major contributor to overall cardiac metabolism, especially after meal intake, where both circulating glucose and insulin levels rise (11, 21). Furthermore, a number of studies have demonstrated that myocardial glucose oxidation is perturbed during the progression of a variety of cardiovascular diseases, including ischemic heart disease, diabetic cardiomyopathy, and heart failure (11, 39). Intriguingly, interventions that promote glucose oxidation have been shown to improve the pathophysiology of these cardiovascular diseases, which are potentially due to enhancing cardiac efficiency, as glucose is the more efficient fuel compared with fatty acids such as oleate and palmitate with regard to ATP produced per mole of oxygen consumed (18, 34, 35).

Pyruvate dehydrogenase (PDH) is the rate-limiting enzyme for glucose oxidation, and a number of studies have shown that impaired PDH activity is a key contributor to the low glucose oxidation rates observed in the diseased myocardium (11). Indeed, obesity-induced cardiac inefficiency and insulin resistance lead to marked declines in PDH activity and insulin-stimulated glucose oxidation rates (34, 37). Likewise, experimental models of diastolic heart failure are associated with a reduction in glucose oxidation rates during aerobic perfusion of

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the isolated working heart (19). PDH is inactivated after phosphorylation via upstream PDH kinases (PDHK), of which there are four isoforms, with PDHK1/PDHK2/PDHK4 being highly expressed but PDHK3 minimally expressed in the heart (24). As glucose and fatty acids compete for entry into the tricarboxylic acid cycle and subsequent oxidative metabolism through a substrate cycle identified by Randle et al. and referred to as the Randle cycle, physiological states associated with increased fatty acid oxidation often result in increased PDHK4 expression and reduced PDH activity and subsequent glucose oxidation rates (11). This is most readily apparent during fasting/prolonged starvation, where circulating free fatty acids (FFAs) and FFA delivery to the heart are increased, which elevates myocardial fatty acid oxidation and leads to a corresponding decrease in glucose oxidation (11). In the setting of obesity, circulating FFAs are also markedly elevated, resulting in a myocardial metabolic profile that mimics that observed in the heart during fasting/prolonged starvation (11, 16). Hence, there is great interest in understanding how PDHK4 expression and activity are regulated, as this may lead to the discovery of novel targets that can be manipulated to increase glucose oxidation rates and attenuate the development of cardiovascular diseases, such as cardiomyopathy resulting from obesity and/or type 2 diabetes (T2D) (39).

In our current understanding, a number of transcription factors that regulate various aspects of intermediary energy metabolism control expression of various *Pdk* isoforms, including peroxisome proliferator-activated receptor (PPAR)-α, hypoxia-inducible factor-1α, estrogen-related receptor-α, and forkhead box O1 (FoxO1) protein (13, 14, 36). Of these, we focused on the role of FoxO1 in mediating *Pdk4* expression and subsequent PDH inactivation in the heart. FoxO1 has been shown to play a key role in the pathophysiology of obesity-induced cardiac insulin resistance (1, 30). Furthermore, FOXO1 mRNA expression was increased in vastus lateralis muscles of humans fed a high-fat diet for 2 days, which corresponded more tightly with PDK4 mRNA expression than PPARA mRNA expression (6). It has also been demonstrated that experimental cardiomyopathy in mice with a cardiac-specific deletion of *Foxo1* is attenuated, and this is associated with a reduction in myocardial Pdk4 mRNA expression (1). Hence, improving our understanding of how FoxO1 regulates Pdk4 mRNA expression and subsequent inactivation of PDH may lead to the development of novel therapies for diabetic cardiomyopathy, a condition with no specific therapies currently approved for use in humans.

METHODS

Animal care. All animals received care in accordance with the regulations of the Canadian Council on Animal Care and with the approval of the University of Alberta Health Sciences Animal Welfare Committee. C57BL/6J mice (The Jackson Laboratory) were bred in our animal facility, and 12-wk-old female offspring were used for experimentation. Female mice were fasted for either 20 or 16 h followed by a 4-h refeeding period. A separate group of animals was treated with dexamethasone (100 mg/kg) 1 h before the 4-h refeeding period or with the FoxO1 antagonist AS1842856 (100 mg/kg via oral gavage) during either a 20- or 16-h fast followed by a 4-h refeed. At the end of the fast or refeed, animals were euthanized via an intraperitoneal injection of pentobarbital sodium (12 mg), and tissues were excised and immediately frozen in liquid N_2 for biochemical analyses.

Cell culture. All reagents were obtained from Sigma. H9c2 ventricular myoblasts (American Type Culture Collection) were cultured in six-well plates in DMEM containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. Cells were incubated in a water-jacketed CO_2 incubator maintained at 37°C with 95% O_2-5% CO_2 (vol/vol). Upon confluence, H9c2 myoblasts were differentiated into cardiac myocytes via growth in DMEM containing 1% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin, and 10 nM retinoic acid, as previously described (33). After 1 wk of differentiation, H9c2 cardiac myocytes were treated with either 10 μM dexamethasone to activate FoxO1 or 100 nM angiotensin II (ANG II) to induce cellular injury, or transfected with scramble, Foxo1, or Pdk4 siRNA (Qiagen) for 48 h using Lipofectamine RNAiMAX (Thermo Scientific) as per the manufacturer's instructions.

Western blot analysis. Frozen myocardial tissue (20 mg) or differentiated H9c2 cardiac myocytes were homogenized in buffer containing 50 mM Tris·HCl (pH 8 at 4°C), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (wt/vol) Brij-35, 1 mM DTT, and protease and phosphatase inhibitors (Sigma). Protein samples were prepared and subjected to Western blot protocols, as previously described (31).

Real-time PCR analysis. First-strand cDNA was synthesized from total RNA using the SuperScript III synthesis system (Invitrogen, Carlsbad, CA). Real-time PCR was carried out with the CFX connect real-time PCR machine (Bio-Rad Laboratories) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative mRNA transcript levels were quantified with the $2^{-\Delta\Delta C_T}$ method (where C_T is threshold cycle) (15), using cyclophilin as our housekeeping internal control gene.

PDH activity assay. PDH activity was measured in protein extracted from differentiated H9c2 cardiac myocytes (50 μl of 1 μg/ml) using the MitoProfile Dipstick Assay Kit (MitoSciences, Eugene, OR), as previously described (29).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSAs) were conducted by using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) as per the manufacturer's instructions. Differentiated H9c2 cardiac myocytes were transfected with a *Foxo1* wild-type (WT) plasmid (Addgene plasmid no. 12148) for 24 h using Lipofectamine 2000 (Thermo Scientific) as per the manufacturer's instructions. Nuclear fractions were prepared with a Nuclear Extract Kit (Active Motif, Carlsbad, CA) as per the manufacturer's instructions. The oligonucleotides used were *Pdk4* WT [containing FoxO1 DNA-binding element (DBE), represented by bold/underlined letters (5′-TTGAGATGGCTCCTGAGTT**GTAAACAA**GGACAAGTCTGGGCGG-3′)] and *Pdk4* mutant (*Pdk4* Mut; mutated FoxO1 DBE; 5′-TTGAGATGGCTCCTGAGTTGTCGGGGAGGACAAGTCTGGGCGG-3′). For DNA-protein binding reactions, 20 fmol of biotin-labeled oligonucleotides were incubated with ≥ 3 μg of nuclear extracts, and competition experiments were performed with 200-fold excess of unlabeled oligonucleotides. All experiments were repeated four times with representative images shown.

