BJ www.biochemj.org

BJ Metabolism

Biochem. J. (2011) **433**, 225–233 (Printed in Great Britain) doi:10.1042/BJ20101104

online data

225

Androgen stimulates glycolysis for *de novo* lipid synthesis by increasing the activities of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 in prostate cancer cells

Jong-Seok MOON*, Won-Ji JIN*, Jin-Hye KWAK*, Hyo-Jeong KIM*, Mi-Jin YUN†, Jae-Woo KIM*, Sahng Wook PARK*[1] and Kyung-Sup KIM*[1]

*Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Center for Chronic Metabolic Disease Research, Brain Korea 21 Project for Medical Science, Yonsei University, College of Medicine, Seodaemungu, Seoul, Korea, and †Department of Diagnostic Radiology and Research Institute of Radiological Science, Yonsei University College of Medicine, Seodaemungu, Seoul, Korea

Up-regulation of lipogenesis by androgen is one of the most characteristic metabolic features of LNCaP prostate cancer cells. The present study revealed that androgen increases glucose utilization for *de novo* lipogenesis in LNCaP cells through the activation of HK2 (hexokinase 2) and activation of the cardiac isoform of PFKFB2 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase). Activation of PKA (cAMP-dependent protein kinase) by androgen increased phosphorylation of CREB [CRE (cAMP-response element)-binding protein], which in turn bound to CRE on the promoter of the *HK2* gene resulting in transcriptional activation of the *HK2* gene. Up-regulation of PFKFB2 expression was mediated by the direct binding of ligand-activated androgen receptor to the *PFKFB2* promoter. The activated PI3K (phosphoinositide 3-kinase)/Akt signalling pathway in LNCaP cells contributes to the phosphorylation of PFKFB2 at Ser^{466} and Ser^{483}, resulting in the constitutive activation of PFK-2 (6-phosphofructo-2-kinase) activity. Glucose uptake and lipogenesis were severely blocked by knocking-down of PFKFB2 using siRNA (small interfering RNA) or by inhibition of PFK-2 activity with LY294002 treatment. Taken together, our results suggest that the induction of *de novo* lipid synthesis by androgen requires the transcriptional up-regulation of HK2 and PFKFB2, and phosphorylation of PFKFB2 generated by the PI3K/Akt signalling pathway to supply the source for lipogenesis from glucose in prostate cancer cells.

Key words: androgen, glycolysis, hexokinase 2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2), prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer in males, where androgen is a critical factor in regulating cell proliferation and growth. The most characteristic metabolic change by androgen in prostate cancer cells is the induction of *de novo* lipid synthesis through the up-regulation of lipogenic enzyme expression [1–5]. Since the metabolites from glycolysis are the main carbon sources for lipid synthesis, glycolysis should be controlled along with lipogenesis. Although the function of androgen in lipogenesis has been extensively investigated, little is known about the role of androgen in glycolysis for *de novo* lipid synthesis. In the present study, we carried out a set of experiments to elucidate the mechanism by which androgen controls glycolysis for support of vigorous lipogenesis in LNCaP prostate cancer cells.

A critical step for a high glycolytic state is the phosphorylation of glucose, which is catalysed by HK (hexokinase) [6,7]. Among the four mammalian HK types (HK 1–4), HK2 is frequently the predominant overexpressed form in tumours [8–10]. Furthermore, mounting evidence indicates that HK2 plays a pivotal role in highly malignant cancer cells by promoting cell growth, survival, enhancing biosynthesis and helping immortalization of the cells under hypoxic conditions. Another key step in controlling the glycolytic flux is the conversion of Fru-6-P (fructose 6-phosphate) into Fru-1,6-P_2 (fructose 1,6-bisphosphate) by PFK-1 (phosphofructokinase). The activity of PFK-1 is allosterically controlled by Fru-2,6-P_2 (fructose 2,6-bisphosphate), which is produced by the bifunctional PFKFB (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), which catalyses both the biosynthesis and degradation of Fru-2,6-P_2 [11]. In mammals, the isoforms of PFKFB are encoded by four separate genes (*PFKFB1*, *PFKFB2*, *PFKFB3* and *PFKFB4*), which are characterized by tissue expression pattern, the ratio of kinase to phosphatase activity, and the response to protein kinases [12]. In cancer cells, the level of Fru-2,6-P_2 is generally elevated [11,13,14] due to overexpression and the activation of PFKFB3 and PFKFB4 [15,16]. PFKFB2, the cardiac isoform of PFKFB, is an essential enzyme in the regulation of glycolysis in the heart. Adrenaline, insulin and a work load increase the level of Fru-2,6-P_2 by elevated PFK-2 (6-phosphofructo-2-kinase) activity of PFKFB2 [17–19]. The C-terminal regulatory domain of PFKFB2, which is absent in the liver isoform PFKFB1, can be phosphorylated on both Ser^{466} and Ser^{483} by PDK-1 (3-phosphoinositide-dependent kinase-1), PKA (cAMP-dependent protein kinase), p70^{S6K} (p70 ribosomal S6 kinase) and MAPK-1 (mitogen-activated protein kinase-1) [19,20]. These findings suggest that increased

Abbreviations used: ACACA, acetyl-CoA carboxylase α; ACLY, ATP citrate lyase; ARE, androgen-response element; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREB, CRE-binding protein; DTT, dithiothreitol; FASN, fatty acid synthase; [^{18}F]FDG, [^{18}F]fluoro-2-deoxyglucose; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; HK, hexokinase; KLK3, kallikrein-related peptidase 3; PFK-1, phosphofructokinase; PFK-2, 6-phosphofructo-2-kinase; PFKFB, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PKA-Cβ2, PKA catalytic subunit β transcript variant 2; *PTEN*, phosphatase and tensin homologue; RNAi, RNA interference; siRNA, small interfering RNA; RT-PCR, real-time PCR.

[1] Correspondence may be addressed to either of these authors (email swpark64@yuhs.ac or kyungsup59@yuhs.ac).

Fru-2,6-P_2 production by elevated PFK-2 activity of PFKFB2 may play an important role in transformation of non-malignant cells or proliferation of cancer cells.

In the present study, we demonstrate that androgen up-regulates the expression of HK2 and PFKFB2, resulting in elevation of the utilization of glucose for *de novo* lipid synthesis in LNCaP prostate cancer cells. We show that androgen-induced HK2 expression is dependent on PKA signalling, and the induction of PFKFB2 expression is achieved by a direct binding of the androgen receptor to the *PFKFB2* promoter. Constitutive activation of the PI3K (phosphoinositide 3-kinase)/Akt signalling pathway in LNCaP cells plays a critical role in the activation of PFK-2 activity of PFKFB2. In conclusion, these results suggest that androgen stimulates the utilization of glucose to undergo a metabolic conversion for both production of ATP and lipogenesis in androgen-dependent prostate cancer cells.

MATERIALS AND METHODS

Reagents

R1881 (methyltrienolone), a synthetic androgen, was purchased from Dupont-New England Nuclear and dissolved in DMSO. Forskolin, H-89 and LY294002 were purchased from Sigma–Aldrich and dissolved in DMSO.

Cell culture and routine procedures

Details of cell culture condition and routine procedures, including transfection, reporter assays, construction of plasmids, quantitative RT-PCR (real-time PCR), Western blotting and ChIP (chromatin immunoprecipitation) assays, are described in the Supplementary Materials and methods section (at http://www.BiochemJ.org/bj/433/bj4330225add.htm).

RNAi (RNA interference)

siRNA (small interfering RNA) oligonucleotides targeting PFKFB2 and control siRNA were purchased from Shanghai Genepharma. The siRNA oligonucleotide sequences were: PFKFB2 #1, 5′-AGAGCAAGAUAGUCUACUATT-3′; PFKFB2 #2, 5′-AGGAAAUAACAGACCUCAATT-3′; PFKFB2 #3, 5′-GUGGAAACAAUUAAACUUATT-3′; and control siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′. Transfections were performed twice over 2 days using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. Details of RNAi are shown in the Supplementary Materials and methods section.

PFK-2 activity assay

PFK-2 activity was measured according to the method described by Manzano et al. [23]. Briefly, cleared cell lysates were obtained by homogenization in 500 μl of buffer A [50 mM Tris/HCl (pH 7.4), 0.1 M KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 50 mM potassium fluoride, 1 mM DTT (dithiothreitol), 10 mg/ml leupeptin, 10 mg/ml aprotinin and 0.5 mM PMSF], and centrifuged at 13000 ***g*** at 4 °C for 10 min. Supernatants were mixed with 1 vol. of buffer B [100 mM Tris/HCl (pH 7.1), 200 mM KCl, 10 mM ATP, 4 mM $MgCl_2$, 2 mM KH_2PO_4, 10 mM Fru-6-P, 35 mM glucose 6-phosphate and 2 mM DTT] and incubated at 30 °C for 30 min. Reactions (200 μl volume) were stopped by the addition of 1 vol. of 0.2 M NaOH followed by heating at 80 °C for 10 min, then neutralized with 40 μl of ice-cold 1 M acetic acid in 20 mM Hepes. After centrifugation (13000 ***g*** for 10 min at 4 °C), 350 μl aliquots of supernatant was mixed with 500 μl of reaction mixture [100 mM Tris/HCl (pH 8.0), 4 mM magnesium acetate, 2 mM Fru-6-P and 0.3 mM NADH], and then the mixtures were incubated with 100 μl of enzyme stock solution [1 unit/ml PP_i-PFK (Fru-6-P 1-phosphotransferase), 500 units/ml triosephosphate isomerase, 170 units/ml α-glycerophosphate dehydrogenase and 45 units/ml aldolase] at 25 °C for 5 min. All enzymes were purchased from Sigma–Aldrich. The assay was started by the addition of 50 μl of 10 mM PP_i solution in the mixtures. Absorbance at 340 nm was measured at room temperature (25 °C) every 1 min for a period of 10 min with an Ultrospec 1100 pro-spectrometer as described previously [22,23]. Assays were performed in triplicate.

