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Fatty acid-bearing albumin but not fatty acid-depleted albumin induces HIF-1 activation in human renal proximal tubular epithelial cell line HK-2

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ABSTRACT

Recently, we found that albumin overload induces expression of the transcription factor hypoxia-inducible factor-1α (HIF-1α) protein and several HIF-1 target genes in human renal proximal tubular epithelial cell line HK-2. In this study, the role of albumin-bound fatty acids in the albumin-induced HIF-1 activation was studied. The enhancing effect of fatty acid-bearing human serum albumin [FA(+)HSA] treatment on HIF-1α protein expression was much greater than that of fatty acid-depleted human serum albumin [FA(−)HSA] treatment. The FA(+)HSA treatment induced HIF-1 target gene mRNAs such as those of glucose transporter 1 (GLUT1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and breast cancer resistance protein (BCRP) in concentration-dependent manners, while FA(−)HSA caused no significant increases in these mRNAs. Consistent with increased GLUT1 mRNA, GLUT1 protein expression and GLUT inhibitor cytochalasin B-sensitive D-[^{3}H]glucose uptake activity were significantly enhanced by treatment with FA(+)HSA, but not with FA(−)HSA. These findings indicate that fatty acids bound to albumin play a crucial role in albumin-induced HIF-1 activation followed by changes in HIF-1 target gene expression and protein product activity.

1. Introduction

Highly enhanced glomerular permeability to albumin is observed in kidney diseases such as diabetic nephropathy, which leads to an increased concentration of albumin in the proximal tubular lumen. Abundant urinary albumin filtered through the glomerular capillaries is suggested to induce intrinsic renal toxicity, which is involved in progression of tubulointerstitial damage followed by end-stage renal disease [1–3]. Exposure of renal proximal tubular cells to excessive amounts of albumin initiates various harmful responses in the proximal tubular cells [1–3].

Under proteinuric conditions, renal tubular cells are suggested to become apoptotic, leading to eventual tubulointerstitial fibrosis through some cell signaling pathways. Several transcription factors, especially nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are linked to inflammatory and immune responses, are involved in the pathogenesis of tubulointerstitial damage with proteinuria [4–7]. Exposure of proximal tubular cells to albumin increases the cellular hydrogen peroxide content, which serves as a signal for the activation of NF-κB [6].

Fatty acids bound to albumin have been reported to play important roles in various effects of albumin overload [8,9]. Proximal tubules exposed to fatty acid-bearing albumin, but not fatty acid-depleted albumin, produced a lipid chemoattractant that has a potentially key role in induction of interstitial inflammation [10]. In addition, albumin-bound fatty acids are able to activate peroxisome proliferator-activated receptor (PPAR) subtype γ, which results in apoptotic cell death [11]. Furthermore, fatty acid-bearing albumin, but not fatty acid-depleted albumin, induced the expression of vascular cell adhesion molecule-1 (VCAM-1), a cell adhesion molecule that mediates the inflammatory process, via transcription factors such as NF-κB and AP-1 [7]. Thus, it is likely

Abbreviations: AP-1, activator protein-1; BCRP, breast cancer resistance protein; FA(+)HSA, fatty acid-bearing human serum albumin; FA(−)HSA, fatty acid-depleted human serum albumin; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT1, glucose transporter 1; HIF-1, hypoxia-inducible factor-1; HSA, human serum albumin; LDH, lactate dehydrogenase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; VEGF, vascular endothelial growth factor; VHL, von Hippel Lindau.

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that albumin-bound fatty acids might be important mediators of renal tubulointerstitial injury.