Luciferase assay. Differentiated H9c2 cardiac myocytes were transfected with either *Foxo1* WT (Addgene plasmid no. 12148), *Foxo3* WT (Addgene plasmid no. 8360), *Foxo4* WT (Addgene plasmid no. 17549), *Foxo1* ADA (Addgene plasmid no. 12149), or *Foxo1* D256 (Addgene plasmid no. 12145) plasmids along with −461 *Pdk4* promoter-luciferase or −100 *Pdk4* promoter-luciferase constructs (17) for 24 h. Cells were lysed in reporter lysis buffer, and a luciferase assay was performed using the Luciferase Assay System (Promega Corporation, Madison, WI) as per the manufacturer's instructions.

Chromatin immunoprecipitation. The EZ chromatin immunoprecipitation (ChIP) kit (EMD Millipore) was used according to the manufacturer's protocol. Briefly, differentiated H9c2 cardiac myocytes were transfected with *Foxo1* WT plasmid for 24 h using Lipofectamine 2000 and treated with 1% formaldehyde to cross-link proteins to DNA. Cells were lysed with protease inhibitors, sonicated

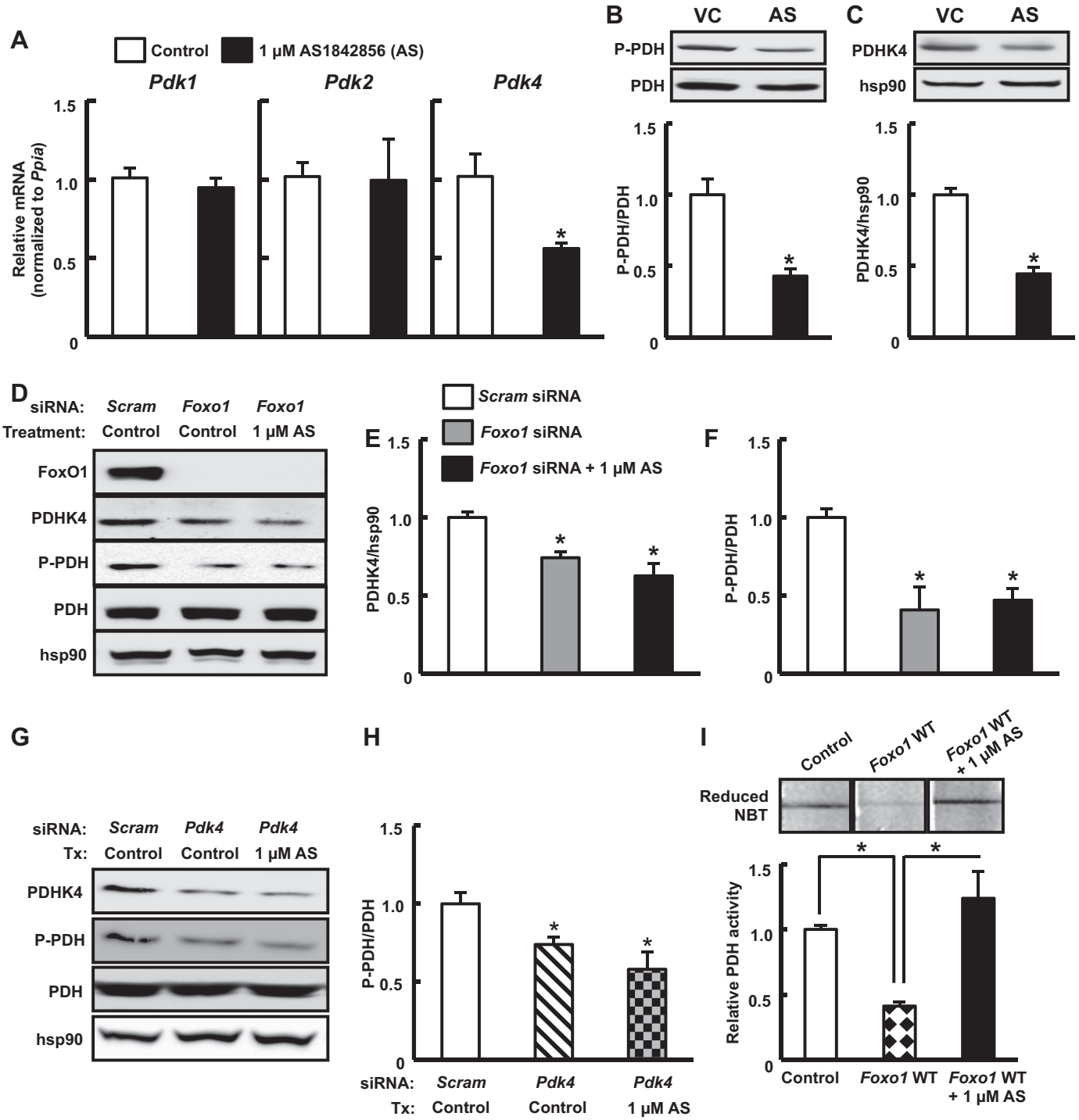

Fig. 1. Forkhead box O1 (FoxO1) protein inhibition increases pyruvate dehydrogenase (PDH) activity in cardiac myocytes. *A*: H9c2 cells were differentiated into cardiac myocytes and treated with 1 μM AS1842856 (AS) for 24 h for the quantification of PDH kinase (Pdk)1/Pdk2/Pdk4 mRNA expression via real-time PCR ($n = 3–6$). *B* and *C*: Pdk4 (PDHK4) expression and PDH phosphorylation were evaluated by Western blot analysis in vehicle control (VC) and 1 μM AS-treated H9c2 cardiac myocytes ($n = 6$). *D–F*: PDHK4 expression and PDH phosphorylation were evaluated by Western blot analysis in H9c2 cardiac myocytes 48 h posttransfection with either scrambled (Scram) control or Foxo1 siRNA ($n = 3$) followed by treatment (Tx) with either vehicle control or 1 μM AS1842856 for 24 h. *G* and *H*: PDH phosphorylation was evaluated by Western blot analysis in H9c2 cardiac myocytes 48 h posttransfection with either Scram control or Pdk4 siRNA followed by treatment with either vehicle control or 1 μM AS1842856 for 24 h ($n = 3$). *I*: PDH activity was assessed using the MitoProfile Dipstick assay in lysates from H9c2 cardiac myocytes transfected with *Foxo1* wild-type (WT) plasmid for 24 h concurrently treated with either vehicle control or 1 μM AS1842856. The assay measures PDH-derived, NADH-mediated reduction of nitroblue tetrazolium (NBT; $n = 3$). Values represent means ± SE. Differences were determined with an unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by a Bonferroni post hoc analysis. $^*P < 0.05$.

to shear DNA into fragments, and incubated with antibody against FoxO1 (Abcam) or IgG (negative control) overnight. The purified DNA and input genomic DNA were analyzed by real-time PCR. The primer sequences for the *Pdk4* promoter used in this experiment were as follows: forward 5′-TAAGGCTATTTAGGCAGTTT-3′ and reverse 5′-CCAGACTTGTCCTTGTTTAC-3′.

Isolated working heart perfusions and assessment of glucose oxidation. Mice were anesthetized with pentobarbital sodium (60 mg/kg ip), and their hearts were subsequently excised and immersed in ice-cold Krebs-Henseleit bicarbonate solution, after which the aorta was cannulated and equilibrated in the Langendorff mode. Hearts were switched to and perfused in the working mode, as previously described (32, 35). Oxygenated Krebs-Henseleit solution consisting of 0.8 mM palmitate bound to 3% fatty acid-free BSA and 5.5 mM [U-^{14}C]glucose was delivered to the left atrium at a preload pressure of 15 mmHg, whereas perfusate was ejected from hearts into the aortic outflow line against a hydrostatic afterload pressure of 50 mmHg. Hearts were perfused aerobically for 30 min, and glucose oxidation was assessed as previously described (32, 35). At the end of perfusion, hearts were immediately frozen in liquid N_2 with Wollenberger tongs and stored at −80°C.

