Regular Article

Hypoxia Decreases Glucagon-Like Peptide-1 Secretion from the GLUTag Cell Line

Yoshitaka Kihira,*[,a,#] Ariunzaya Burentogtokh,[a,#] Mari Itoh,[a] Yuki Izawa-Ishizawa,[a] Keisuke Ishizawa,[b,†] Yasumasa Ikeda,[a] Koichiro Tsuchiya,[b] and Toshiaki Tamaki[a]

[a] *Department of Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School; 3–18–15 Kuramoto-cho, Tokushima 770–8503, Japan: and* [b] *Department of Medical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School; 3–18–15 Kuramoto-cho, Tokushima 770–8503, Japan.*
Received August 25, 2014; accepted January 12, 2015

Glucagon-like peptide-1 (GLP-1), an incretin hormone, is secreted from L cells located in the intestinal epithelium. It is known that intestinal oxygen tension is decreased postprandially. In addition, we found that the expression of hypoxia-inducible factor-1α (HIF-1α), which accumulates in cells under hypoxic conditions, was significantly increased in the colons of mice with food intake, indicating that the oxygen concentration is likely reduced in the colon after eating. Therefore, we hypothesized that GLP-1 secretion is affected by oxygen tension. We found that forskolin-stimulated GLP-1 secretion from GLUTag cells, a model of intestinal L cells, is suppressed in hypoxia (1% O_2). Forskolin-stimulated elevations of preproglucagon (ppGCG) and proprotein convertase 1/3 (PC1/3) mRNA expression were decreased under hypoxic conditions. The finding that H89, a protein kinase A (PKA) inhibitor, inhibited the forskolin-stimulated increase of ppGCG and PC1/3 indicated that the cAMP-PKA pathway is involved in the hypoxia-induced suppression of the genes. Hypoxia decreased hexokinase 2 mRNA and protein expression and increased lactate dehydrogenase A mRNA and protein expression. Concomitantly, lactate production was increased and ATP production was decreased. Together, the results indicate that hypoxia decreases glucose utilization for ATP production, which probably causes a decrease in cAMP production and in subsequent GLP-1 production. Our findings suggest that the postprandial decrease in oxygen tension in the intestine attenuates GLP-1 secretion.

Key words glucagon-like peptide-1; hypoxia; cAMP; hypoxia-inducible factor-1α; L cell

Glucagon-like peptide-1 (GLP-1), an incretin hormone, acts on pancreatic β cells by enhancing insulin secretion from the cells.[1,2] In addition to directly stimulating insulin secretion, GLP-1 also helps to confer glucose sensitivity to β cells by stimulating glucose transporters and glucokinase.[3,4] Thus, GLP-1 is important for insulin-mediated glucose homeostasis. Indeed, pharmacological GLP-1 analogues and inhibitors of dipeptidyl peptidase-4, which inactivates GLP-1, have been approved for the treatment of type 2 diabetes.

GLP-1 is secreted from L cells located in the intestinal epithelium. The density of the L cells increases from the duodenum to the rectum.[5–7] Nutrient intake acts as a stimulus to the L cells to result in a biphasic pattern of GLP-1 secretion. An initial rapid rise of the circulating GLP-1 levels occurs 15–30 min after a meal, and this is followed by a second minor peak 90–120 min thereafter.[8] An important trigger of the GLP-1 secretion is the increase of cytoplasmic cAMP concentration.[9] Lipids stimulate GPR119, a Gs-coupled receptor, and ligand binding to GPR119 activates adenylyl cyclase to result in an increase in cAMP levels and enhanced GLP-1 secretion.[10,11] In addition, it has been shown that L-glutamine also stimulates GLP-1 secretion through the elevation of cytoplasmic cAMP concentration.[12,13]

The intestine is a unique organ in terms of oxygen tension. As the intestinal lumen is anoxic, the intestinal epithelium is in the hypoxic condition.[14] In addition, it is known that postprandial oxygen tension in the villus decreases from resting values of 14–17 to 4–7 mmHg.[15] As intestinal L cells are localized in the intestinal epithelium, GLP-1 secretion is probably influenced by changes of the oxygen tension. Therefore, we hypothesized that GLP-1 secretion is affected by oxygen tension.

In the present study, we examined the effect of hypoxia on GLP-1 secretion from intestinal L cells using GLUTag cell line, a mouse intestinal L cell model. The results suggest that intestinal oxygen concentrations are decreased after food intake, because hypoxia-inducible factor-1α (HIF-1α) is postprandially increased. We also showed that hypoxia decreased GLP-1 secretion from the L cells. This is probably caused by the decrease of GLP-1 production. Thus, GLP-1 secretion was probably affected by oxygen tension.

MATERIALS AND METHODS

Antibodies The following antibodies were used for the analyses: rabbit polyclonal anti-HIF-1α antibody (catalog no. 10006421, Cayman Chemicals, U.S.A.); mouse monoclonal anti-pimonidazole antibody (HP1-100, Natural Pharmacia International, Inc., U.S.A.); rabbit polyclonal anti-cAMP response element binding protein (CREB) antibody (#9197, Cell Signaling Technology, U.S.A.); rabbit polyclonal anti-phospho-CREB (pCREB) antibody (#9189, Cell Signaling Technology); rabbit polyclonal anti-hexokinase 2 (HK2) antibody (Cell Signaling Technology); rabbit polyclonal anti-lactate dehydrogenase A (LDHA) antibody (#2012, Cell Signaling Technology); and

[#] These authors contributed equally to this work.