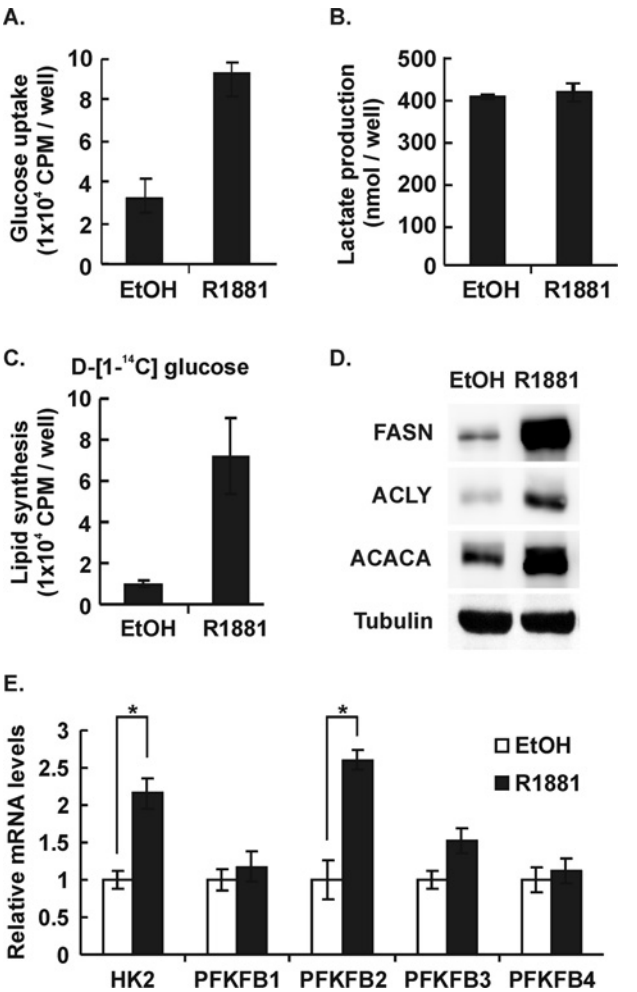

Figure 1 Androgen increases glucose uptake and lipid synthesis in LNCaP cells

Glucose uptake (**A**), lactate production (**B**) and lipid synthesis from D-[1-^{14}C]glucose (**C**) were measured in LNCaP cells in the absence or the presence of 10 nM R1881 for 72 h. Results shown are the means ± S.D. of triplicate samples from a representative experiment. (**D**) In each group, the amount of FASN, ACLY and ACACA were measured by Western blot analysis, using tubulin as an internal control. (**E**) Quantitative RT-PCR analysis was performed to measure the level of mRNAs for *HK2* and *PFKFB* isoforms in LNCaP cells in the absence (open columns) or the presence (solid columns) of 10 nM R1881 for 72 h. Relative amounts of mRNAs were represented using *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA as an invariant control. $*P < 0.05$. EtOH, ethanol.

Glucose uptake and lactate assay

LNCaP cells (6×10^5 cells/well) or PC3 cells (2×10^5 cells/well) were plated on six-well plates and treated as indicated. For the

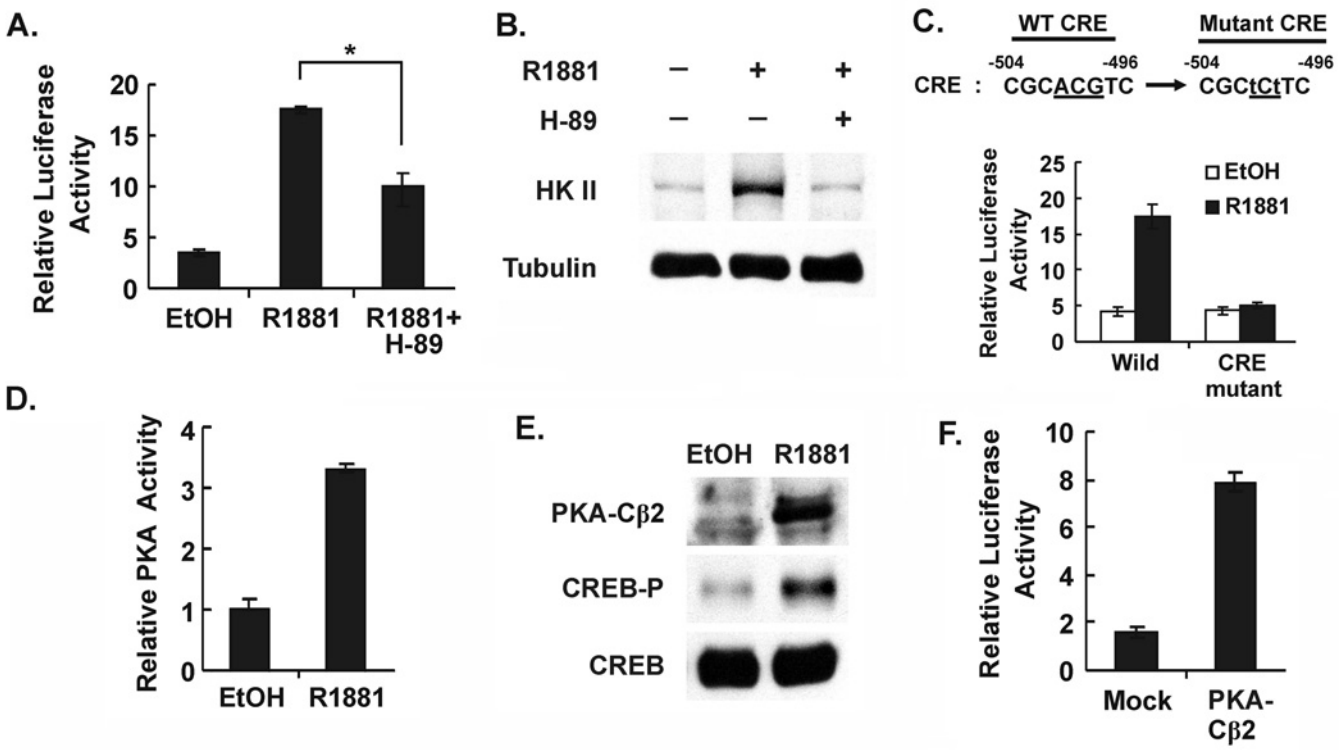

Figure 2 Androgen activates the transcription of the *HK2* gene via cAMP/PKA signalling in LNCaP cells

(**A**) The luciferase activity of the *HK2* promoter was measured in LNCaP cells transfected with the pHK2-Luc construct, after incubation for 72 h in the absence or the presence of 10 nM R1881. At 24 h before harvest, when used, H-89 was added at a final concentration of 10 μM. Luciferase activities were normalized to the activities of SV40 (simian virus 40) promoter-driven *Renilla* luciferase. Values are the means $\pm$ S.D. of triplicate samples from a representative experiment. *$P < 0.05$. (**B**) The levels of HK2 were analysed by Western blot analysis with the protein extracts of LNCaP cells treated as described in (**A**). Tubulin was used as an invariant control. (**C**) The conserved CRE in the *HK2* promoter located at –504 from the ATG codon was mutated as shown. LNCaP cells transfected with promoter–Luc constructs harbouring wild-type or mutant CRE were incubated in the absence (open column) or the presence (solid column) of 10 nM R1881 for 72 h. Values are the means $\pm$ S.D. of triplicate samples from a representative experiment. (**D**) PKA activities were measured as described in Supplementary Materials and methods section (at http://www.BiochemJ.org/bj/433/bj4330225add.htm) in LNCaP cells treated as described in (**A**). Data shown are relative to that of the group not treated with R1881. (**E**) In each group, the levels of PKA-Cβ2, phospho-CREB and total CREB in nuclear fractions were measured by Western blot analyses. (**F**) The activities of the *HK2* promoter were assayed in PC3 cells exogenously overexpressing PKA-Cβ2. Cells were transfected with the pHK2-Luc construct in combination with the empty vector or pSG5-PKA-Cβ2. The relative luciferase activities were analysed 48 h after transfection. Values are the means $\pm$ S.D. of triplicate samples from a representative experiment. EtOH, ethanol.

glucose-uptake assay, cells were incubated in glucose-free RPMI 1640 medium (Invitrogen) for 6 h, then supplied with approx. 0.3 MBq (10 μCi) [^{18}F]FDG ([^{18}F]fluoro-2-deoxyglucose) per well. After incubation at 37 °C for 20 min, cells were washed twice with PBS. The cells were harvested in PBS, and the radioactivity was measured using a Wallac 148 Wizard 3 γ-counter (PerkinElmer Life and Analytical Science). All experiments were performed in triplicate. The lactate levels in the culture medium were measured using a lactate assay kit according to the manufacturer's instructions (Biovision).