Statistical analysis. All values are presented as means ± SE. Significant differences were determined by the use of an unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by a Bonferroni post hoc analysis. Differences were considered significant when $P < 0.05$.

RESULTS

Modulation of FoxO1 in differentiated H9c2 cardiac myocytes regulates PDH activity. To determine whether FoxO1 controls PDH activity in the heart, we treated differentiated H9c2 cardiac myocytes with the FoxO1 antagonist AS1842856 (1 μM). We observed a significant decrease in Pdk4 mRNA expression but not Pdk1 or Pdk2 after treatment with AS1842856 (Fig. 1*A*). These mRNA changes were associated with a reduction in both PDHK4 protein expression and PDH phosphorylation (Fig. 1, *B* and *C*). To confirm that the effect of AS1842856 on the PDHK4-PDH axis was dependent on reduced FoxO1 activity, we used siRNA to knockdown *Foxo1* in H9c2 cardiac myocytes. *Foxo1* knockdown also resulted in a significant decrease in both PDHK4 protein expression and PDH phosphorylation, and no further reduction in PDHK4 expression or PDH phosphorylation was observed with concurrent AS1842856 treatment (Fig. 1, *D–F*). Similarly, siRNA-mediated knockdown of *Pdk4* also reduced PDH phosphorylation, and no further decrease in PDH phosphorylation was seen with concurrent AS1842856 treatment (Fig. 1, *G* and *H*). Moreover, using an enzyme activity assay that immunocaptures PDH and measures PDH-dependent NADH production, we observed a significant reduction in PDH activity in differentiated H9c2 cardiac myocytes after transfection of a plasmid overexpressing *Foxo1* WT, which was attenuated via pretreatment with 1 μM AS1842856 (Fig. 1*I*). Taken together, AS1842856's actions on PDH activity in H9c2 cardiac myocytes appear to be dependent on reducing FoxO1-mediated expression of *Pdk4*/PDHK4.

Because previous studies have reported that the glucocorticoid dexamethasone activates FoxO1 (14, 28), we treated differentiated H9c2 cardiac myocytes with 10 μM dexamethasone, which increased *Pdk4*/PDHK4 expression and subsequent PDH phosphorylation (Fig. 2, *A* and *B*). Conversely, pretreatment with 1 μM AS1842856 negated these dexamethasone-mediated outcomes, indicating that FoxO1 is involved in glucocorticoid responsiveness in the heart (Fig. 2, *A* and *B*). In addition, we demonstrated that dexamethasone increased activity of a *Pdk4* promoter-regulated luciferase reporter construct, which was completely abrogated via transfection with a plasmid vector encoding for a dominant negative FoxO1 (Fig. 2*C*). Hence, FoxO1 appears to regulate the PDHK4-PDH axis in vitro in differentiated H9c2 cardiac myocytes.

Pdk4 is a direct transcriptional target of FoxO1 in differentiated H9c2 cardiac myocytes. Although a number of studies have observed an association between increased FoxO1 activity and Pdk4 expression (6, 27, 28), we determined whether FoxO1 directly induces the expression of the *Pdk4* gene in cardiac myocytes. Indeed, a conserved consensus FoxO1 DBE is located at 352, 326, and 339 nucleotides upstream of the transcriptional start site in the promoter region of the human,

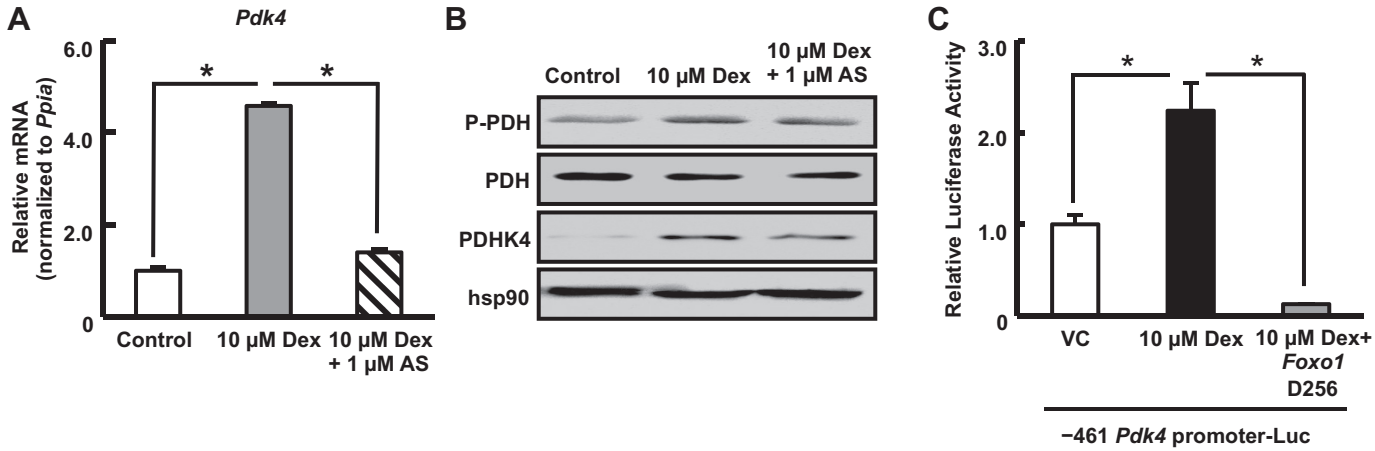

Fig. 2. FoxO1 regulates *Pdk4* expression in H9c2 cardiac myocytes. *A*: Pdk4 mRNA was measured via real-time PCR in H9c2 cardiac myocytes treated with 10 μM dexamethasone (Dex) in the presence or absence of 1 μM AS1842856 ($n = 3$). *B*: PDHK4 expression and PDH phosphorylation were evaluated by Western blot analysis, and representative images are shown. *C*: luciferase reporter assays were performed using H9c2 cardiac myocytes transfected with or without *Foxo1* D256 overexpression plasmids and reporter constructs, including a 461-bp promoter region of the rat *Pdk4* gene [containing FoxO1 DNA-binding element (DBE)] fused to a luciferase gene and treated with 10 μM Dex or vehicle control for 24 h. The luciferase activity was corrected for protein content and fold induction compared with control vector ($n = 4$). Values represent means ± SE. Differences were determined using one-way ANOVA followed by a Bonferroni post hoc analysis. *$P < 0.05$.