[†] Present address: *Department of Clinical Pharmacology, Institute of Health Biosciences, The University of Tokushima Graduate School; 3–18–15 Kuramoto-cho, Tokushima 770–8503, Japan.*

* To whom correspondence should be addressed. e-mail: kihira@tokushima-u.ac.jp

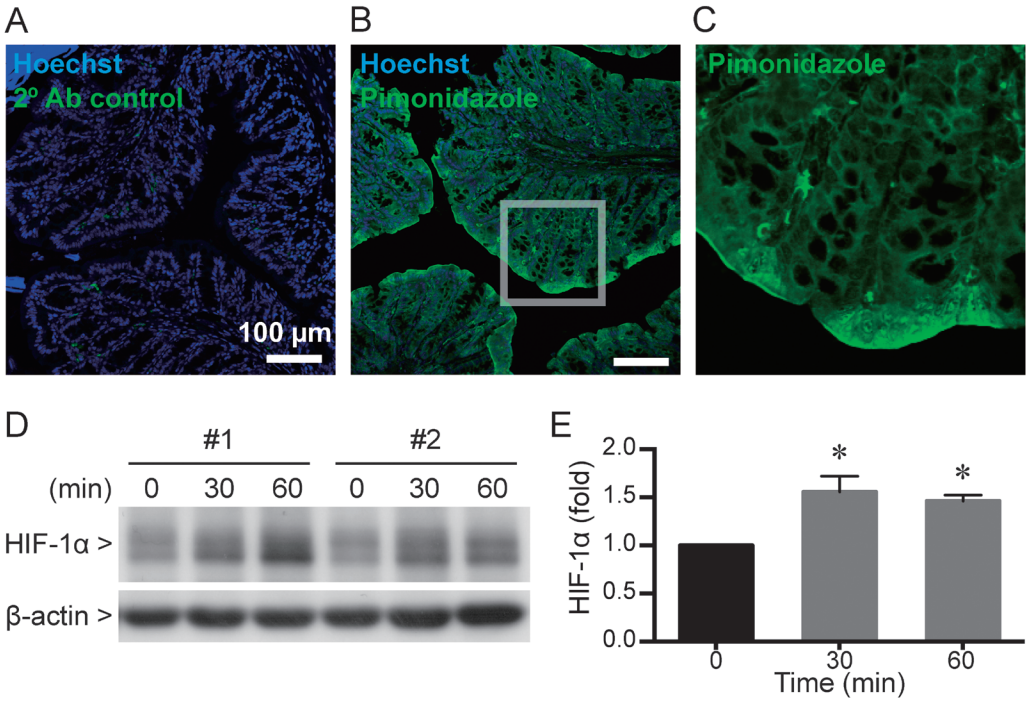

Fig. 1. Hypoxic Environment in Mouse Colon

Mice fasted for 24 h were injected with pimonidazole. One hour after the injection, the colon was resected and stained without (A) or with (B and C) the antibody against pimonidazole. (A) Representative merged image of nuclear staining with Hoechst (blue) and control staining with secondary antibody only (2° Ab control; green). (B) Representative merged image of nuclear staining with Hoechst (blue) and pimonidazole staining (green). (C) Magnified image of the boxed area in B. Bars indicate 100 μm. (D) Representative Western blot image of the time course of HIF-1α expression in colons after food intake. The image shows temporal changes of HIF-1α expression after food intake in two mice (#1 and #2). (E) Quantification of the immunostaining intensities of HIF-1α. Values are means±S.E.M. (n=5). *p<0.05.

rabbit monoclonal anti-β-actin antibody (#4970, Cell Signaling Technology).

Mice and Tissue Samples All studies with mice were carried out in strict accordance with the guidelines of the Animal Research Committee of the University of Tokushima. Male C57BL/6J mice were from Japan SLC, Inc. The animals were maintained in a 12-h light/dark cycle with free access to water and a standard diet. For immunostaining, eight-week-old mice were fasted for 24 h and then 60 mg/kg pimonidazole was injected intraperitoneally. One hour after the pimonidazole injection, the colon was resected and fixed with 4% paraformaldehyde. For Western blot, eight-week-old mice were fasted for 24 h and 500 μL of liquid food (Enjoy climeal; energy 200 kcal, protein 7.5 g, fat 5.6 g, carbohydrates 31.3 g, sugars 29.4 g, dietary fiber 1.9 g, sodium 141 mg; Clinico Co., Ltd., Japan) was given orally through a feeding tube. Thirty or sixty minutes after the food intake, the colon was resected and lysed in lysis buffer (20 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, protease inhibitor mixture (2 g/mL aprotinin, 1 μg/mL leupeptin, 2 μg/mL antipain, and 10 μg/mL benzamidine), and phosphatase inhibitor mixture (10 mM NaF, 60 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate)).

Immunostaining Frozen sections of the colon were intermittently boiled in 10 mM citrate buffer for 15 min and treated with 0.3% Triton X-100 in TBS for 10 min. Then, the sections were blocked with TBS containing 10% normal goat serum for 1 h at room temperature. The sections were incubated with blocking solution containing primary antibodies and Hoechst 33342 overnight at 4°C. Then, the sections were washed and probed with Alexa-conjugated secondary reagents (Life Technologies). Fluorescence images were taken with an A1 confocal microscope configured with a Ti-E inverted microscope and an objective lens having 20× magnification (Nikon).

Cell Treatment Mouse intestinal L cell line GLUTag cells[16] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were plated at 50000 cells/cm^2 in 12-well culture plates and allowed to grow for 48 h. The cells were preincubated with KRH buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and 0.1% bovine serum albumin (BSA)) for 30 min. Then, the cells were incubated with 10 μM forskolin (Fsk)/1-methyl-3-(2-methylpropyl)-7*H*-purine-2,6-dione (IBMX) in KRH buffer containing 2.5 mM glucose for 4 h at 37°C. For treatment with H89, a protein kinase A (PKA) inhibitor, or dimethyloxalylglycine (DMOG), a prolyl hydroxylase (PHD) inhibitor, the GLUTag cells were preincubated with KRH buffer for 30 min, and then the cells were incubated with 10 μM Fsk/IBMX and 10 μM H89 or 500 μM DMOG in KRH buffer containing 2.5 mM glucose for 4 h at 37°C.

Western Blot Cells or a piece of colon were lysed in the lysis buffer. Proteins were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked and incubated with a primary antibody overnight at 4°C. Then, the membranes were probed with an horseradish peroxidase (HRP)-conjugated secondary antibody produced in goat (KPL). Immunoreactive bands were detected with ECL (GE Healthcare) and visualized by exposing the membranes to X-ray films (GE Healthcare). The proteins were quantified by densitometric analysis using ImageJ analysis software.