Lipid synthesis assay

Lipid synthesis from glucose was assayed by measuring the incorporation of ^{14}C from D-[1-^{14}C]glucose (PerkinElmer Life and Analytical Science) into lipid. Cells in six-well plates were incubated for 6 h in glucose-free RPMI 1640 medium and then were treated with 72 μM of D-[1-^{14}C]glucose (4 μCi/ml) for 2 h. Cells were harvested and washed three times with PBS, and disrupted by adding 400 μl of 0.5 % Triton X-100. The lipids were extracted with 500 μl of a hexane/isopropanol solution [3:2 (v:v)]. Aliquots of solvent layer were collected and dried under nitrogen gas. The dried samples were resuspended in 50 μl of chloroform, and the radioactivity of ^{14}C was counted on a LS 6500 scintillation counter (Beckman Coulter).

Statistical analysis

SPSS for Windows version 17.0 was used for all statistical analyses.

RESULTS

Androgen activates glycolysis and lipid synthesis in LNCaP cells

To observe the changes in glycolysis by androgen in androgen-dependent LNCaP prostate cancer cells, glucose uptake and lactate production were measured after treatment with R1881, an androgen agonist. The uptake of [^{18}F]FDG, which accumulates as 2-deoxyglucose 6-phosphate, over 20 min was markedly elevated up to 3-fold in cells treated with R1881 (Figure 1A), whereas lactate production was remained unchanged (Figure 1B). To determine whether the increase in glucose uptake serves as a substrate for the synthesis of lipids in LNCaP cells, we measured the amount of hexane/isopropanol-extractable lipids synthesized with D-[1-^{14}C]glucose. R1881 drastically increased

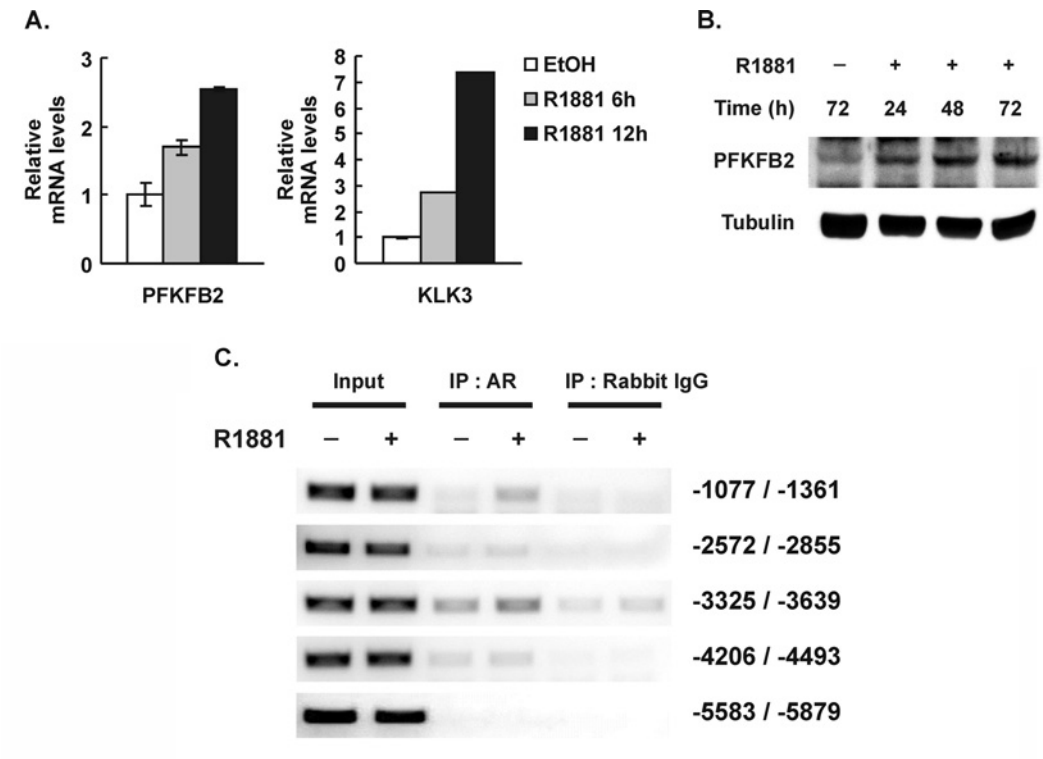

Figure 3 Androgen increases the expression of PFKFB2 in LNCaP cells by recruiting liganded androgen receptor to the *PFKFB2* promoter

(**A**) The relative amounts of mRNA for *PFKFB2* and *KLK3* were determined by quantitative RT-PCR in a time course after treatment with 10 nM R1881. Each amount of mRNA was normalized using *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA as an invariant control. (**B**) The levels of protein for PFKFB2 were measured by Western blot analysis in the extracts of LNCaP cells treated with R1881 for the indicated times. Tubulin was used as an invariant control. (**C**) ChIP analyses were performed on LNCaP cells treated with 10 nM R1881 for 6 h using an androgen-receptor(AR)-specific antibody or non-specific IgG. The indicated chromosomal regions, denoted as nucleotide numbers from the transcription start of the *PFKFB2* gene, were amplified by PCR and analysed in 1.2 % agarose-gel electrophoresis. EtOH, ethanol; IP, immunoprecipitation.

the incorporation of ^{14}C from glucose into lipids up to 7-fold (Figure 1C), and this result was supported by marked induction of lipogenic enzymes, such as FASN (fatty acid synthase), ACLY (ATP citrate lyase) and ACACA (acetyl-CoA carboxylase α) (Figure 1D). These results suggest that the androgen-dependent activation of glucose uptake provides an important carbon source for androgen-dependent lipogenesis.

Androgen induces the expression of *HK2* and *PFPKB2* in LNCaP cells

On the basis of the assumption that the increase in glycolysis supports androgen-dependent lipogenesis, microarray analyses were carried out to reveal glycolytic genes whose expression is under the control of androgen. The expression of *HK2* and *PFKFB2* genes were increased 2.6- and 3.0-fold by R1881 treatment. Quantitative RT-PCR confirmed these significant increases in *HK2* and *PFKFB2*, whereas other *PFKFB* isoforms remained unchanged (Figure 1E). These results indicate that HK2 and PFKFB2 could have an important role in the androgen-dependent increase in glycolysis in LNCaP cells.

PKA signalling activated by androgen stimulates the expression of *HK2*

CREB [CRE (cAMP-response element)-binding protein] plays a critical role in the strong transcriptional activation of the *HK2* promoter, which contains a well-characterized CRE [24–26]. CREB is the transcriptional activator associated with the cAMP/PKA signalling pathway, which is stimulated in LNCaP cells after androgen treatment [26a]. On the basis of these previous observations, we examined whether the induction of HK2 by androgen in LNCaP cells is achieved by transcriptional activation of the *HK2* promoter (–2054/+21) via the cAMP/PKA signalling pathway. The activity of the *HK2* promoter was increased 8-fold by androgen and this activation was significantly attenuated by treatment with H-89, an inhibitor of PKA (Figure 2A). Consistent with the *HK2* promoter activity, the HK2 protein level was markedly increased by androgen. However, whereas H-89 only partially inhibited luciferase activity (Figure 2A), it fully blocked the induction of protein accumulation (Figure 2B). The difference between the HK2 protein mass and luciferase activity might be due to different stabilities of these proteins in cells treated with H-89. To determine whether androgen-mediated activation of the *HK2* promoter is dependent on CRE, the CRE was mutated as shown in Figure 2(C). Mutation of CRE completely suppressed the responsiveness to androgen, indicating that CRE plays a critical role in the regulation of the *HK2* promoter by androgen (Figure 2C). We have shown previously that androgen induces PKA activity by increasing the expression of PKA-Cβ2 (PKA catalytic subunit β transcript variant 2) in LNCaP cells [26a]. In the present study, we confirmed that PKA activity, the PKA-Cβ2 protein level and phosphorylation of CREB were significantly increased by R1881 treatment in LNCaP cells (Figures 2D and 2E). Exogenous overexpression of PKA-Cβ2 markedly activated the *HK2* promoter in PC3 cells (Figure 2F). These results suggest that the induction of HK2 expression

by androgen in LNCaP cells resulted from activation of PKA signalling.