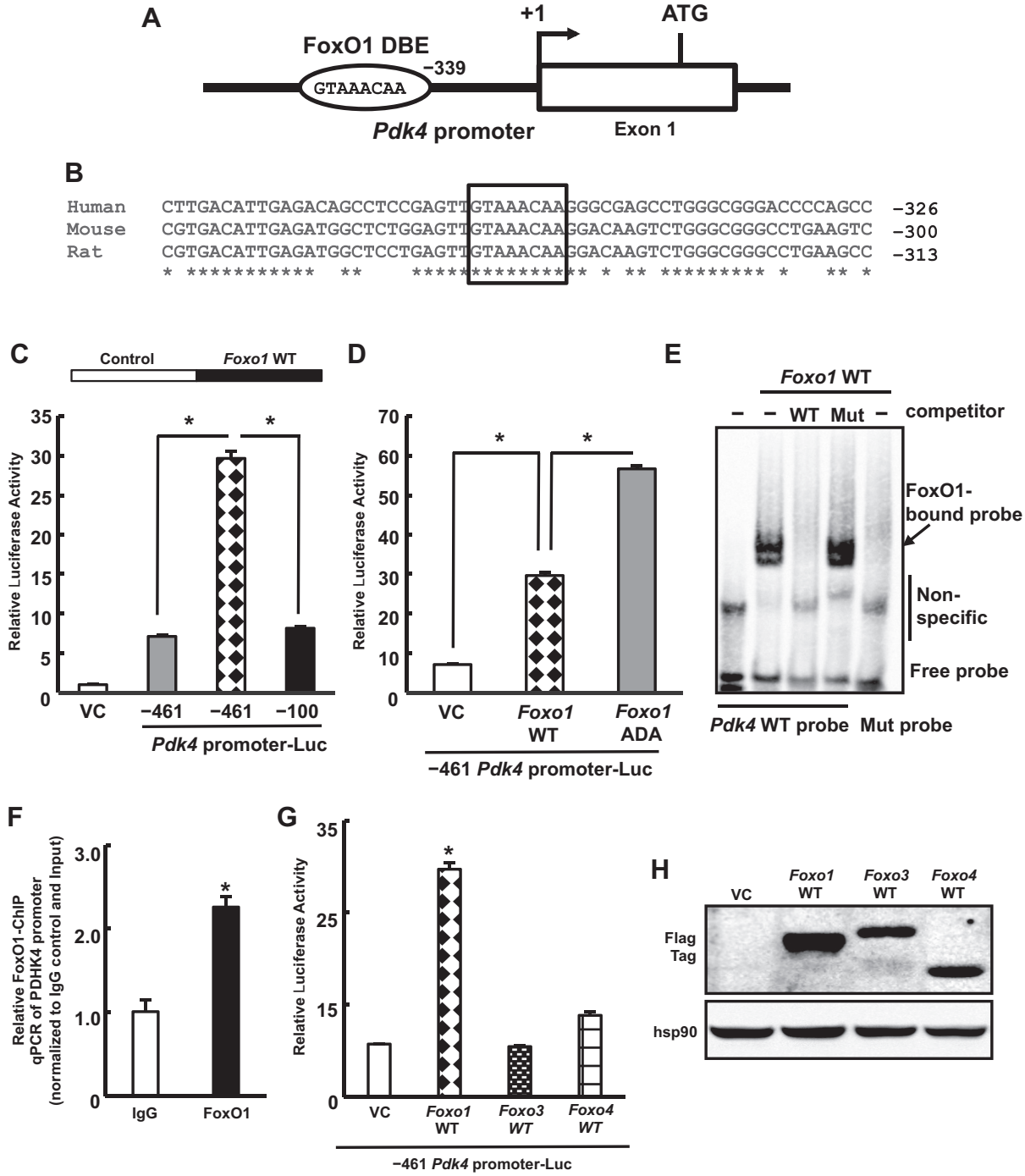

Fig. 3. *Pdk4* is a direct transcriptional target of FoxO1. *A*: model of the 5′-untranslated region of the rat *Pdk4* gene showing FoxO1 DBE at −339 position. *B*: clustal W sequence alignment showing the conserved FoxO1 DBE (in box) in human, mouse, and rat *Pdk4* promoters. *C* and *D*: luciferase reporter assays were performed using H9c2 cardiac myocytes transfected with or without *Foxo1* WT or *Foxo1* ADA overexpression plasmids and reporter constructs, including a 461-bp promoter region of the rat *Pdk4* gene (containing FoxO1 DBE) fused to a luciferase gene or one with a 100-bp promoter region (lacking the FoxO1 DBE). Luciferase activity was corrected for protein content and fold induction compared with control vector (n = 3–5). *E*: electrophoretic mobility shift assay was performed with a biotin-labeled *Pdk4* promoter element containing FoxO1 DBE (WT) and mutant DBE (Mut) oligonucleotides using nuclear fractions from H9c2 cardiac myocytes transfected with a *Foxo1* WT plasmid. Unlabeled WT and Mut oligonucleotides were used as competitor probes (n = 4). *F*: chromatin immunoprecipitation/quantitative (q)PCR was performed with IgG control/FoxO1 antibody using H9c2 cardiac myocytes transfected with a *Foxo1* WT plasmid, and the *Pdk4* promoter was detected by qPCR using specific primers. *G*: luciferase reporter assays were performed using H9c2 cardiac myocytes transfected with or without *Foxo1/Foxo3/Foxo4* WT plasmids and reporter constructs, including a 461-bp promoter region of the rat *Pdk4* gene (containing FoxO DBE) fused to a luciferase gene. *H*: expression of FoxO1/FoxO3/FoxO4 was evaluated by Western blot analysis, and representative images are shown. Values represent means ± SE. Differences were determined with an unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by a Bonferroni post hoc analysis. $^*P < 0.05$.

mouse, and rat *Pdk4/PDK4* genes, respectively (Fig. 3, *A* and *B*) (7, 17). To address this, we transfected differentiated H9c2 cardiac myocytes with *Foxo1* WT plasmid along with a luciferase reporter construct encoding the *Pdk4* promoter. We found that *Foxo1* overexpression significantly increased luciferase activity using this *Pdk4* reporter construct encoding the FoxO1 DBE (−461) compared with a *Pdk4* reporter construct lacking the FoxO1 DBE (−100) (Fig. 3*C*). Moreover, transfection with a plasmid encoding a constitutively active FoxO1 (*Foxo1 ADA*) further increased luciferase activity of the transfected −461 *Pdk4* reporter construct (Fig. 3*D*). Conversely, transfection of a plasmid encoding for dominant negative FoxO1 abolished the increase in luciferase activity of the −461 *Pdk4* reporter vector construct induced via overexpression of constitutively active FoxO1 (data not shown).

To further examine the direct FoxO1-mediated regulation of *Pdk4* transcription, we assessed the DNA-binding efficiency of FoxO1 to the *Pdk4* promoter by EMSAs with various *Pdk4* promoter FoxO1 DBE probes applied to nuclear fractions from differentiated H9c2 cardiac myocytes transfected with the *Foxo1* WT plasmid. We observed a prominent shift indicating binding of FoxO1 after application of a *Pdk4* promoter FoxO1 WT DBE biotinylated probe to nuclear fractions from H9c2 cells transfected with the *Foxo1* WT plasmid (Fig. 3*E*). This specific shifted band completely disappeared after coapplication of 200-fold excess of a *Pdk4* promoter FoxO1 WT DBE nonbiotinylated probe, but not after coapplication of 200-fold excess of a *Pdk4* promoter FoxO1 mutant DBE nonbiotinylated probe (Fig. 3*E*). Moreover, no shift was detected in nuclear fractions from differentiated H9c2 cardiac myocytes transfected with the *Foxo1* WT plasmid after application of a *Pdk4* promoter FoxO1 mutant DBE biotinylated probe (Fig. 3*E*). To further confirm the binding of FoxO1 to the *Pdk4* promoter region, we performed a ChIP-quantitative PCR assay. We observed a significant increase in the detection of the *Pdk4* promoter region within FoxO1 antibody-pulled cardiac myocyte chromatin extracts compared with IgG control using specific primers for the *Pdk4* promoter region containing FoxO1 DBE (Fig. 3*F*).