Real-Time Polymerase Chain Reaction (PCR) Total

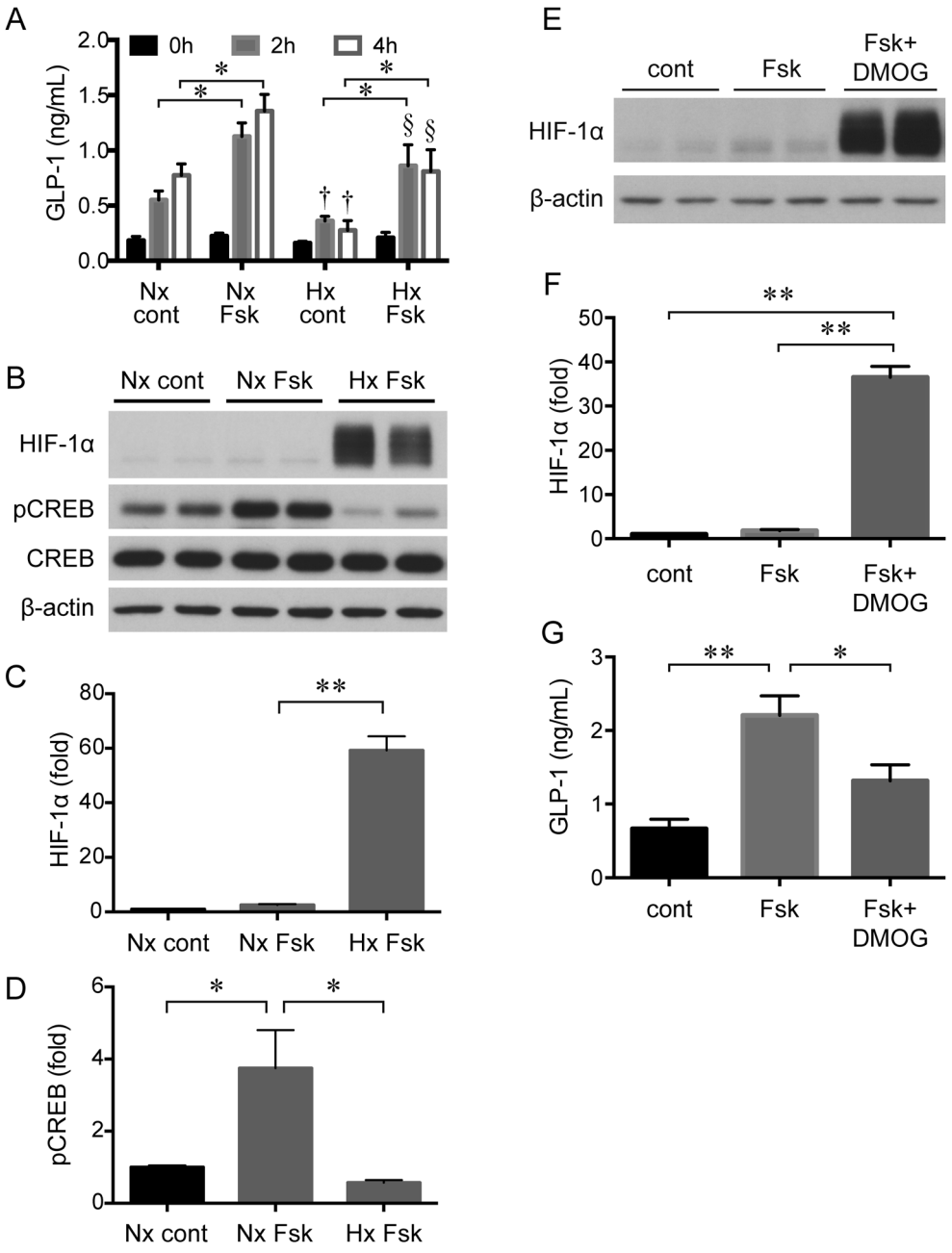

Fig. 2. GLP-1 Secretion from GLUTag Cells

GLUTag cells were treated with 10 μM Fsk/IBMX under the normoxic (Nx) or hypoxic (Hx) condition. (A) Time course of GLP-1 secretion from GLUTag cells. Black bars indicate 0 h, gray bars indicate 2 h, and white bars indicate 4 h after the Fsk/IBMX treatment. Values are means±S.E.M. (n=6). *p<0.01. $^{\dagger}p$<0.05 *vs.* Nx cont in the same period of the Fsk/IBMX treatment. $^{\S}p$<0.05 *vs.* Nx Fsk in the same period of the Fsk/IBMX treatment. (B) Western blots of HIF-1α, pCREB, CREB, and β-actin are shown. The samples were prepared at 4 h after Fsk/IBMX treatment. (C, D) Quantification of the immunostaining intensities of HIF-1α (C) and pCREB (D). Values are means±S.E.M. (n=6). **p<0.01 and *p<0.05. GLUTag cells were treated with 10 μM Fsk/IBMX or 10 μM Fsk/IBMX and 500 μM DMOG for 4 h. (E) Western blots of HIF-1α and β-actin are shown. (F) Quantification of the immunostaining intensities of HIF-1α. Values are means±S.E.M. (n=6). **p<0.01. (G) GLP-1 secretion from GLUTag cells treated with Fsk/IBMX and DMOG for 4 h. Values are means±S.E.M. (n=6). **p<0.01 and *p<0.05.