Androgen induces the recruitment of androgen receptors to the *PFKFB2* promoter resulting in induction of PFKFB2

To determine whether *PFKFB2* is a direct target gene of the androgen receptor, we compared the regulation of *PFKFB2* with the *KLK3* (kallikrein-related peptidase 3) gene, which is a known target of the androgen receptor in LNCaP cells. As shown in Figure 3(A), both *KLK3* and *PFKFB2* mRNA accumulated in a similar manner by treatment with R1881. *PFKFB2* mRNA was significantly induced after treatment with R1881 by 1.8- and 2.5-fold at 6 and 12 h respectively. The amount of *KLK3* mRNA was increased to a slightly higher level by 2.7- and 7-fold at 6 and 12 h respectively (Figure 3A). In accordance with the mRNA data, PFKFB2 protein was markedly increased 24 h after R1881 treatment and continued to increase until 72 h (Figure 3B). To elucidate whether the androgen receptor directly binds to the *PFKFB2* promoter, a ChIP assay was carried out in LNCaP cells 6 h after R1881 treatment using an antibody against the androgen receptor. Five regions located at approx. 1 kb intervals on the *PFKFB2* promoter were amplified by PCR using chromatin immunoprecipitates as templates (Figure 3C). Treatment with R1881 increased the recruitment of the androgen receptor only on the –1361 to –1077 region from the transcription start site (GenBank® accession number NM_006212.2), suggesting that a functional ARE (androgen-response element) is located at this region and is involved in the androgen-mediated regulation of PFKFB2 expression.

Next, we examined the effects of androgen on *PFKFB2* promoter activity, using a pPFKFB2–Luc construct which contains the *PFKFB2* promoter fragment (–1949/+92) complexed with the luciferase gene. Androgen increased the luciferase activity by 3-fold (Figure 4A), which correlates with the androgen-induced increase in endogenous *PFKFB2* gene expression. In order to find the key sequence element involved in androgen-mediated regulation, the putative ARE in the *PFKFB2* promoter was searched using the MatInspector program of Genomatix software (http://www.genomatix.de). Only one putative ARE was found at –1204 and its mutation resulted in complete unresponsiveness to R1881 (Figure 4B), suggesting that the ARE at –1204 mediates the androgen-dependent activation of the *PFKFB2* promoter. Taken together, these results indicate that the *PFKFB2* gene is a direct binding target of the androgen receptor and is activated by androgen in the LNCaP cells.

PI3K/Akt signalling is involved in the phosphorylation of PFKFB2 in LNCaP cells

The unique C-terminal region of PFKFB2, which is not conserved in the other isoforms of PFKFB, contains the critical residues (Ser^{466} and Ser^{483}) that control the enzyme activity through their phosphorylation status [17,19,20,27]. R1881 increased the phosphorylation at Ser^{466} and Ser^{483} of PFKFB2 as well as increasing the total enzyme level (Figure 5A). To determine the signalling pathway involved in the phosphorylation of these residues, LNCaP cells were treated with forskolin (a PKA activator), H-89 (a PKA inhibitor) or LY294002 (a PI3K inhibitor) for 40 min after treatment with R1881 for 72 h. The level of phosphorylation at Ser^{466} and Ser^{483} was not affected by treatment with forskolin or H-89 (Figure 5B), whereas LY294002 significantly decreased the phosphorylation of both Ser^{466} and Ser^{483} (Figure 5C). Consistent with this decreased level

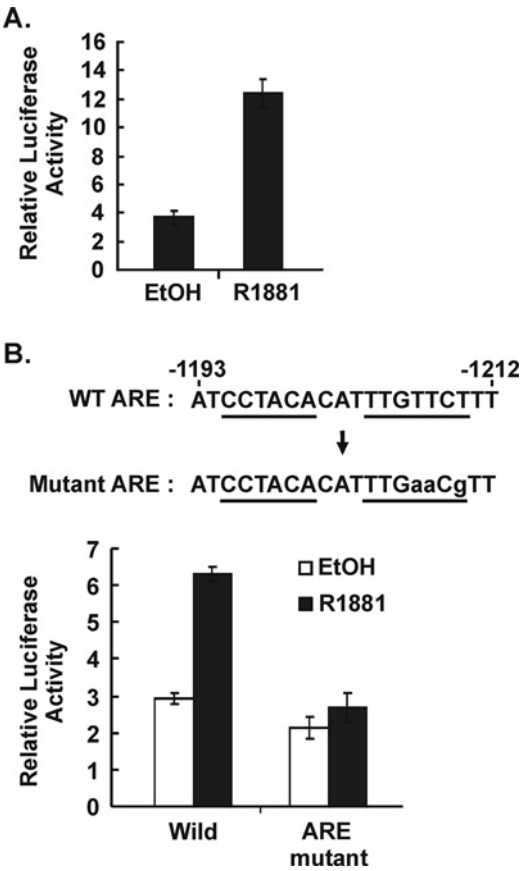

Figure 4 Androgen activates the *PFKFB2* promoter in LNCaP cells

(**A**) LNCaP cells were transfected with the pPFKFB2–Luc construct, then incubated for 72 h in the absence or the presence of 10 nM R1881. The relative luciferase activities were analysed as described in Figure 1(A). Data shown are the means ± S.D. of triplicate samples from a representative experiment. (**B**) A putative consensus ARE is underlined in the *PFKFB2* promoter, and the mutant construct was generated by the destruction of this consensus as indicated by lowercase characters. The responsiveness to R1881 was measured in LNCaP cells transfected with the wild-type pPFKFB2–Luc construct or the mutant construct as described in (**A**). Data shown are the means ± S.D. of triplicate samples from a representative experiment. EtOH, ethanol.

of phosphorylation, LY294002 markedly inhibited the PFK-2 activity (Figure 5D) and glucose uptake (Figure 5E) was increased by R1881. In LNCaP cells, it has been reported that the PI3K/Akt signalling pathway is constitutively activated by mutations in the *PTEN* (phosphatase and tensin homologue) gene and is likely to participate in activation of PFK-2 activity. Inhibition of glucose uptake by LY294002 resulted in the block of the flux of carbon from [^{14}C]glucose into lipid synthesis (Figure 5F) without significant changes in amounts of lipogenic enzymes and the androgen receptor (Figure 5G). These results suggest that the PFK-2 activity of the bifunctional enzyme PFKFB2 should be activated by PI3K/Akt signalling for the high rate of glycolysis to support androgen-induced lipogenesis.

PFKFB2 has a critical role in glucose uptake and glucose-dependent lipid synthesis

Inhibition of the PI3K/Akt signalling pathway drastically decreased PFK-2 activities and glucose uptake. Because the PI3K/Akt signalling pathway affects diverse metabolic pathways, the direct contribution of the PFK-2 activity of PFKFB2 to glucose uptake should be confirmed. Overexpression of PFKFB2 by

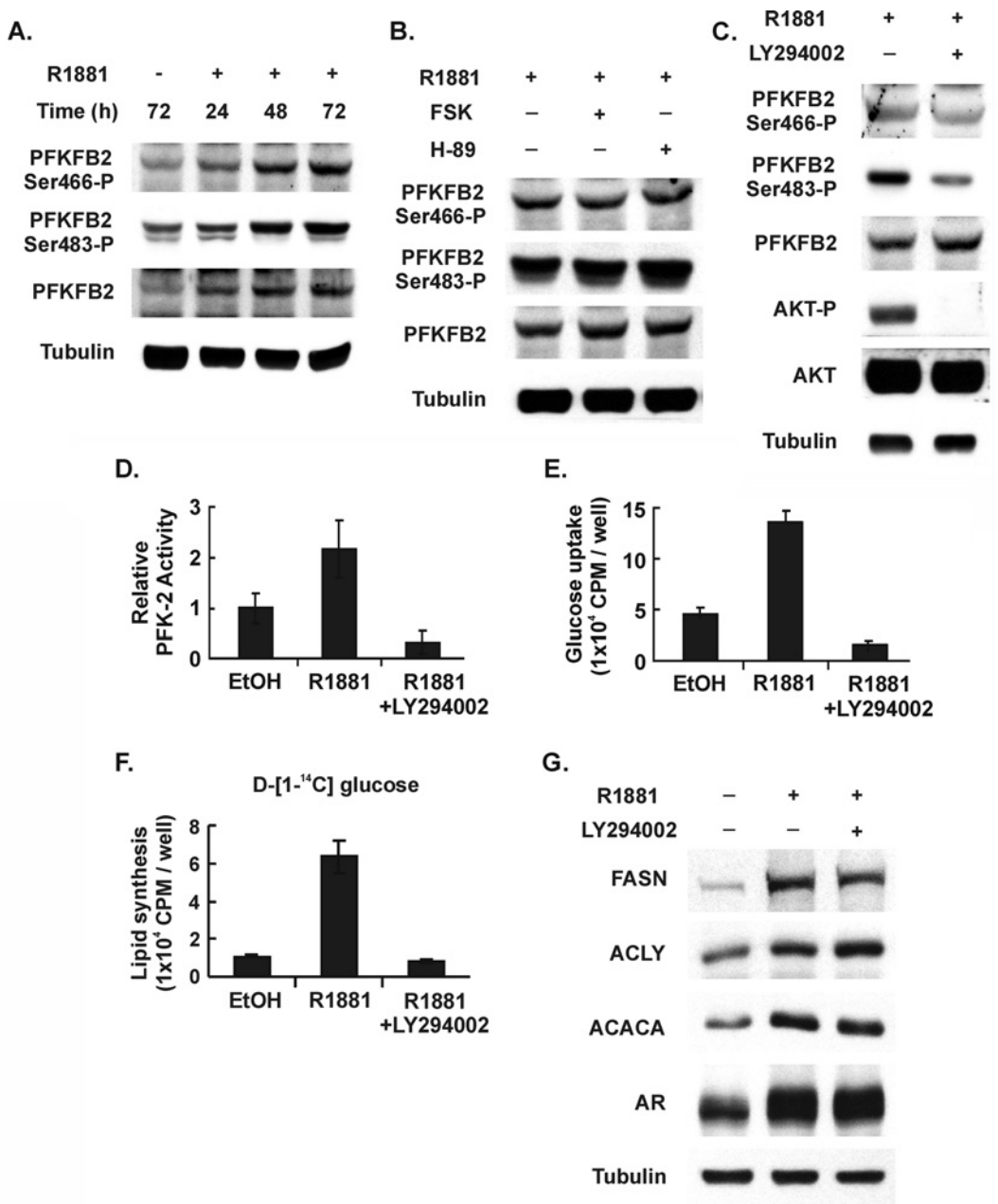

Figure 5 The PI3K signalling pathway is involved in the phosphorylation of Ser^{466} and Ser^{483} of PFKFB2