Because all FoxO transcription factors share the same DNA-binding consensus sequence and can thus act redundantly in the control of their target gene expression, we anticipated that

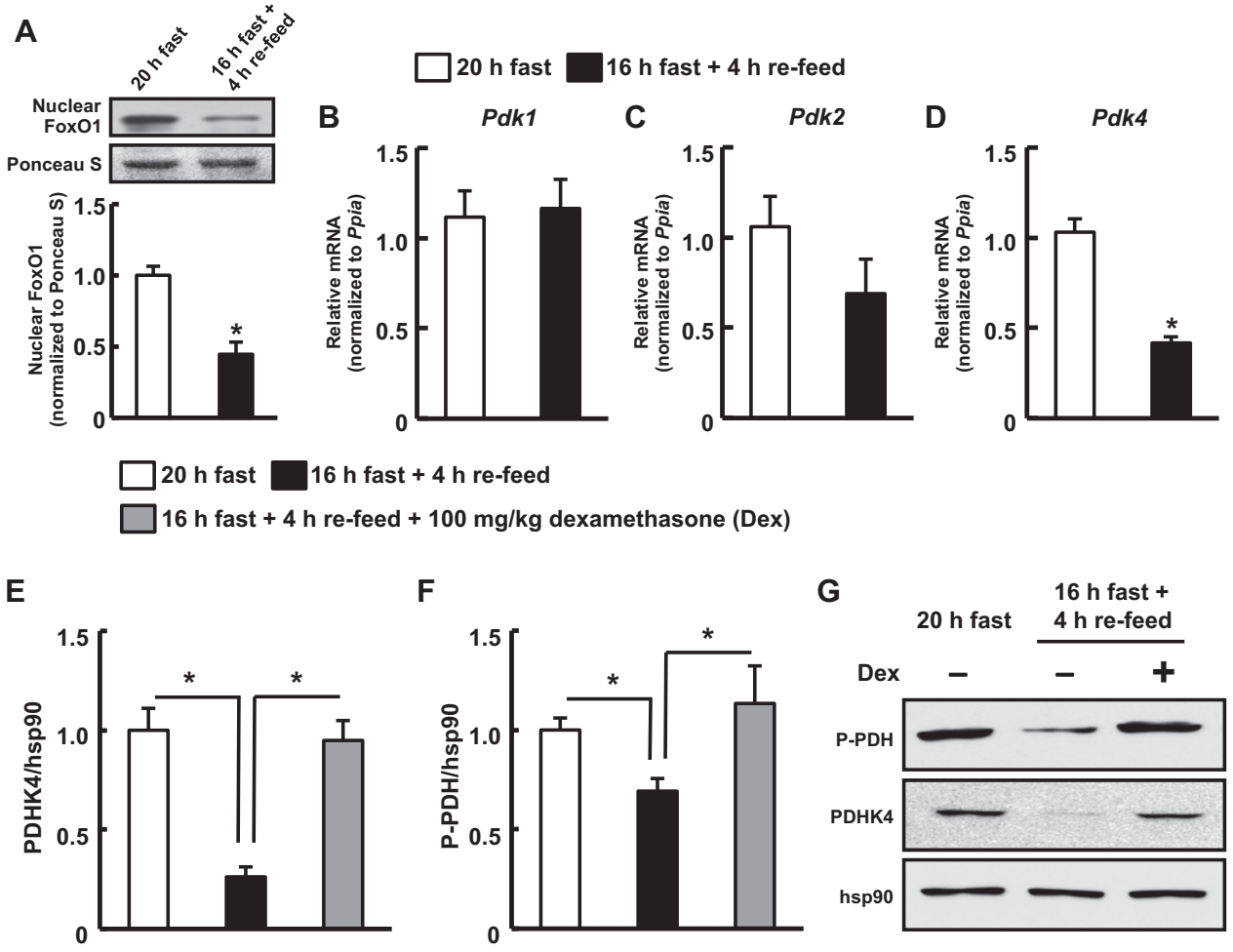

Fig. 4. FoxO1 regulates *Pdk4* expression during fasting/refeeding. *A*: nuclear FoxO1 expression in myocardial extracts from C57BL/6J mice fasted for 20 or 16 h followed by a 4-h refeeding period ($n = 5$). *B–D*: Pdk1/Pdk2/Pdk4 mRNA was measured via real-time PCR in myocardial RNA extracts from C57BL/6J mice fasted for 20 or 16 h followed by a 4-h refeeding period ($n = 8$). *E–G*: a separate cohort of mice was treated with either vehicle control or dexamethasone (100 mg/kg) 1 h before the 4-h refeeding period, and myocardial protein extracts were prepared for the evaluation of PDHK4 expression and PDH phosphorylation by Western blot analysis ($n = 5–8$). Values represent means ± SE. Differences were determined with an unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by a Bonferroni post hoc analysis. $^*P < 0.05$.

overexpression of FoxO1, FoxO3, or FoxO4 in differentiated H9c2 cardiac myocytes would all lead to subsequent increases in the transcription of *Pdk4*. To our surprise, we observed an increase only in luciferase activity of our transfected −461 *Pdk4* reporter construct after plasmid-mediated FoxO1 overexpression and not in response to FoxO3 or FoxO4 overexpression (Fig. 3, *G* and *H*).

FoxO1 activity controls Pdk4 expression during fasting/refeeding. Because fasting is known to increase FoxO1 expression in the liver and contribute to fasting-mediated increases in gluconeogenesis (4), we fasted mice for either 20 or 16-h followed by refeeding for the final 4 h and assessed PDH status in the heart. As expected, nuclear FoxO1 protein and Pdk4 mRNA expression were higher in myocardial extracts of mice fasted for 20 h versus those of mice fasted for 16-h followed by a 4-h refeeding period, although no changes were observed for Pdk1 and Pdk2 mRNA expression (Fig. 4, *A–D*). Of interest is that treatment of mice with dexamethasone (100 mg/kg) 1-h before the 4-h refeeding period in 16-h-fasted mice prevented the refeeding-mediated reduction in PDHK4 protein expression and PDH phosphorylation (Fig. 4, *E–G*). These findings suggest that FoxO1 is a key regulator of *Pdk4*/PDHK4 expression, PDH activity, and subsequent glucose oxidation rates during the transition from fasting to feeding and vice versa.

In addition, we also demonstrated that pharmacological FoxO1 inhibition with AS1842856 (3 treatments of 100 mg/kg via oral gavage; Fig. 5*A*) during a 20-h fast reduces PDHK4 protein expression and PDH phosphorylation (Fig. 5, *B* and *C*). Conversely, FoxO1 antagonism combined with a 16-h fast and 4-h refeed did not produce any further reductions in PDHK4 protein expression and PDH phosphorylation versus FoxO1 inhibition alone (Fig. 5, *B* and *C*). Hence, it is likely that feeding-induced reductions in PDHK4 expression and PDH phosphorylation involve a reduction in FoxO1 activity.

Pharmacological FoxO1 inhibition increases glucose oxidation rates in the isolated working mouse heart. We repeated our experiments involving inhibition of FoxO1 in 20-h fasted mice via three treatments of AS1842856 every 8 h. Four hours after the last treatment, mice were euthanized and had their hearts extracted for aerobic isolated working heart perfusions (Fig. 6*A*). We observed that FoxO1 inhibition during a 20-h fast led to a significant increase in glucose oxidation rates compared with vehicle control-treated mice fasted for 20 h