RNA was prepared with ISOGEN (Nippon Gene). One microgram of total RNA was subjected to reverse transcription (RT)-PCR using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Quantitative RT-PCR was performed using the iCycler MyiQ2 Real-Time PCR Detection System (BIO-RAD Laboratories Inc.) with THUNDERBIRD SYBR qPCR Mix (TOYOBO Co., Ltd.). The following primers were used for the analyses: preproglucagon (ppGCG) forward (5′-TGA CGT TTG GCA ATG TTG TT-3′), ppGCG reverse (5′-CAG AGG AGA ACC CCA GAT CA-3′); proprotein convertase 1/3 (PC1/3) forward (5′-AGC TGG TGT GTC TCT GAT CTT G-3′), PC1/3 reverse (5′-ACC AAA CGC AAA AGA AGG CG-3′); HK2 forward (5′-AGA ACC GTG GAC TGG ACA AC-3′), HK2 reverse (5′-GCC AGA TCT CTC ACC GTC TC-3′); LDHA forward (5′-CAA AGT CCA AGA TGG CAA CCC-3′), LDHA reverse (5′-AGC ACC AAC CCC AAC AAC TGT-3′); and 36B4 forward (5′-GCT CCA AGC AGA TGC AGC A-3′), 36B4 reverse (5′-CCG GAT GTG AGG CAG CAG-3′).

GLP-1 Secretion and Metabolite Assay The incubation buffer was collected and centrifuged to remove any floating cells. Assays for GLP-1 and lactate were carried out with an ELISA kit (Shibayagi) and a Lactate Assay Kit II (BioVision),

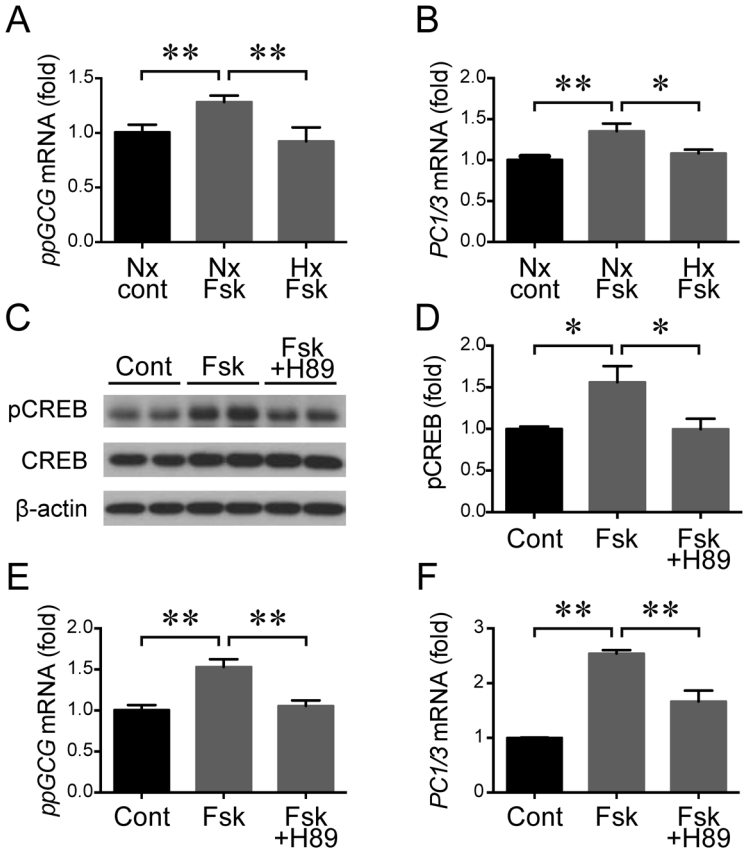

Fig. 3. mRNA Levels of ppGCG and PC1/3

GLUTag cells were treated with 10 μM Fsk/IBMX for 4 h under the normoxic (Nx) or hypoxic (Hx) condition. (A) mRNA levels of ppGCG are shown. Values are means±S.E.M. (n=4). (B) mRNA levels of PC1/3 are shown. Values are means±S.E.M. (n=6). GLUTag cells were treated with 10 μM Fsk/IBMX and 10 μM H89, a PKA inhibitor, for 4 h under the normoxic condition. (C) Western blots of pCREB, CREB, and β-actin. (D) Quantification of the immunostaining intensities of pCREB. (E) mRNA levels of ppGCG are shown. Values are means±S.E.M. (n=4). (F) mRNA levels of PC1/3 are shown. Values are means±S.E.M. (n=4). $^{**}p<0.01$ and $^{*}p<0.05$.

respectively. ATP concentrations in GLUTag cells were measured with an EnzyLight ATP Assay Kit (BioAssay Systems).

Statistical Studies One-way ANOVA was conducted with Prism 6 (GraphPad Software, Inc.). A $p<0.05$ was considered statistically significant.

RESULTS

Food Intake Increases Colonic HIF-1α Expression First, the hypoxic environment of the colon was investigated. After a 24-h fast, mice were injected with pimonidazole intraperitoneally. One hour after the injection, the colon was resected and immunostained with anti-pimonidazole antibody. As can be seen from Figs. 1B and C, the colon showed pimonidazole signals on the luminal surface, whereas control staining without the primary antibody showed no signal (Fig. 1A). The results suggest that the luminal epithelium in colon is hypoxic. Next, changes in the degree of hypoxia in the colons after food intake were investigated. To this end, 500 μL of liquid food was orally given to mice fasted for 24 h, and the expression levels of HIF-1α were measured because HIF-1α is accumulated in cells under the hypoxic condition.[17] HIF-1α expression was significantly increased in the colon with food intake (Figs. 1D, E). Therefore, oxygen concentration is probably reduced in the colon after food intake, as shown in a previous study.[15]