(**A**) The amount of total or phosphorylated forms of PFKFB2 (Ser^{466} and Ser^{483}) were measured by Western blot analyses in LNCaP cells treated with 10 nM R1881 for the times indicated. (**B** and **C**) LNCaP cells grown in medium containing 10 nM R1881 for 72 h were treated with 10 μM forskolin or H-89 (**B**) or with 25 μM LY294002 for 40 min before harvest (**C**). The amounts of indicated proteins were determined by Western blot analyses using tubulin as an invariant control. (**D**–**G**) LNCaP cells were grown in the absence or the presence of 10 nM R1881 for 72 h, then treated with 25 μM LY294002 for 40 min before harvest. PFK-2 activity (**D**), glucose uptake (**E**) and lipid synthesis from D-[1-^{14}C]glucose (**F**) were assayed in each group. PFK-2 activity was denoted as the relative value to that of the group without any treatment. Data shown are the means $\pm$ S.D. of triplicate samples from a representative experiment. The amount of FASN, ACLY, ACACA and androgen receptor (AR) were measured by Western blot analyses in each group, using tubulin as an invariant control (**G**). EtOH, ethanol.

transient transfection in PC3 cells (Figure 6C) increased the PFK-2 activity 3-fold (Figure 6A), which was accompanied by an increase in glucose uptake (Figure 6B). In contrast with the overexpression experiment, knocking-down of the PFKFB2 expression using siRNAs (Figures 6D and 6E) blocked the androgen-induced increase in glucose uptake (Figure 6F). Accordingly, glucose-dependent lipid synthesis was inhibited by knocking-down PFKFB2 (Figure 6G); however, the increased levels of lipogenic enzymes induced by androgen were not affected (Figure 6H). These results indicate that an increase in glucose uptake mediated by up-regulation of PFKFB2 is an important requirement for androgen-dependent induction of lipid synthesis in LNCaP cells.

DISCUSSION

The androgen receptor has been implicated in proliferation and progression of prostate tumours [5,28,29]. Androgen-induced

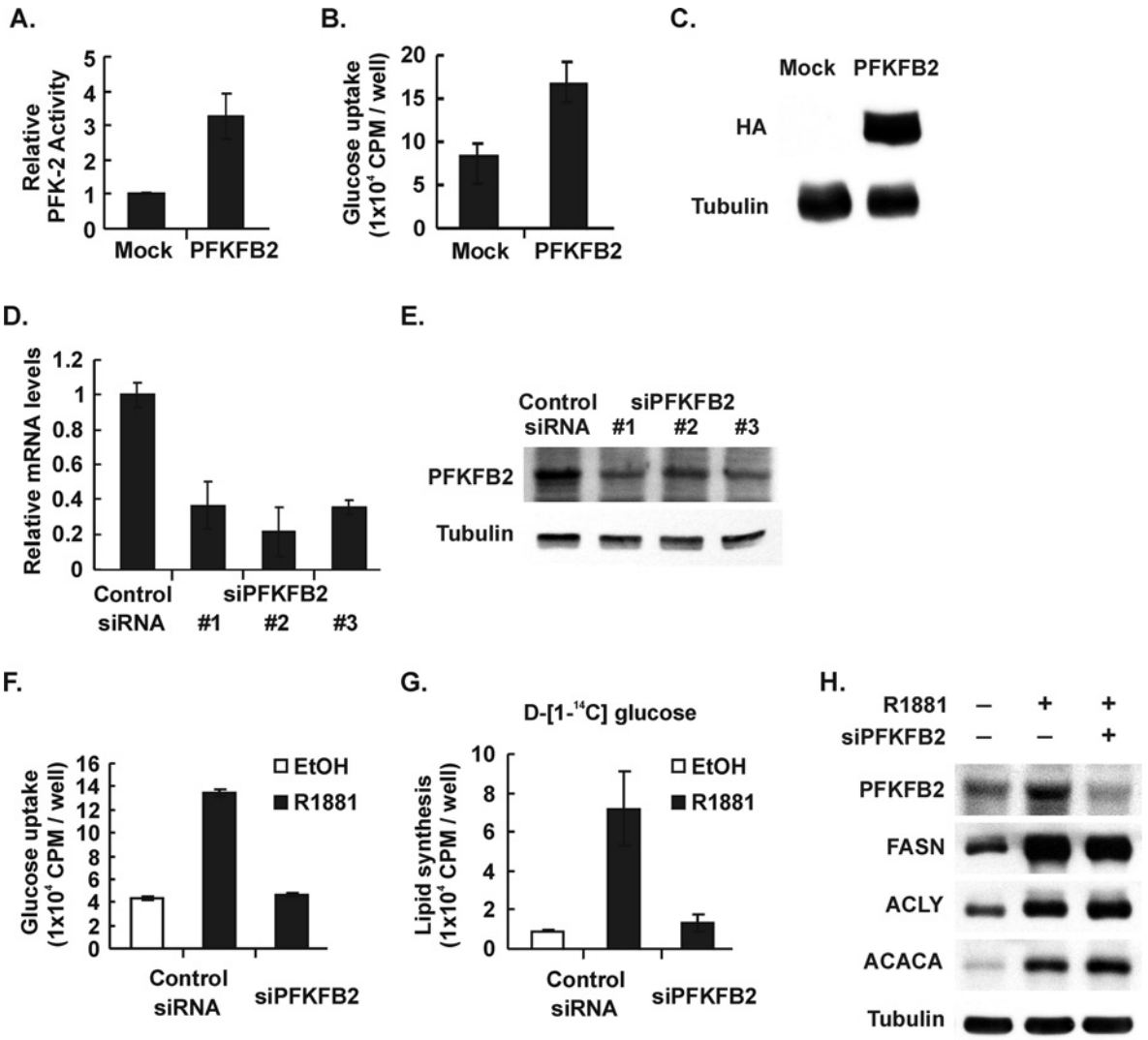

Figure 6 PFKFB2 has a critical role in glucose uptake and lipid production in prostate cancer cells

PFK-2 activity (**A**) and glucose uptake (**B**) were measured in PC3 cells transfected with an empty vector or the pSG5-PFKFB2 expression vector. PFK-2 activity was denoted as the relative value to that of mock-transfected cells. Data shown are the means ± S.D. of triplicate samples from a representative experiment. The expression of exogenous PFKFB2 was confirmed by Western blot analysis using an antibody against the HA-tag. Tubulin was used as an invariant control (**C**). (**D** and **E**) The levels of PFKFB2 mRNA (**D**) and protein (**E**) were measured in LNCaP cells transfected with the control or three independent PFKPB2 siRNAs (#1–#3) and then grown in the presence of R1881 for 72 h. Glucose uptake (**F**) and lipid synthesis from D-[1-^{14}C]glucose (**G**) were determined in LNCaP cells which were transfected with a control or the PFKPB2 siRNA #3 and then grown in the absence or presence of R1881 for 72 h as indicated. Values shown are the means ± S.D. of triplicate samples from a representative experiment. The amount of FASN, ACLY and ACACA were measured by Western blot analyses in each group using tubulin as an invariant control (**H**). EtOH, ethanol.

lipogenesis is an important metabolic change necessary for survival and proliferation of these tumours and the mechanisms responsible for transcriptional activation of lipogenic enzyme genes by the androgen receptor have been extensively studied [1,3]. Glucose utilization has also been reported to be controlled in an androgen-dependent manner in these tumours. In a clinical study, androgen-ablation therapy suppressed glucose utilization in prostate cancers [30] and most metastatic prostate cancers that express high levels of androgen receptors demonstrate high glucose uptake [31]. In general, glycolysis is known to be the main carbon source for vigorous lipogenesis, but the studies regarding how glycolysis is controlled by androgen in prostate cancer cells are strictly limited. In the present study, we focused on how androgen functions to induce glycolysis in LNCaP cells and how this induced glycolysis is linked to changes in lipogenesis required for cancer cell proliferation.