HIF-1, a basic helix-loop-helix transcription factor, is composed of an inducible α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β) [12,13]. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases and binds to the von Hippel Lindau (VHL) protein, which leads to HIF-1α ubiquitination and degradation by 26S proteasomes. In hypoxia, the activities of prolyl hydroxylases decrease, resulting in the prevention of HIF-1α binding to VHL protein. The stabilization of HIF-1α is followed by nuclear translocation, dimerization with HIF-1β and binding to hypoxia-response elements in the promoters of target genes such as those of glucose transporter 1 (GLUT1), erythropoietin and vascular endothelial growth factor (VEGF) mRNAs [12–14]. Thus, HIF-1α is an important regulatory factor that allows individual cells to adapt to hypoxia. In addition, the expression of HIF-1α protein is reported to be affected by insulin, insulin-like growth factor, angiotensin II, thrombin and platelet-derived growth factor in oxygen-independent manners [15,16]. Recently, we found that fatty acid-bearing albumin overload induces expression of HIF-1α protein and its target genes, such as those of GLUT1 and GAPDH, in human renal proximal tubular cell line HK-2 [17]. However, it has not been clarified yet whether or not fatty acids are essential for the HIF-1 activation by albumin overload. In this study, we investigated the roles of fatty acids bound to albumin in the expression of HIF-1α and its target genes in HK-2 cells.

2. Materials and methods

2.1. Materials

Fatty acid-bearing human serum albumin [FA(+)HSA] (catalog No. A9511), fatty acid-depleted human serum albumin [FA(−)HSA] (catalog No. A3782), and XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cytochalasin B was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cobalt(II) chloride hexahydrate was obtained from Nacalai Tesque (Kyoto, Japan). D-[2-^{3}H]glucose (310.8 GBq/mmol) was purchased from Moravek Biochemicals, Inc. (Bera, CA, USA). All other chemicals used in the experiments were commercial products of the highest purity available.

2.2. Cell culture

HK-2 cells, a human proximal tubular cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (MP Biomedicals, Santa Ana, CA, USA), containing 10% fetal bovine serum (FBS), under an atmosphere of 5% CO_2–95% air at 37 °C, and were subcultured every 7 days using 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin. The medium was replaced with fresh medium every 2 days.

2.3. Albumin treatment

HK-2 cells were cultured in medium containing 10% FBS. At 4 days after seeding, the medium was changed to serum-free medium, and the cells were maintained for a further 24 h. Then, the cells were treated with FA(+)HSA or FA(−)HSA for 48 h at concentrations up to 40 mg/ml. FA(+)HSA or FA(−)HSA were dissolved in serum-free medium.

2.4. Measurement of long chain fatty acids in human serum albumin

The long chain fatty acid content in FA(+)HSA and FA(−)HSA solutions that were prepared with a concentration of 50 mg/ml in saline were determined by gas chromatography in a commercial laboratory (SRL, Inc., Tokyo, Japan).

2.5. Oil red O staining

Oil red O staining was performed to examine cellular lipid deposits in HK-2 cells and was performed by the method of Thomas et al. [18] with several modifications. Briefly, at 48 h after treatment with FA(+)HSA or FA(−)HSA as described above, the cells were washed twice and then fixed with 4% paraformaldehyde at room temperature for 10 min. After the cells had been washed twice, they were incubated with 60% 2-propanol at room temperature for 1 min. Then, the cells were stained with 0.18% Oil Red O (Sigma–Aldrich) in 60% 2-propanol at 37 °C for 15 min, and then incubated with 60% 2-propanol at room temperature for 1 min. After the cells had been washed twice, the nuclei were counterstained with Mayer's hematoxylin solution (Sigma–Aldrich) at room temperature for 5 min. The cells were rinsed in distilled water until background staining was removed, and then were mounted and observed under a phase-contrast microscope (Olympus CKX41 microscope, Tokyo, Japan).

2.6. XTT assay

HK-2 cells were treated with FA(+)HSA or FA(−)HSA as described above. After the treatment, the cells were washed twice, 0.2 ml of a 250 μM XTT solution containing 10 μM phenazine methosulfate was added to each dish, and then the cells were incubated at 37 °C for 30 min. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was determined using a spectrophotometric plate reader to measure absorbance at a wavelength of 490 nm. The reference absorbance (nonspecific readings) was measured at a wavelength of 650 nm.