Hypoxia Decreases GLP-1 Secretion from L Cells To investigate the effect of hypoxia on GLP-1 secretion, the intestinal L cell line GLUTag was treated with 10 μM Fsk/IBMX under the hypoxic condition, and then GLP-1 secretion time course was measured under the hypoxic condition (1% O_2). As shown in Fig. 2A, the Fsk/IBMX treatment stimulated GLP-1 secretion from GLUTag cells compared with the normoxic control using KRH buffer containing 2.5 mM glucose at 2 h and 4 h after the treatment. The basal and Fsk/IBMX-stimulated GLP-1 secretion was significantly lower at 2 h and 4 h after the treatment under the hypoxic condition than under the normoxic condition. It is known that the elevation of intracellular cAMP activates CREB by phosphorylation.[18,19] In addition, it is known that hypoxia induces the accumulation of HIF-1α.[17] Therefore, pCREB and HIF-1α expression was investigated. GLUTag cells were treated with 10 μM Fsk/IBMX under the normoxic or hypoxic condition for 4 h. The levels of pCREB were elevated by the Fsk/IBMX treatment under the normoxic condition, whereas hypoxia had no effect on the pCREB levels. However, the increase of the pCREB levels with the Fsk/IBMX treatment was significantly attenuated under the hypoxic condition, although there were no changes in the total CREB protein levels (Figs. 2B, D). On the other hand, HIF-1α expression was increased only under the hypoxic condition (Figs. 2B, C). In addition, the co-incubation of Fsk/IBMX and DMOG, which inhibits PHD and induces the accumulation of HIF-1α independent of hypoxia, increased HIF-1α and attenuated GLP-1 secretion from GLUTag cells (Figs. 2E–G), indicating that HIF-1α *per se* participates in the reduction of the GLP-1 secretion. Therefore, it is suggested that pCREB reduc-

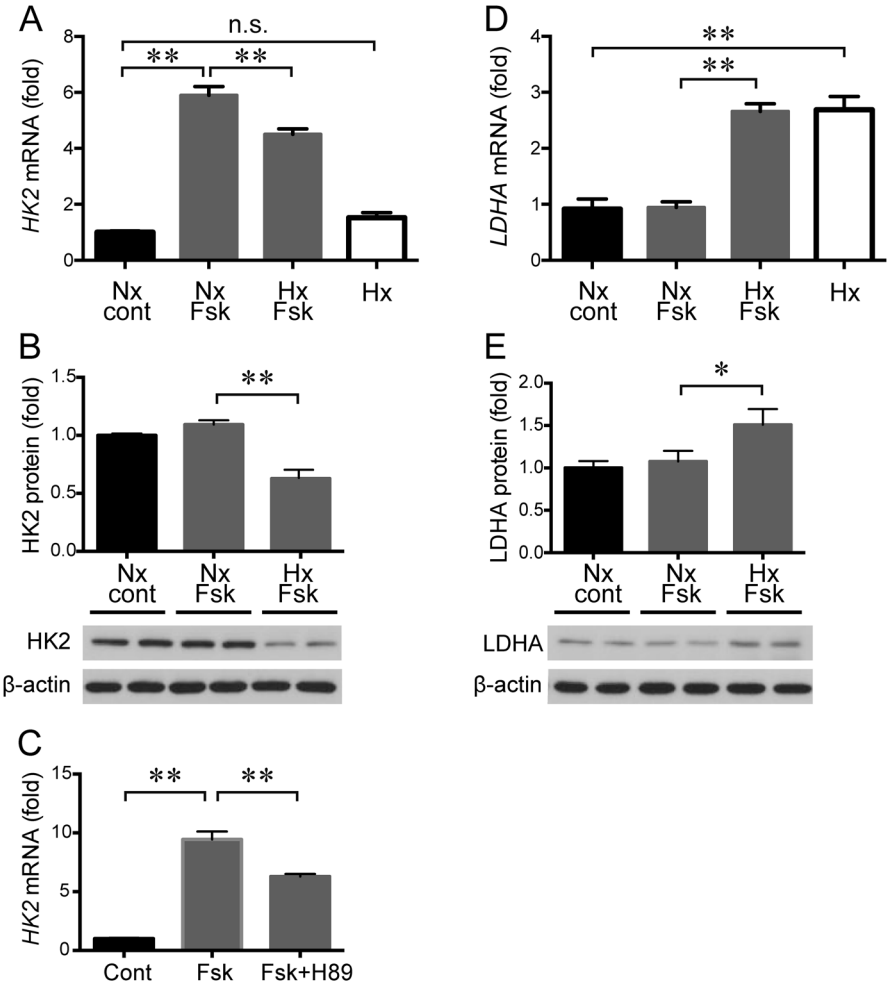

Fig. 4. Effects of Hypoxia on ATP Production

GLUTag cells were treated with 10 μM Fsk/IBMX for 4 h under the normoxic (Nx) or hypoxic (Hx) condition. mRNA (A) and protein (B) levels of HK2 are shown. Values are means±S.E.M. (n=6). (C) Effect of H89 on the mRNA expression of HK2 is shown. GLUTag cells were treated with 10 μM H89 and 10 μM Fsk/IBMX for 4 h under the normoxic condition. Values are means±S.E.M. (n=4). mRNA (D) and protein (E) levels of LDHA are shown. Values are means±S.E.M. (n=6). Western blots are shown under the graphs in B and E. $^{**}p<0.01$ and $^{*}p<0.05$.

tion and/or HIF-1α accumulation are involved in the gradual secretion of GLP-1 under the hypoxic condition.

GLP-1 Production Is Reduced in the Hypoxic Condition GLP-1 is derived from ppGCG.[20] In addition, ppGCG mRNA expression is mediated by cAMP signal comprising CREB.[21,22] Because we found that hypoxia decreased pCREB, we examined ppGCG expression. Fsk/IBMX significantly increased ppGCG mRNA expression in the normoxic condition. Hypoxia reduced the increase of ppGCG mRNA expression by Fsk/IBMX (Fig. 3A). GLP-1 is cleaved from ppGCG by PC1/3.[23] In addition, PC1/3 gene has two CREB binding sites,[24] suggesting that CREB regulates PC1/3 expression. Therefore, we measured PC1/3 expression as well. The PC1/3 mRNA expression was increased by Fsk/IBMX treatment (Fig. 3B). The increase of the PC1/3 mRNA expression with Fsk/IBMX treatment was significantly attenuated under the hypoxic condition (Fig. 3B). Then, to study the involvement of the PKA pathway in the regulation of ppGCG and PC1/3, H89, a PKA inhibitor, was tested. The inhibitory effect of H89 on PKA was confirmed by measuring the phosphorylation of CREB, a target of PKA. H89 co-incubation with Fsk/IBMX markedly reduced the phosphorylation of CREB (Figs. 3C, D), suggesting that H89 inhibited PKA activity. As shown in Figs. 3E and F, elevations of the mRNA levels of ppGCG and PC1/3 with Fsk/IBMX treatment were cancelled by the addition of H89. The results suggest that hypoxia reduces GLP-1 production *via* suppression of GLP-1 synthesis regulated by cAMP signal.