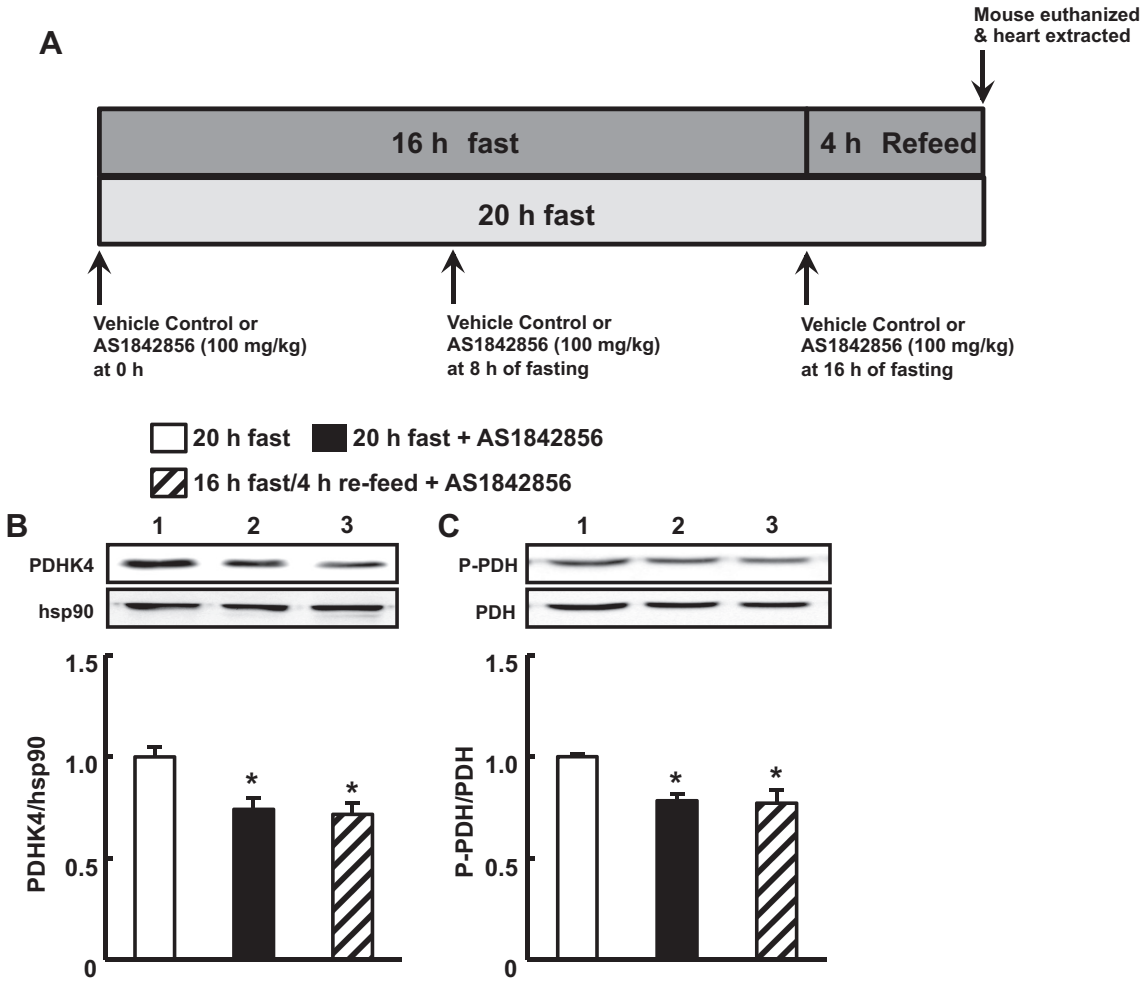

Fig. 5. In vivo FoxO1 inhibition decreases expression of PDHK4 and PDH phosphorylation in the heart. *A*: C57BL/6J mice were treated with vehicle control or AS1842856 3 times (100 mg/kg) during a 20-h fast or for 16 h followed by a 4-h refeeding period. *B*: PDHK4 expression and PDH phosphorylation were evaluated by Western blot analysis in myocardial extracts ($n = 4–6$). Values represent means ± SE. Differences were determined using one-way ANOVA followed by a Bonferroni post hoc analysis. *$P < 0.05$.

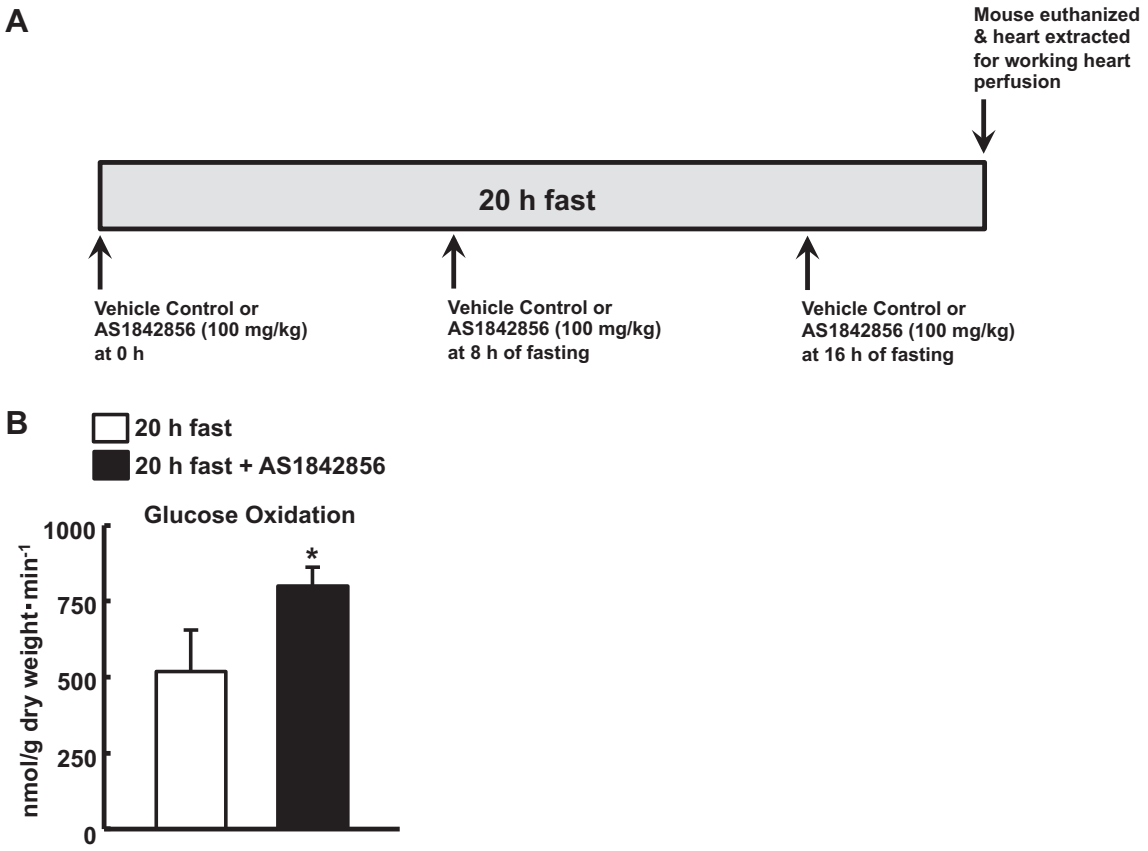

Fig. 6. In vivo FoxO1 inhibition increases glucose oxidation rates in the isolated working heart. *A*: C57BL/6J mice were treated with vehicle control or AS1842856 (100 mg/kg) three times during a 20-h fast. *B*: assessment of glucose oxidation rates during aerobic working heart perfusions from hearts isolated from 20 h-fasted mice, as shown in *A* ($n = 4$). Values represent means ± SE. Differences were determined using an unpaired, two-tailed Student's *t*-test. *$P < 0.05$.

(Fig. 6*B*), which was entirely consistent with our in vitro signaling data.

FoxO1 activation exacerbates ANG II-induced apoptosis of differentiated H9c2 cardiac myocytes. T2D is associated with increased circulating levels of ANG II (22), a molecular mediator of pressure overload-induced heart failure (23), whereas FoxO1 has been shown to regulate cell survival/apoptosis. Because altering glucose oxidation rates has been demonstrated to influence cardiac myocyte apoptosis (11, 12), we presumed that FoxO1-mediated regulation of PDH/glucose oxidation may also influence markers of H9c2 cardiac myocyte viability. Likewise, this premise would be consistent with the impaired myocardial glucose oxidation rates and increased cardiac myocyte apoptosis that are observed in the heart during T2D (39). Accordingly, we treated differentiated H9c2 cardiac myocytes with ANG II (100 nM) and observed increased caspase-3 cleavage and apoptosis-inducing factor protein expression in cells also transfected with our *Foxo1* WT plasmid (Fig. 7, *A* and *B*). These observations are consistent with FoxO1 augmenting ANG II-induced cardiac myocyte apoptosis through increases in mitochondrial depolarization, and both of these effects were abolished in H9c2 cardiac myocytes pretreated with the broad PDHK inhibitor dichloroacetate [DCA (1.5 mM); Fig. 7, *A* and *B*].