To address which glycolytic enzymes were transcriptionally regulated by androgen in prostate cancer cells, we performed a microarray analysis using total RNA isolated from LNCaP cells treated with or without R1881. The transcripts for *HK2* and *PFKFB2* among glycolytic enzymes were selectively elevated by androgen treatment. Cancer cells display a high rate of glucose uptake owing to elevated expression of glucose transporters and increased HK activity [32]. Among the four mammalian HK isoforms, it is proposed that the elevated HK activity in cancer cells is mainly due to high expression of HK2 [25]. Even if prostate cancer is known to have a relatively low glucose catabolic rate, the high rate of *de novo* lipid synthesis induced by androgen requires a carbon source from glycolysis. For this reason, the increase in glycolysis by androgen should be accompanied with a high rate of lipid synthesis. The present study revealed that *HK2* and *PFKFB2* are the major target genes for inducing glycolysis

by androgen in LNCaP cells. HK2 is the principal isoform of HKs in skeletal muscle, heart and adipose tissue [33], and its activity is stimulated by exercise and β-adrenergic agonists, which involve the cAMP/PKA signalling pathway [34,35]. The 5′ flanking region of the *HK2* promoter is rich in putative response elements, including six GC boxes, an E2F element, a CRE and a CCAAT box [25,26]. Our results showed that the CRE in this region plays a critical role in androgen-dependent activation of HK2 in LNCaP (Figures 2B and 2C). In a previous report, we have shown that R1881 enhanced the activity of PKA, which plays an important role in proliferation [26a,36], and this increase in PKA activity was correlated with the up-regulation of PKA-Cβ2 by androgen treatment in LNCaP cells [26a]. We further elucidated that the androgen-dependent induction of PKA-Cβ2 in the nucleus coincides with an increase in phosphorylation of CREB, which binds to CRE and activates transcription of the *HK2* gene (Figure 2E). Taken together, it is strongly suggested that the *HK2* gene is regulated in an androgen-dependent manner via up-regulation of the PKA signalling pathway in LNCaP cells.

In addition to an increase in HK activity, persistent consumption of glucose 6-phosphate into the glycolytic pathway is necessary to achieve a high rate of glucose uptake. The fate of glucose 6-phosphate to catabolic glycolysis is determined by the amount of Fru-2,6-P_2, whose synthesis and degradation are catalysed by the bifunctional enzyme PFKFB. Mammals possess the distinct isoforms of PFKFB encoded by four different genes (*PFKFB1*, *PFKFB2*, *PFKFB3* and *PFKFB4*) [12]. Rapidly proliferating cancer cells produce markedly elevated levels of Fru-2,6-P_2, which is generally thought to be mediated by high expression of PFKFB3 and PFKFB4 isoforms. However, in the present study, we found that LNCaP cells uniquely express PFKFB2 which shows a high ratio of kinase/phosphatase activity, as PFKFB3 does [15]. Expression of PFKFB2 was induced by the direct recruitment of the ligand-activated androgen receptor to the *PFKFB2* promoter in LNCaP cells. The *PFKFB2* promoter was activated by R1881 in androgen-independent PC3 cells when the androgen receptor was exogenously overexpressed (results not shown). Androgen-dependent induction of PFKFB2 is assumed to induce glucose uptake and glucose-dependent lipid synthesis in LNCaP cells, because knocking-down *PFKFB2* by siRNA severely impaired both processes (Figures 6F and 6G). These results demonstrate that the expression of PFKFB2 is directly regulated by the ligand-activated androgen receptor and plays a key role in androgen-induced glucose uptake and glucose-dependent lipid synthesis in LNCaP cells.

In addition to the changes in the amount of PFKFB2, the changes in kinase activity of PFKFB2 were addressed in the present study, because the concentration of Fru-2,6-P_2 can be elevated only when its PFK-2 activity is activated [11]. The regulation of PFK-2 activity is achieved by phosphorylation or dephosphorylation of PFKFB2 at Ser^{466} and Ser^{483}, mediated by several kinases, such as calcium/calmodulin-dependent protein kinase [27], PKA [37] or PI3K and Akt [19,20]. We have revealed in the present study that the PFK-2 activity of PFKFB2 was regulated by the PI3K/Akt signalling pathway, which is constitutively activated in LNCaP cells as a result of mutations of *PTEN*, an important regulator of the PI3K/Akt signalling pathway [38,39]. However, despite PKA activation by androgen, PKA did not affect the phosphorylation status of PFKFB2. PFKFB4, which is one of the isoforms expressed in LNCaP cells and known to be overexpressed in various malignancies [16,40], was not induced by androgen (results not shown). However, PFKFB4 might contribute little to glucose flux in prostate cancer cells, considering that the inhibition of phosphorylation of PFKFB2 by LY294002 inhibited androgen-induced PFK-2 activity to below the basal level (Figure 5E).

In conclusion, the present study demonstrates that the stimulation of HK2 and PFKFB2 by androgen induces high glucose uptake and the activation of glucose utilization, resulting in increased lipogenesis in prostate cancer.

AUTHOR CONTRIBUTION

Jong-Seok Moon, Won-Ji Jin, Jin-Hye Kwak, Hyo-Jeong Kim, Sahng Wook Park and Kyung-Sup Kim performed the experiments. Jong-Seok Moon, Won-Ji Jin, Jin-Hye Kwak, Hyo-Jeong Kim, Sahng Wook Park and Kyung-Sup Kim conceived and designed the experiments. Mi-Jin Yun and Jae-Woo Kim performed the experiments of glucose uptake using [^{18}F]FDG. Jong-Seok Moon, Sahng Wook Park and Kyung-Sup Kim analysed the data and wrote the paper. All of the contributing authors critically reviewed the manuscript.

FUNDING

This work was supported by the National Research Foundation funded by the Korea government (MEST) [grant number 2010-0028364]; and by the 2007 Korean National Cancer Control Program, Ministry of Health and Welfare, Republic of Korea [grant number 0720400].

REFERENCES

1 Swinnen, J. V., Esquenet, M., Goossens, K., Heyns, W. and Verhoeven, G. (1997) Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. Cancer Res. **57**, 1086–1090
2 Swinnen, J. V., Ulrix, W., Heyns, W. and Verhoeven, G. (1997) Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. Proc. Natl. Acad. Sci. U.S.A. **94**, 12975–12980
3 Heemers, H., Maes, B., Foufelle, F., Heyns, W., Verhoeven, G. and Swinnen, J. V. (2001) Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. Mol. Endocrinol. **15**, 1817–1828
4 Lee, M. Y., Moon, J. S., Park, S. W., Koh, Y. K., Ahn, Y. H. and Kim, K. S. (2009) KLF5 enhances SREBP-1 action in androgen-dependent induction of fatty acid synthase in prostate cancer cells. Biochem. J. **417**, 313–322
5 Swinnen, J. V., Heemers, H., van de Sande, T., de Schrijver, E., Brusselmans, K., Heyns, W. and Verhoeven, G. (2004) Androgens, lipogenesis and prostate cancer. J. Steroid Biochem. Mol. Biol. **92**, 273–279
6 Bustamante, E. and Pedersen, P. L. (1977) High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase. Proc. Natl. Acad. Sci. U.S.A. **74**, 3735–3739
7 Bustamante, E., Morris, H. P. and Pedersen, P. L. (1981) Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. J. Biol. Chem. **256**, 8699–8704
8 Johansson, T., Berrez, J. M. and Nelson, B. D. (1985) Evidence that transcription of the hexokinase gene is increased in a rapidly growing rat hepatoma. Biochem. Biophys. Res. Commun. **133**, 608–613
9 Shinohara, Y., Ichihara, J. and Terada, H. (1991) Remarkably enhanced expression of the type II hexokinase in rat hepatoma cell line AH130. FEBS Lett. **291**, 55–57
10 Rempel, A., Bannasch, P. and Mayer, D. (1994) Differences in expression and intracellular distribution of hexokinase isoenzymes in rat liver cells of different transformation stages. Biochim. Biophys. Acta **1219**, 660–668
11 Pilkis, S. J., Claus, T. H., Kurland, I. J. and Lange, A. J. (1995) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu. Rev. Biochem. **64**, 799–835
12 Okar, D. A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R. and Lange, A. J. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem. Sci. **26**, 30–35
13 Mojena, M., Bosca, L. and Hue, L. (1985) Effect of glutamine on fructose 2,6-bisphosphate and on glucose metabolism in HeLa cells and in chick-embryo fibroblasts. Biochem. J. **232**, 521–527
14 Yamamoto, M. (1990) Glycolytic activity and fructose 2, 6-bisphosphate changes in rat brain during ischemia. No To Shinkei **42**, 405–411
15 Bando, H., Atsumi, T., Nishio, T., Niwa, H., Mishima, S., Shimizu, C., Yoshioka, N., Bucala, R. and Koike, T. (2005) Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer. Clin. Cancer Res. **11**, 5784–5792