2.7. LDH assay

HK-2 cells were treated with FA(+)HSA or FA(−)HSA as described above. After the treatment, lactate dehydrogenase (LDH) activity in the medium and the lysed cells was measured with a Cytotoxicity Detection Kit^{plus} (LDH) (Roche Diagnostics GmbH, Mannheim, Germany) according to the instruction manual. The percentage of LDH released from the cells was calculated by determining the ratio of LDH in the medium compared to that in the lysed cells plus the culture medium.

2.8. Western blot analysis

Whole cell lysates derived from HK-2 cells were used for Western blot analysis, which was performed as described previously [17]. The primary antibodies used in this study were as follows: anti-HIF-1α rabbit antibody (Novus Biologicals, Littleton, CO) (1:250), anti-GLUT1 rabbit antibody (1:1000) (Millipore Corporation, Billerica, MA, USA), and monoclonal anti-β-actin mouse antibody (Sigma–Aldrich) (1:2000). The corresponding secondary antibodies were as follows: anti-rabbit IgG linked with horseradish peroxidase (GE Healthcare, Buckinghamshire, England) (1:5000), and anti-mouse IgG linked with horseradish peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) (1:5000).

2.9. Real-time PCR

Total RNA was extracted from HK-2 cells using an RNeasy®Plus Mini Kit (QIAGEN GmbH, Hilden, Germany). Real-time PCR was performed using THUNDER BIRD SYBR qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan). The primer pair of GLUT1 sense and antisense (sense: 5′-ATACTCATGACCATCGCGCTAG-3′; antisense: 5′-AAAGAAGGCCA CAAAGCCAAAG-3′) used was specific for a 93-bp fragment of GLUT1 transcripts. The primer pair of GAPDH sense and antisense (sense: 5′-CCACCCATGGCAAATTCC-3′; antisense: 5′-TGGGATTTCCATT GATGACAA-3′) used was specific for a 69-bp fragment of GAPDH transcripts. The primer pair of breast cancer resistance protein (BCRP) sense and antisense (sense: 5′-GGATGAGCCTACAACTG GCTT-3′; antisense: 5′-CTTCCTGAGGCCAATAAGGTG-3′) used was specific for a 162 bp fragment of BCRP transcripts. The primer pair of PPARγ sense and antisense (sense: 5′-TGTCGGTTTCAGAAAT GCCTTG-3′; antisense: 5′-CTCGCCTTTGCTTTGGTCAG-3′) used was specific for a 209 bp fragment of PPARγ transcripts. The primer pair of β-actin sense and antisense (sense: 5′-CCTGGCACCCAGCACAAT-3′; antisense: 5′-GCCGATCCACACGGAGTACT-3′) used was specific for a 70-bp fragment of β-actin transcripts. The PCR conditions consisted of initial denaturation at 95 °C for 30 s, followed by amplification for 45 cycles of 10 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. The threshold cycle (Ct) value for each mRNA was determined using the second derivative maximum method.

2.10. Glucose uptake studies

Uptake studies were performed on the confluent cells attached to 24-well plates. Briefly, HK-2 cells were cultured in medium containing 10% FBS. At 4 days after seeding, the medium was changed to serum-free medium, and the cells were maintained for a further 24 h. Then, the cells were treated with FA(+)HSA, FA(−)HSA or cobalt chloride at the indicated concentrations, and maintained for 48 h. After removal of the culture medium, each well was washed and preincubated with phosphate-buffered saline (PBS) (in mM, 137 sodium chloride, 3 KCl, 8 K_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$) containing 5 mM D-glucose. Then, the uptake of D-[^{3}H]glucose was determined as previously described [19]. In order to estimate sodium-independent uptake of D-[^{3}H]glucose (GLUT activity), sodium-free phosphate buffer (in mM, 137 choline chloride, 3 KCl, 8 K_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$) containing 5 mM D-glucose was used as an incubation buffer. Protein contents were determined by the Bradford method with bovine serum albumin as a standard [20]. The uptake of the radioactive substrate was normalized as to the protein content of the cells in each well.