Hypoxia Decreases ATP Production in L Cells ATP is the source of cAMP. In addition, ATP production in L cells is an important factor affecting GLP-1 secretion.[25,26] Because hypoxia influences glycolysis,[27,28] it is speculated that ATP production in L cells is affected by hypoxia. Therefore, we measured the expression of HK2 and LDHA, which are key regulators of glycolysis and are regulated by both HIF-1α and CREB,[29–31] under the hypoxic condition. Treatment with Fsk/IBMX increased HK2 mRNA expression in the L cells (Fig. 4A). In contrast, HK2 protein expression was not significantly affected by the Fsk/IBMX treatment compared with the control (Fig. 4B). Hypoxia decreased HK2 mRNA and protein expression (Figs. 4A, B). Incubation of the cells with Fsk/IBMX and H89 decreased the upregulation of HK2 mRNA expression by Fsk/IBMX, indicating that HK2 expression was regulated through the PKA pathway. LDHA mRNA and protein expression was not altered by Fsk/IBMX under the normoxic condition, but was significantly increased under the hypoxic

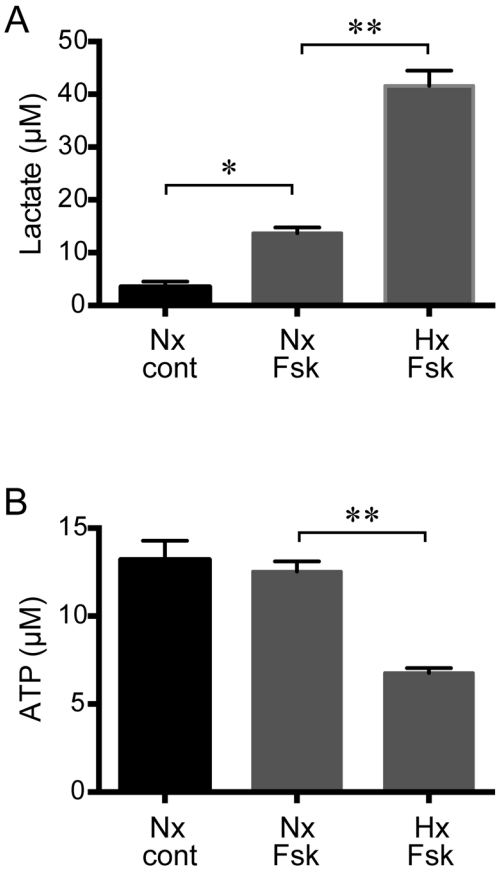

Fig. 5. Production of Lactate and ATP

GLUTag cells were treated with 10 μM Fsk/IBMX for 4 h under the normoxic (Nx) or hypoxic (Hx) condition. (A) Lactate concentrations in the culture medium were measured. Values are means±S.E.M. (n=8). (B) ATP concentrations in the cells were measured. Values are means±S.E.M. (n=4). $^{**}p<0.01$ and $^{*}p<0.05$.

condition (Figs. 4D, E). The results indicate that glucose utilization for ATP synthesis is decreased and instead, lactate production is increased under the hypoxic condition. Therefore, lactate and ATP production was measured. Lactate concentration in the culture medium was increased by the Fsk/IBMX treatment under the normoxic condition, and the increase became much more prominent under the hypoxic condition (Fig. 5A). Although ATP concentration in the GLUTag cells was not significantly changed after the Fsk/IBMX treatment under the normoxic condition, it was significantly decreased under the hypoxic condition (Fig. 5B). The decreased ATP production under the hypoxic condition is perhaps a vital contributor to the reduction of GLP-1 secretion.

DISCUSSION

In the present study, we showed that oxygen concentration affected GLP-1 secretion. Intestinal circulation is approximately 20% of the total cardiac output during fasting conditions, and is increased by twofold after food intake.[32–34] Intestinal blood flow and oxygen extraction locally increase. However, it is known that oxygen tension in the villus decreases from resting values of 14–17 to 4–7 mmHg.[15] In our experiment, pimonidazole staining clearly showed that the colonic epithelium is hypoxic (Fig. 1). In addition, it was clarified that HIF-1α was postprandially increased in colon (Fig. 1). These results indicate that oxygen tension in colon was decreased after food intake. Thus, food intake probably induces hypoxia in the intestine, as shown in a previous study.[15]

We hypothesized that the fluctuation of oxygen tension in colon affects GLP-1 secretion. It is known that the pO_2 threshold for pimonidazole binding is 10 mmHg (1–2% O_2),[35–37] indicating that oxygen tension in the intestinal epithelium is less than 10 mmHg. Therefore, GLP-1 secretion was studied at 1% O_2. Fsk/IBMX-stimulated GLP-1 secretion from GLUTag cells was suppressed under the hypoxic condition (Fig. 2). GLP-1 secretion is stimulated immediately after food intake to reach maximal levels 15–30 min thereafter, and this is followed by a second minor peak 90–120 min thereafter.[8] The attenuation of the initial secretion and second minor peak may be a result of the hypoxia-induced attenuation of GLP-1 secretion.

Fsk/IBMX-induced CREB phosphorylation was decreased to the basal level under the hypoxic condition (Fig. 3). The activity of PKA, which phosphorylates CREB, was decreased under the hypoxic condition[38] and as such, CREB phosphorylation was probably decreased under the hypoxic condition. In contrast, HIF-1α was induced only in the hypoxic condition (Fig. 2). As both proteins are transcription factors, the decrease of GLP-1 secretion under the hypoxic condition is probably regulated in part by changes of the gene expression. GLP-1 is produced by cleavage from ppGCG[23] and ppGCG mRNA expression is mediated by cAMP signal comprising CREB.[21,22] In addition, it is suggested that PC1/3, which cleaves GLP-1 from ppGCG, is transactivated by CREB.[24] Therefore, it is possible that ppGCG and PC1/3 expression is affected by hypoxia. Our results showed that the upregulation of those genes with Fsk/IBMX was cancelled under the hypoxic condition (Fig. 3). In addition, H89 also attenuated the elevation of those genes with Fsk/IBMX (Fig. 3). Therefore, the PKA-CREB pathway is probably important for the regulation of GLP-1 production, although PC1/3 mRNA levels were not completely suppressed by the H89 treatment, suggesting that other factors are involved in PC1/3 regulation.