16 Minchenko, O. H., Ochiai, A., Opentanova, I. L., Ogura, T., Minchenko, D. O., Caro, J., Komisarenko, S. V. and Esumi, H. (2005) Overexpression of 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase-4 in the human breast and colon malignant tumors. Biochimie **87**, 1005–1010
17 Kitamura, K., Kangawa, K., Matsuo, H. and Uyeda, K. (1988) Phosphorylation of myocardial fructose-6-phosphate,2-kinase: fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. Activation by phosphorylation and amino acid sequences of the phosphorylation sites. J. Biol. Chem. **263**, 16796–16801
18 Rousseau, G. G. and Hue, L. (1993) Mammalian 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase: a bifunctional enzyme that controls glycolysis. Prog. Nucleic Acid Res. Mol. Biol. **45**, 99–127
19 Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. and Rider, M. H. (1997) Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J. Biol. Chem. **272**, 17269–17275
20 Bertrand, L., Alessi, D. R., Deprez, J., Deak, M., Viaene, E., Rider, M. H. and Hue, L. (1999) Heart 6-phosphofructo-2-kinase activation by insulin results from Ser-466 and Ser-483 phosphorylation and requires 3-phosphoinositide-dependent kinase-1, but not protein kinase B. J. Biol. Chem. **274**, 30927–30933
21 Reference deleted
22 Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H. G. (1982) A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2,6-bisphosphate. Eur. J. Biochem. **129**, 191–195
23 Manzano, A., Rosa, J. L., Ventura, F., Perez, J. X., Nadal, M., Estivill, X., Ambrosio, S., Gil, J. and Bartrons, R. (1998) Molecular cloning, expression, and chromosomal localization of a ubiquitously expressed human 6-phosphofructo-2-kinase/ fructose-2, 6-bisphosphatase gene (PFKFB3). Cytogenet. Cell Genet. **83**, 214–217
24 Mathupala, S. P., Rempel, A. and Pedersen, P. L. (1995) Glucose catabolism in cancer cellsIsolation, sequence, and activity of the promoter for type II hexokinase. J. Biol. Chem. **270**, 16918–16925
25 Lee, M. G. and Pedersen, P. L. (2003) Glucose metabolism in cancer: importance of transcription factor–DNA interactions within a short segment of the proximal region of the type II hexokinase promoter. J. Biol. Chem. **278**, 41047–41058
26 Osawa, H., Robey, R. B., Printz, R. L. and Granner, D. K. (1996) Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat hexokinase II gene. J. Biol. Chem. **271**, 17296–17303
26a Moon, J. S., Lee, M. Y., Park, S. W., Han, W. K., Hong, S. W., Ahn, J. H. and Kim, K. S. (2008) Androgen-dependent activation of human cytomegalovirus major immediate-early promoter in prostate cancer cells. Prostate **68**, 1450–1460
27 Depre, C., Rider, M. H., Veitch, K. and Hue, L. (1993) Role of fructose 2,6-bisphosphate in the control of heart glycolysis. J. Biol. Chem. **268**, 13274–13279
28 Culig, Z., Klocker, H., Bartsch, G. and Hobisch, A. (2002) Androgen receptors in prostate cancer. Endocr. Relat. Cancer **9**, 155–170
29 Heinlein, C. A. and Chang, C. (2004) Androgen receptor in prostate cancer. Endocr. Rev. **25**, 276–308
30 Oyama, N., Akino, H., Suzuki, Y., Kanamaru, H., Ishida, H., Tanase, K., Sadato, N., Yonekura, Y. and Okada, K. (2001) FDG PET for evaluating the change of glucose metabolism in prostate cancer after androgen ablation. Nucl. Med. Commun. **22**, 963–969
31 Larson, S. M., Morris, M., Gunther, I., Beattie, B., Humm, J. L., Akhurst, T. A., Finn, R. D., Erdi, Y., Pentlow, K., Dyke, J. et al. (2004) Tumor localization of 16β-^{18}F-fluoro-5α-dihydrotestosterone versus ^{18}F-FDG in patients with progressive, metastatic prostate cancer. J. Nucl. Med. **45**, 366–373
32 Pauwels, E. K., Sturm, E. J., Bombardieri, E., Cleton, F. J. and Stokkel, M. P. (2000) Positron-emission tomography with [^{18}F]fluorodeoxyglucose. Part I Biochemical uptake mechanism and its implication for clinical studies. J. Cancer Res. Clin. Oncol. **126**, 549–559
33 Katzen, H. M. and Schimke, R. T. (1965) Multiple forms of hexokinase in the rat: tissue distribution, age dependency, and properties. Proc. Natl. Acad. Sci. U.S.A. **54**, 1218–1225
34 Young, P., Cawthorne, M. A., Levy, A. L. and Wilson, K. (1984) Reduced maximum capacity of glycolysis in brown adipose tissue of genetically obese, diabetic (db/db) mice and its restoration following treatment with a thermogenic beta-adrenoceptor agonist. FEBS Lett. **176**, 16–20
35 O'Doherty, R. M., Bracy, D. P., Osawa, H., Wasserman, D. H. and Granner, D. K. (1994) Rat skeletal muscle hexokinase II mRNA and activity are increased by a single bout of acute exercise. Am. J. Physiol. **266**, E171–E178
36 Desiniotis, A., Schafer, G., Klocker, H. and Eder, I. E. (2010) Enhanced antiproliferative and proapoptotic effects on prostate cancer cells by simultaneously inhibiting androgen receptor and cAMP-dependent protein kinase A. Int. J. Cancer **126**, 775–789
37 Rider, M. H., Vandamme, J., Lebeau, E., Vertommen, D., Vidal, H., Rousseau, G. G., Vandekerckhove, J. and Hue, L. (1992) The two forms of bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase result from alternative splicing. Biochem. J. **285**, 405–411
38 Dahia, P. L. (2000) PTEN, a unique tumor suppressor gene. Endocr. Relat. Cancer **7**, 115–129
39 Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W. B. and Bova, G. S. (1998) Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res. **58**, 204–209
40 Minchenko, O., Opentanova, I., Minchenko, D., Ogura, T. and Esumi, H. (2004) Hypoxia induces transcription of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene via hypoxia-inducible factor-1α activation. FEBS Lett. **576**, 14–20

Received 22 July 2010/14 October 2010; accepted 19 October 2010
Published as BJ Immediate Publication 19 October 2010, doi:10.1042/BJ20101104

Biochem. J. (2011) **433**, 225–233 (Printed in Great Britain) doi:10.1042/BJ20101104

online data

SUPPLEMENTARY ONLINE DATA

Androgen stimulates glycolysis for *de novo* lipid synthesis by increasing the activities of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 in prostate cancer cells

Jong-Seok MOON*, Won-Ji JIN*, Jin-Hye KWAK*, Hyo-Jeong KIM*, Mi-Jin YUN†, Jae-Woo KIM*, Sahng Wook PARK*[1] and Kyung-Sup KIM*[1]

*Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Center for Chronic Metabolic Disease Research, Brain Korea 21 Project for Medical Science, Yonsei University, College of Medicine, Seodaemungu, Seoul, Korea, and †Department of Diagnostic Radiology and Research Institute of Radiological Science, Yonsei University College of Medicine, Seodaemungu, Seoul, Korea

MATERIALS AND METHODS

Cell culture

LNCaP and PC3 cell lines were purchased from the A.T.C.C. and were maintained in RPMI 1640 (Invitrogen) containing 10 % FBS (fetal bovine serum; Invitrogen), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen). For the experiment assessing the effect of androgen, cells were pre-incubated in medium containing 5 % CT-FBS (charcoal-treated FBS) instead of FBS for 48 h and then further incubated in medium containing 1 % CT-FBS in the presence or absence of 10 nM R1881 for 72 h. CT-FBS was prepared by incubating FBS with dextran-coated charcoal at 4°C overnight and then removing the charcoal by centrifugation [1].

Construction of recombinant plasmids

The 5′ flanking regions of the human *HK2* and *PFKFB2* gene were amplified by PCR using the genomic DNA isolated from LNCaP cells and the following primers: for the *HK2* promoter from − 2054 to +21 (arbitrarily numbered according to the first base of the translated ATG annotated as +1), 5′-GTAATCCCAG-CTATTTGGGACGCT-3′ and 5′-AAGCAGATGCGAGGCAA-TTATCCTG-3′; for the *PFKFB2* promoter from − 1949 to +92 (numbers deduced from transcription start site as +1, GenBank® accession number NM_006212.2), 5′-TTAAATTGGGGCT-TCCACACTACACC-3′ and 5′-CCTACGATCTCTCCTAAGA-TCCCTCCC-3′. The PCR products were subcloned into the SmaI site of pGL3-basic vector (Promega). The resulting plasmids were designated as pHK2–Luc and pPFKFB2–Luc for HK2 and PFKFB2 respectively. The CRE mutant construct of pHK2–Luc and the ARE mutant construct of pPFKFB2–Luc were generated using a QuikChange® Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. The primers used for mutagenesis were: CRE, 5′-AATGGGC-GTGCGCTCTTCACTGATCC-3′ and 5′-GGATCAGTGAAG-AGCGCACGCCCATT-3′; ARE, 5′-AATCCATACTCATTGAA-CGTTATCCAGCC-3′ and 5′-GGCTGGATAACGTTCAATGA-GTATGGATT-3′. The expression vector for PKA-Cβ2 has been described previously [1]. For the generation of the PFKFB2 expression vector, the full-length cDNA was amplified from LNCaP cDNA using a primer set of 5′-GTCTGGGGCATC-TTCCTCAGAACA-3′ and 5′-GCTAGTCGGCCCCTTCTTG-CAT-3′, and was subcloned into the SmaI site of pSG5-HA-tagged expression vector. The integrity of each construct was verified by DNA sequencing.