DISCUSSION

In this study, we observed that FoxO1 directly mediates transcription of the *Pdk4* gene in H9c2 cardiac myocytes, likely explaining how increased FoxO1 activity can reduce glucose oxidation in the heart, as *Pdk4* encodes for the protein PDHK4, a major kinase phosphorylating and subsequently inactivating PDH, the rate-limiting enzyme of glucose oxidation (11, 24). We confirmed the latter by demonstrating that FoxO1 overexpression reduces PDH enzymatic activity (24), and this inhibition was negated via concurrent treatment with the FoxO1 antagonist AS1842856. Interestingly, we also demonstrated that reduced FoxO1 activity may be a key mediator of cardiac metabolic flexibility with the switch from fatty acid to glucose oxidation during the transition from fasting to feeding, as nuclear FoxO1 localization and subsequent Pdk4 expression and PDH phosphorylation are reduced during refeeding after a prolonged fast. Furthermore, pharmacological FoxO1 inhibition in vivo in fasted mice enhanced glucose oxidation rates in the isolated working heart ex vivo. Finally, FoxO1 overexpression exacerbated ANG II-induced cardiac myocyte injury, and this effect was likely dependent on increased PDHK4 expression/activity, as it was negated via PDHK inhibition with DCA.

Our study is not the first to suggest that FoxO1 regulates *Pdk4* transcription, as studies in HepG2 cells have demon-

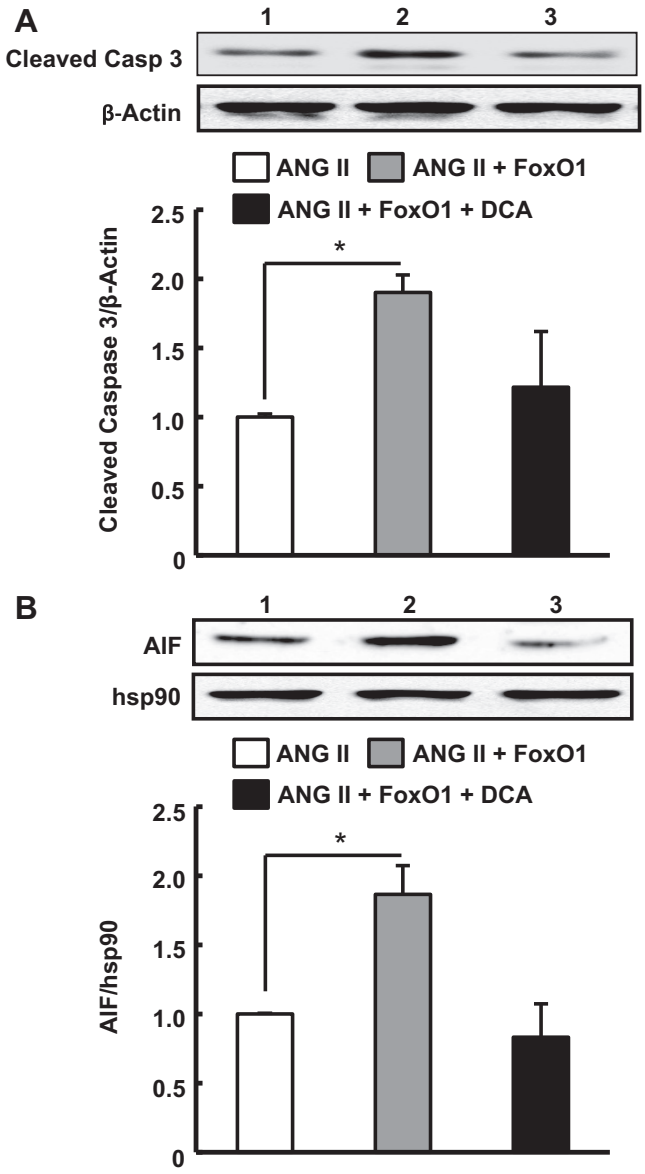

Fig. 7. FoxO1 exacerbates angiotensin II (ANG II)-induced apoptosis of cardiac myocytes. Differentiated H9c2 cardiac myocytes were transfected with either an empty vector or *Foxo1* WT plasmid for 24 h followed by treatment with ANG II (100 nM) with/without dichloroacetate (DCA; 1.5 mM) for the next 24 h. Cells were lysed, and protein extracts prepared to measure cleaved caspase-3 (*A*) and apoptosis-inducing factor (*B*) via Western blot analysis ($n = 3$). *Lane 1*, 100 nM ANG II; *lane 2*, 100 nM ANG II + FoxO1 overexpression; *lane 3*, 100 nM ANG II + FoxO1 overexpression + 1.5 mM DCA. Values represent means ± SE. Differences were determined by the use of one-way ANOVA followed by a Bonferroni post hoc analysis. $*P < 0.05$.

strated that transfection with constitutively active FoxO1 increased luciferase activity driven by a human *PDK4* promoter construct (14). Similar to our observations, treatment with dexamethasone also increased PDK4 expression in HepG2 cells, and these effects were abolished by insulin. However, insulin failed to repress dexamethasone-induced PDK4 expression in HepG2 cells transfected with the constitutively active FoxO1 (14). Likewise, dexamethasone also increased *Pdk4* expression in rat primary hepatocytes (5). These findings are consistent with the role that FoxO1 plays in hepatic gluconeogenesis, since FoxO1-induced PDHK4 would inhibit PDH and conserve pyruvate for pyruvate carboxylase and subsequent gluconeogenesis. Unlike the liver, the skeletal muscle and heart are not gluconeogenic organs, and a number of prior studies have suggested that PPAR-α is the key transcription factor controlling PDHK4 expression in these organs (8, 20, 36), although a study by Furuyama et al. (9) also suggested that FoxO1 directly regulates *Pdk4* transcription during starvation in skeletal muscle. Despite other studies showing an association between increased FoxO1 activity and PDHK4 expression in the muscle (6, 9) and heart (1, 27), respectively, our study directly demonstrates in cardiac myocytes that FoxO1 activation results in increased transcription of *Pdk4* and subsequent phosphorylation-induced inactivation of PDH. Puthanveetil et al. (28) also demonstrated in adult rat cardiac myocytes that dexamethasone regulates the PDHK4-PDH axis in a FoxO1-dependent manner, but this study did not provide conclusive evidence that FoxO1 directly regulates the transcription of *Pdk4* to modify PDH activity and glucose oxidation. In this study, we used a variety of approaches to confirm that FoxO1 directly regulates *Pdk4* transcription, including genetic techniques to elevate or reduce FoxO1 expression/activity followed by luciferase, EMSA, and ChIP assays to assess *Pdk4* transcription. Furthermore, in our in vivo experiments, we observed that increased myocardial PDHK4 expression and subsequent PDH phosphorylation were tightly associated with increased myocardial nuclear FoxO1 expression. Thus, alterations in FoxO1-mediated *Pdk4* transcription may be a key factor controlling metabolic flexibility and the switch from fatty acid oxidation to glucose oxidation during the transition from fasting to refeeding and vice versa. We also observed no additional reductions in PDHK4 expression and subsequent PDH phosphorylation in hearts from fasted/refed mice versus hearts from fasted/refed mice treated with the FoxO1 antagonist AS1842856. Hence, the mechanism of refeeding-induced increases in myocardial glucose oxidation likely involves a decrease in FoxO1 activity, since refeeding plus FoxO1 inhibition did not provide any further reductions in PDHK4 expression or PDH phosphorylation compared with refeeding alone. In addition, we did observe a trend toward reduced Pdk2 mRNA expression in hearts from fasted/refed mice, suggesting that PDHK2 may also be important in controlling the transition to glucose oxidation in the fed state and entirely consistent with PDHK2 being an abundantly expressed and important PDHK isoform in the heart (24, 25). However, PDHK2 protein expression was unchanged in hearts from fasted/refed mice versus mice that were only fasted (Fig. 8*A*). Moreover, siRNA-mediated *Foxo1* knockdown had no effect on PDHK2 protein expression in H9c2 cardiac myocytes (Fig. 8*B*).