ATP is the source of cAMP. In addition, ATP is important for GLP-1 secretion.[25,26] As hypoxia affects glycolysis,[27,28] HK2 and LDHA expression under the hypoxic condition was studied. HK2 mRNA expression was increased by Fsk/IBMX treatment and the increase was cancelled by co-incubation with H89 (Fig. 4), indicating that the regulation of HK2 mRNA expression is PKA-dependent in the intestinal L cells. In addition, hypoxia *per se* did not increase the HK2 expression, indicating that the involvement of HIF-1α in the regulation of HK2 is insignificant in the cells. Although it is known that HK2 is regulated by both CREB and HIF-1α,[29,30] CREB is probably the main regulator of HK2 in the L cells. In contrast to the mRNA expression, HK2 protein expression was not significantly increased by Fsk/IBMX treatment compared with the control, suggesting that HK2 protein degradation was stimulated. However, hypoxia markedly decreased the HK2 protein expression. The decrease of HK2 mRNA expression and the stimulation of HK2 protein degradation may be cooperatively involved in the decrease of HK2 protein expression. In contrast, LDHA mRNA and protein expression was increased under the hypoxic condition (Fig. 4). In addition, the hypoxic condition increased LDHA mRNA levels. These results indicate that HIF-1α regulates LDHA in the L cells. Therefore, LDHA is probably regulated by HIF-1α, but not CREB, in the L cells, although it is reported that CREB

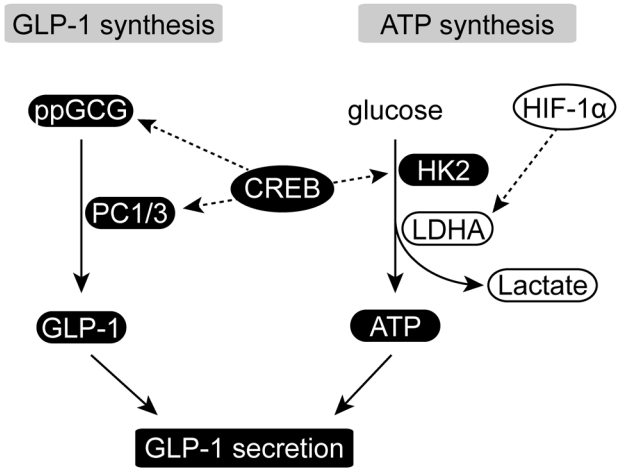

Fig. 6. Schematic Diagram of Our Model

White characters on black background indicate factors downregulated under the hypoxic condition, and black characters on white background indicate factors upregulated under the hypoxic condition. Broken arrows indicate regulation by transcriptional factors. Under the hypoxic condition, GLP-1 production and ATP production are decreased, and these probably lead to the reduction of GLP-1 secretion.

binds to the LDHA gene promoter.[31] The fact that lactate concentration in the culture medium was increased and ATP concentration in the L cells was decreased under the hypoxic condition (Fig. 5) indicated that hypoxia decreased glucose utilization for ATP production. Fsk/IBMX treatment *per se* did not increase ATP concentration in the L cells, indicating that the Fsk/IBMX-stimulated GLP-1 secretion is independent of the ATP production, and the hypoxia-induced decrease of ATP perhaps causes the decreased GLP-1 secretion through the reduction of cAMP.

In summary, Fsk stimulation activates CREB by phosphorylation and concomitantly, ppGCG, PC1/3, and HK2 are upregulated. This probably induces the upregulation of GLP-1 and ATP production. However, under the hypoxic condition, the phosphorylation of CREB is reduced and the expression levels of ppGCG, PC1/3, and HK2 are also reduced. This probably leads to the reduction of GLP-1 and ATP production. In addition, under the hypoxic condition, HIF-1α is accumulated and induces LDHA, which reduces glucose availability for ATP production. The accumulation of HIF-1α is also one of the causes of the reduction of GLP-1 secretion (Fig. 6).