Transient transfections and analyses of the promoter activity

PC3 cells (2×10^5 cells/well) were seeded into six-well plates and were transfected with reporter constructs and expression vectors (500 ng of DNA) using Lipofectamine™ and Plus reagent (Invitrogen) according to the manufacturer's instructions. pRL-SV40 (Promega) was co-transfected for standardization of transfection efficiency. At 48 h after transfection, cells were harvested and luciferase activities in 5 μl of cell lysates were measured using the Dual-Luciferase Reporter Assay System® (Promega) according to the manufacturer's instructions. The androgen-dependent transcriptional activation was assayed in LNCaP cells as follows. LNCaP cells (3×10^6 cells) were seeded in a 100-mm plate in medium containing 5 % CT-FBS. At 48 h after incubation, cells were transfected with 3 μg of HK2–Luc or PFKFB2–Luc mixed with 6 ng of pRL-SV40 (Promega) as described for PC3 cells. At 6 h after incubation, the medium was replaced with fresh medium containing 5 % CT-FBS. On the next day, cells were trypsinized, transferred to each well of a six-well plate, and further incubated in medium containing 1 % CT-FBS with or without 10 nM R1881 for 72 h. After incubation, luciferase activities were assayed as for PC3 cells described above. All experiments were carried out in triplicate, and luciferase activities were normalized to *Renilla* luciferase activity to standardize transfection efficiency.

Quantitative RT-PCR

Total RNA was isolated from cultured cells using the TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. For quantitative RT-PCR, cDNA was synthesized from 4 μg of total RNA using random hexamers and SuperScript reverse transcriptase II® (Invitrogen) according to the manufacturer's protocol. The resulting cDNA was diluted with 80 μl of TE buffer (10 mM Tris/HCl and 0.1 mM EDTA, pH 8.0) and 2 μl was subsequently used for RT-PCR. The mixture in a volume of 10 μl, containing cDNA and various sets of gene-specific primers, was mixed with 10 μl of 2 × SYBR Green PCR Master Mix® (Applied Biosystems) and then subjected to RT-PCR quantification using the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems). The sequences of the primers used in RT-PCR were

[1] Correspondence may be addressed to either of these authors (email swpark64@yuhs.ac or kyungsup59@yuhs.ac).

as follows: PFKFB1, 5′-CCCTGGCCAACTTCATTCAG-3′ and 5′-TGTGACTGGTCCACACCTTCA-3′; PFKFB2, 5′-CCATGAAGATCCGCAAACAGT-3′ and 5′-TCCTCAGTGAGATACGCCTTAACAT-3′; PFKFB3, 5′-CCGCTCATGAGACGCAATAGT-3′ and 5′-TTGATGCGAGGCTTTTTGGT-3′; PFKFB4, 5′-TGACTATGTCAACCGCGACAGT-3′ and 5′-AGCACTCAATGCGCCTCAT-3′; HK2, 5′-ATGGATGCCTAGATGACTTCCGCA-3′ and 5′-TAAGTGTTGCAGGATGGCTCGGA-3′; KLK3, 5′-CAGGTATTTCAGGTCAGCCACA-3′ and 5′-ATGACCTTCACAGCATCCGTGA-3′; and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CCCCTTCATTGACCTTCAACTA-3′ and 5′-GAGTCCTTCCACGATACCAAAG-3′. All reactions were performed in triplicate. The relative amounts of RNA were calculated by using the comparative C_T method. GAPDH was used as an invariant control.

Western blot analyses

Cells were harvested and lysed in 2× SDS loading buffer, then briefly sonicated. Lysates were cleared by centrifugation at 13000 ***g*** for 10 min at 4°C. Supernatants were collected and protein concentrations were determined using the Bradford assay (Bio-Rad). Aliquots of proteins (30 μg for each) were separated by SDS/PAGE (8 % gels) and transferred on to Protran® nitrocellulose transfer membranes (Whatman). Membranes were blocked in TBST (Tris-buffered saline containing 0.1 % Tween 20) and 5 % skimmed milk for 2 h, and incubated for 3 h or overnight with primary antibody diluted in TBST. After washing with TBST, the membrane was incubated with ImmunoPure HRP (horseradish peroxidase)-conjugated goat anti-rabbit or anti-mouse IgG antibody (Pierce) at room temperature for 20 min. The immunoreactive bands were visualized with the SuperSignal West Pico Chemiluminescent System® (Pierce). Antibodies used in the present study were: HK2 (catalogue number SC-28889, Santa Cruz Biotechnology), PFKFB2 (catalogue number SC-50953, Santa Cruz Biotechnology), p-PFKFB2 Ser^{466} (catalogue number SC-32966-R, Santa Cruz Biotechnology), p-PFKFB2 Ser^{483} (catalogue number SC-32967-R, Santa Cruz Biotechnology), androgen receptor (catalogue number SC-815, Santa Cruz Biotechnology) and HA-tag (catalogue number SC-7392, Santa Cruz Biotechnology); CREB (catalogue number #9197, Cell Signaling Technology) and phospho-CREB (catalogue number #9191, Cell Signaling Technology); tubulin (catalogue number CP-06, Calbiochem). The antibody against PKA-Cβ2 has been described previously [1].

PKA activity

Cells treated as indicated were washed with PBS and lysed with 1 ml of lysis buffer (20 mM Mops, 50 mM 2-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodiumvanadate, 5 mM EGTA, 2 mM EDTA, 1 % Nonidet P40, 1 mM DTT, 1 mM benzamidin, 1 mM PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin). The cell lysates were collected 10 min after incubation, and cleared supernatants were collected by centrifugation at 13400 ***g*** for 15 min. PKA activity in lysates was determined using the StressXpress® PKA kinase assay kit (catalogue number EKS-390A, Assay Design).

ChIP assay

ChIP assays were carried out using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, formaldehyde was added directly to cultured cells after treatment with or without 10 nM R1881. After cross-linking, cells were lysed with lysis buffer containing protease inhibitors, nuclei were isolated, and the chromatin was sheared to an average size in 250–500 bp long sections by sonication. The sheared chromatin was pre-cleared with salmon sperm DNA/Protein A–sepharose and then precipitated with the antibody against the androgen receptor (catalogue number SC-815, Santa Cruz Biotechnology). Rabbit IgG control was used to monitor non-specific interactions. Immune complexes were adsorbed on to salmon sperm DNA/Protein A–sepharose beads, washed extensively, and eluted with elution buffer. After precipitation, DNA was resuspended in water and PCR was performed to amplify ARE regions of the *PFKFB2* gene using the following primers: ARE1, 5′-GGCAACTAGAGCACTCTGGGAGTTGT-3′ and 5′-CTTTTACAGTGGAGGTGAGTTTGCACC-3′; ARE2, 5′-ATAGTTTCAAAGCCGACCAGAAAGC-3′ and 5′-TTGCCCATCCAATCTGGTAAGGAA-3′; ARE3, 5′-CCCTGACGTAACTAGAAGCACCACG-3′ and 5′-CACTAAATTGGGATGCAACCTCAAGTG-3′; ARE4, 5′-CTGTCACTCCAGCAGAAAGAGGCAT-3′ and 5′-CAAGGGAACATGCCAAGGAGACAG-3′; and negative control, 5′-CCACCTTCACCAAGGAAAGTGCTAAA-3′ and 5′-CAAGGCACACCCTCTAAAACTTAGCTTC-3′.

RNAi

Briefly, LNCaP cells (3×10^6 cells) were seeded in a 100-mm plate in medium containing 5 % CT-FBS. At 48 h after incubation, cells were transfected with 18 μl of a 20 μM three independent siRNA solution and 5 μl of Lipofectamine™ RNAiMAX were incubated in 500 μl of serum-free Opti-MEM medium (Invitrogen) for 20 min and overlaid on to LNCaP cells cultured in the wells of a six-well plate in 500 μl of serum-free Opti-MEM medium. At 6 h after incubation, the medium was replaced with fresh medium containing 5 % CT-FBS. Cells were transfected again in the same way on the next day. After overnight incubation, cells were treated with or without 10 nM R1881 for 72 h in medium containing 1 % CT-FBS. The mRNA and protein levels of PFKFB2 were measured by quantitative RT-PCR and Western blot analyses.

REFERENCE

1 Moon, J. S., Lee, M. Y., Park, S. W., Han, W. K., Hong, S. W., Ahn, J. H. and Kim, K. S. (2008) Androgen-dependent activation of human cytomegalovirus major immediate-early promoter in prostate cancer cells. Prostate **68**, 1450–1460

Received 22 July 2010/14 October 2010; accepted 19 October 2010
Published as BJ Immediate Publication 19 October 2010, doi:10.1042/BJ20101104