Interestingly, a previous study (10) in rats has demonstrated during fasting and refeeding that *Pdk4* transcription is regulated via a fatty acid-dependent but PPAR-α-independent pathway. Although FoxO1 activity was not considered in this scenario, in light of our findings FoxO1 may be the major transcription factor controlling *Pdk4* expression in the heart during fasting, similar to that in skeletal muscle (9), as activation of FoxO1 may be fatty acid dependent (4). In support of this, pharmacological FoxO1 inhibition in mice mitigated fasting-mediated increases in myocardial PDHK4 expression and subsequent PDH phosphorylation, thereby increasing glucose oxidation rates in the isolated working heart ex vivo. To our

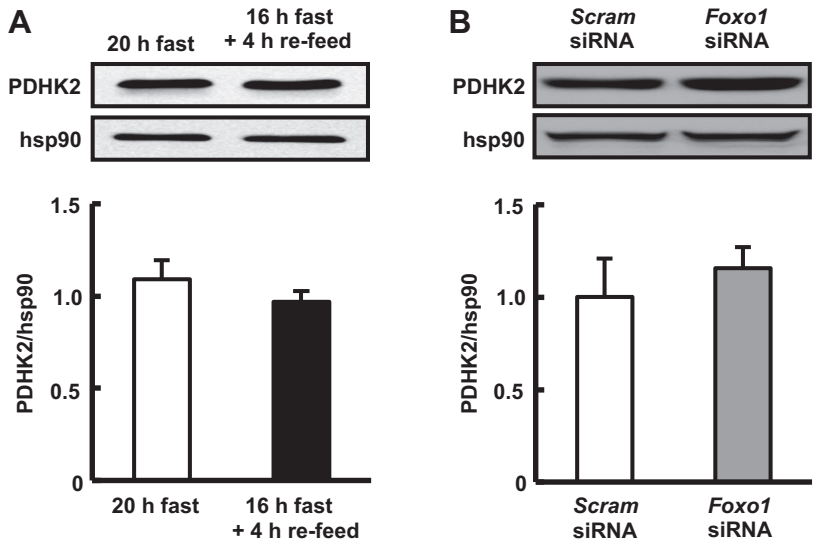

Fig. 8. FoxO1 inhibition does not affect PDHK2 expression. *A*: PDHK2 expression in myocardial extracts from C57BL/6J mice fasted for 20 or 16 h followed by a 4-h refeeding period was evaluated by Western blot analysis ($n = 8$). *B*: expression of PDHK2 was evaluated by Western blot analysis in H9c2 cardiac myocytes 48 h after transfection with either a scrambled (Scram) control siRNA or Foxo1 siRNA ($n = 3$). Values represent means ± SE.

knowledge, our observations are the first report that in vivo FoxO1 antagonism in mice influences myocardial glucose oxidation.

Despite all FoxO transcription factors sharing an identical DNA-binding consensus sequence, both FoxO3 and FoxO4 overexpression in differentiated H9c2 cardiac myocytes failed to increase luciferase activity of our transfected −461 *Pdk4* reporter construct. These findings suggest that FoxO-mediated regulation of their target genes may take place in a cell/tissue type-specific manner, and in the cardiac myocyte/heart, the necessary cofactors required for FoxO3/FoxO4 control of *Pdk4* transcription are absent. Conversely, it is possible that FoxO3 or FoxO4 baseline expression/activity in the cardiac myocyte/heart is high, masking our ability to discern their regulation of *Pdk4* transcription after plasmid-induced overexpression. We did measure mRNA expression of all three FoxO isoforms in H9c2 cardiac myocytes, and in relation to FoxO1, FoxO4 had similar mRNA expression levels, whereas FoxO3 had greater mRNA expression levels (data not shown). However, we do not have data comparing baseline protein expression levels for these three FoxO isoforms in H9c2 cardiac myocytes, and, unfortunately, regular Western blot methods cannot answer this question due to inherent differences in the antibody sensitivity for each FoxO isoform. Hence, further indepth experimentation is necessary to delineate whether FoxO3 and/or FoxO4 regulate the PDHK4-PDH axis in the heart.

Of clinical relevance, we also demonstrated that FoxO1 activation enhanced apoptosis of cardiac myocytes treated with ANG II, a molecular mediator of pressure overload-induced cardiac hypertrophy and heart failure (23). Moreover, these actions of ANG II plus FoxO1 activation were negated via concurrent treatment with DCA, a broad inhibitor of PDHKs that includes PDHK4 (11), thus suggesting that FoxO1-enhanced apoptosis of ANG II-treated differentiated H9c2 cardiac myocytes is dependent on increased PDHK4 expression/activity. These specific experiments do have limitations, however, as ANG II treatment alone of H9c2 cardiac myocytes did not increase caspase-3 cleavage versus vehicle control treatment (data not shown). Although ANG II induces apoptosis of numerous cell types, including neonatal rat cardiac myocytes and vascular smooth muscle cells (26, 38), it is possible that differentiated H9c2 cardiac myocytes are more resistant to ANG II, but cellular toxicity is unmasked in the presence of increased FoxO1 activity. Nevertheless, our observations are of interest, since cardiac-specific *Foxo1*-deficient mice are protected against diabetic cardiomyopathy, and Battiprolu et al. (1) concluded that a reduction in *Pdk4* expression and subsequent increase in glucose metabolism may be partly responsible for the observed benefit. Because obesity and T2D are associated with increased circulating levels of ANG II (22), our findings are entirely consistent with FoxO1-mediated *Pdk4* expression contributing to T2D-associated reductions in myocardial glucose oxidation and subsequent ventricular dysfunction. However, our study is also limited in that many other factors are altered during T2D that may contribute to diabetic cardiomyopathy, including inflammation, oxidative stress, and endoplasmic reticulum stress (2, 3). Thus, further mechanistic work is still required to elucidate how T2D actually promotes increased myocardial FoxO1 activity and subsequent reductions in glucose oxidation and whether this conclusively augments cardiac myocyte apoptosis in vivo in the diabetic heart.

Taken together, our findings illustrate that FoxO1 is a major transcription factor directly regulating the expression of *Pdk4* in the heart. We further demonstrate that FoxO1 is a critical transcription factor controlling PDH activity and metabolic flexibility of glucose oxidation in the heart during the transition from fasting to feeding. Finally, the observation that myocardial FoxO1 activity is increased and contributes to the pathology of diabetic cardiomyopathy, coupled together with our finding that inhibition of PDHK4 negates FoxO1-accelerated apoptosis of cardiac myocytes, suggests that inhibition of FoxO1-induced *Pdk4* transcription may be a novel target to pursue for the potential treatment of T2D-related cardiovascular disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.G. and J.R.U. conceived and designed research; K.G., B.S., R.A.B., H.A., A.E., K.L.H., W.K.M., M.A., F.E., M.G., and G.S. performed experiments; K.G. analyzed data; K.G., B.S., R.A.B., E.A.P., G.S., and J.R.U. interpreted results of experiments; K.G. and J.R.U. prepared figures; K.G. and J.R.U. drafted manuscript; K.G., B.S., R.A.B., H.A., A.E., K.L.H., W.K.M., M.A., F.E., M.G., E.A.P., G.S., and J.R.U. edited and revised manuscript; K.G., B.S., R.A.B., H.A., A.E., K.L.H., W.K.M., M.A., F.E., M.G., E.A.P., G.S., and J.R.U. approved final version of manuscript.

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