Acknowledgments We would like to thank Dr. Daniel Drucker (Mount Sinai Hospital SLRI, Toronto) for permission to use the GLUTag cell line. This work was partially supported by the Kato Memorial Bioscience Foundation, and by KAKENHI (26870409).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Creutzfeldt W. The incretin concept today. *Diabetologia*, **16**, 75–85 (1979).
2) Kieffer TJ, Habener JF. The glucagon-like peptides. *Endocr. Rev.*, **20**, 876–913 (1999).
3) Marchetti P, Bugliani M, Boggi U, Masini M, Marselli L. The pancreatic beta cells in human type 2 diabetes. *Adv. Exp. Med. Biol.*, **771**, 288–309 (2012).
4) Weir GC, Bonner-Weir S. Islet β cell mass in diabetes and how it relates to function, birth, and death. *Ann. N. Y. Acad. Sci.*, **1281**, 92–105 (2013).
5) Evans GS, Potten CS. The distribution of endocrine cells along the mouse intestine: a quantitative immunocytochemical study. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.*, **56**, 191–199 (1988).
6) Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, Goke B. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *Eur. J. Clin. Invest.*, **22**, 283–291 (1992).
7) Pinto HC, Portela-Gomes GM, Grimelius L, Kohnert KD, de Sousa JC, Albuquerque MA. The distribution of endocrine cell types of the gastrointestinal mucosa in genetically diabetic (db/db) mice. *Gastroenterology*, **108**, 967–974 (1995).
8) Rask E, Olsson T, Soderberg S, Johnson O, Seckl J, Holst JJ, Ahren B, Northern Sweden Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA). Impaired incretin response after a mixed meal is associated with insulin resistance in nondiabetic men. *Diabetes Care*, **24**, 1640–1645 (2001).
9) Ezcurra M, Reimann F, Gribble FM, Emery E. Molecular mechanisms of incretin hormone secretion. *Curr. Opin. Pharmacol.*, **13**, 922–927 (2013).
10) Chu ZL, Carroll C, Alfonso J, Gutierrez V, He H, Lucman A, Pedraza M, Mondala H, Gao H, Bagnol D, Chen R, Jones RM, Behan DP, Leonard J. A role for intestinal endocrine cell-expressed g protein-coupled receptor 119 in glycemic control by enhancing glucagon-like peptide-1 and glucose-dependent insulinotropic peptide release. *Endocrinology*, **149**, 2038–2047 (2008).
11) Cox HM, Tough IR, Woolston AM, Zhang L, Nguyen AD, Sainsbury A, Herzog H. Peptide YY is critical for acylethanolamine receptor Gpr119-induced activation of gastrointestinal mucosal responses. *Cell Metab.*, **11**, 532–542 (2010).
12) Reimann F, Williams L, da Silva Xavier G, Rutter GA, Gribble FM. Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells. *Diabetologia*, **47**, 1592–1601 (2004).
13) Tolhurst G, Zheng Y, Parker HE, Habib AM, Reimann F, Gribble FM. Glutamine triggers and potentiates glucagon-like peptide-1 secretion by raising cytosolic Ca^{2+} and cAMP. *Endocrinology*, **152**, 405–413 (2011).
14) Espey MG. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. *Free Radic. Biol. Med.*, **55**, 130–140 (2013).
15) Bohlen HG. Intestinal tissue pO_2 and microvascular responses during glucose exposure. *Am. J. Physiol.*, **238**, H164–H171 (1980).
16) Drucker DJ, Jin T, Asa SL, Young TL, Brubaker PL. Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. *Mol. Endocrinol.*, **8**, 1646–1655 (1994).
17) Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am. J. Physiol.*, **271**, C1172–C1180 (1996).
18) Gonzalez GA, Menzel P, Leonard J, Fischer WH, Montminy MR. Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.*, **11**, 1306–1312 (1991).
19) Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*, **59**, 675–680 (1989).
20) Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF. Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J. Biol. Chem.*, **261**, 11880–11889 (1986).
21) Chepurny OG, Bertinetti D, Diskar M, Leech CA, Afshari P, Tsalkova T, Cheng X, Schwede F, Genieser HG, Herberg FW, Holz GG. Stimulation of proglucagon gene expression by human GPR119

in enteroendocrine L-cell line GLUTag. *Mol. Endocrinol.*, **27**, 1267–1282 (2013).
22) Drucker DJ, Brubaker PL. Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3953–3957 (1989).
23) Hurtado V, Roncero I, Blazquez E, Alvarez E, Sanz C. Glucagon-like peptide-1 and its implications in obesity. *Hot Topics in Endocrine and Endocrine-Related Diseases*. (Fedele M ed.) InTech, U.S.A., pp. 165–195 (2013).
24) Jansen E, Ayoubi TA, Meulemans SM, Van de Ven WJ. Cell type-specific protein-DNA interactions at the cAMP response elements of the prohormone convertase 1 promoter. Evidence for additional transactivators distinct from CREB/ATF family members. *J. Biol. Chem.*, **272**, 2500–2508 (1997).
25) Reimann F, Gribble FM. Glucose-sensing in glucagon-like peptide-1-secreting cells. *Diabetes*, **51**, 2757–2763 (2002).
26) Shiota C, Larsson O, Shelton KD, Shiota M, Efanov AM, Hoy M, Lindner J, Kooptiwut S, Juntti-Berggren L, Gromada J, Berggren PO, Magnuson MA. Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *J. Biol. Chem.*, **277**, 37176–37183 (2002).
27) Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol. Cell*, **40**, 294–309 (2010).
28) Semenza GL. Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*, **3**, 721–732 (2003).
29) Chou CC, Coatney RW. Nutrient-induced changes in intestinal blood flow in the dog. *Br. Vet. J.*, **150**, 423–437 (1994).
30) Matheson PJ, Wilson MA, Garrison RN. Regulation of intestinal blood flow. *J. Surg. Res.*, **93**, 182–196 (2000).
31) Matheson PJ, Wilson MA, Spain DA, Harris PD, Anderson GL, Garrison RN. Glucose-induced intestinal hyperemia is mediated by nitric oxide. *J. Surg. Res.*, **72**, 146–154 (1997).
32) Gross MW, Karbach U, Groebe K, Franko AJ, Mueller-Klieser W. Calibration of misonidazole labeling by simultaneous measurement of oxygen tension and labeling density in multicellular spheroids. *Int. J. Cancer*, **61**, 567–573 (1995).
33) Chou SC, Azuma Y, Varia MA, Raleigh JA. Evidence that involucrin, a marker for differentiation, is oxygen regulated in human squamous cell carcinomas. *Br. J. Cancer*, **90**, 728–735 (2004).
34) Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.*, **293**, E1118–E1128 (2007).
35) Beitner-Johnson D, Leibold J, Millhorn DE. Hypoxia regulates the cAMP- and Ca^{2+}-calmodulin signaling systems in PC12 cells. *Biochem. Biophys. Res. Commun.*, **242**, 61–66 (1998).
36) Moon JS, Jin WJ, Kwak JH, Kim HJ, Yun MJ, Kim JW, Park SW, Kim KS. 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. *Biochem. J.*, **433**, 225–233 (2011).
37) Gwak GY, Yoon JH, Kim KM, Lee HS, Chung JW, Gores GJ. Hypoxia stimulates proliferation of human hepatoma cells through the induction of hexokinase II expression. *J. Hepatol.*, **42**, 358–364 (2005).
38) Wollberg P, Söderqvist H, Nelson BD. Mitogen activation of human peripheral T lymphocytes induces the formation of new cyclic AMP response element-binding protein nuclear complexes. *J. Biol. Chem.*, **269**, 19719–19724 (1994).