2.11. Data analysis

Statistically significant differences were determined by Tukey–Kramer's test for post hoc analysis. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Quantification of long chain fatty acids bound to albumin

Long chain fatty acid contents in saline solutions including 50 mg/ml FA(+)HSA or FA(−)HSA were measured by gas chromatography (Supplementary Table 1). The main components of fatty acids in the solution with FA(+)HSA were linoleic acid (C18:2ω6), oleic acid (C18:1ω9), and palmitoleic acid (C16:0), which amounted to 75.1, 64.7 and 61.3 μg/ml, respectively. On the other hand, the concentrations of fatty acids in the solution with FA(−)HSA were much lower than those with FA(+)HSA.

3.2. Effects of albumin with and without fatty acids on intracellular lipid droplets in HK-2 cells

To examine neutral lipid deposits in HK-2 cells treated with FA(+)HSA or FA(−)HSA, oil red O staining was performed (Supplementary Fig. 1). Lipid deposits were observed in the cytoplasm of HK-2 cells treated with FA(+)HSA, and the accumulated lipid droplets increased in a concentration-dependent manner. In contrast, few lipid droplets were detected in control cells (0 mg/ml) and the cells treated with FA(−)HSA.

3.3. Effects of albumin with and without fatty acids on cell viability and cytotoxicity in HK-2 cells

After HK-2 cells had been treated with various concentrations of FA(+)HSA or FA(−)HSA, cell viability and cytotoxicity were estimated. The XTT assay showed that FA(+)HSA decreased the cell viability in a concentration-dependent manner (Supplementary Fig. 2A), while FA(−)HSA did not cause significant changes in the cell viability. In the LDH assay, a significant increase in LDH leakage into the medium was observed with FA(+)HSA, but not with FA(−)HSA (Supplementary Fig. 2B).

3.4. Effects of albumin with and without fatty acids on HIF-1α protein expression in HK-2 cells

To compare the effects of FA(+)HSA and FA(−)HSA on the expression of HIF-1α protein, Western blot analysis was performed. As shown in Fig. 1, the expression of HIF-1α protein in HK-2 cell lysates was enhanced by FA(+) HSA treatment for 48 h in a concentration-dependent manner, while the increase in HIF-1α protein expression caused by FA(−)HSA treatment for 48 h was much weaker than that with FA(+)HSA. Since we previously found that the mRNA expression of HIF-1α was significantly enhanced in HK-2 cells treated with FA(+)HSA [17], the effect of FA(−)HSA on the mRNA of HIF-1α was investigated. There were no significant changes in HIF-1α mRNA expression in HK-2 cells treated with FA(−)HSA at concentrations of 5–40 mg/ml (Supplementary Fig. 3).

3.5. Effects of albumin with and without fatty acids on mRNA and protein expression of HIF-1 target genes in HK-2 cells

We investigated the effects of FA(+)HSA and FA(−)HSA on the mRNA expression of HIF-1 target genes such as GLUT1, GAPDH and BCRP [21–23]. As shown in Fig. 2, real-time PCR analysis revealed that FA(+)HSA treatment for 48 h increased the mRNA expression of these target genes. In contrast, FA(−)HSA treatment

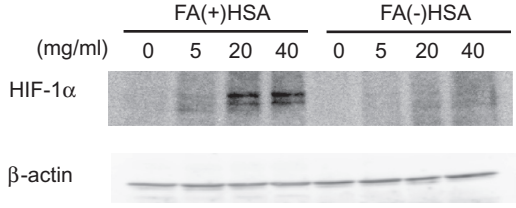

Fig. 1. Expression of HIF-1α protein in HK-2 cells treated with fatty acid-bearing albumin [FA(+)HSA] and fatty acid-depleted albumin [FA(−)HSA]. Western blot analysis of HIF-1α and β-actin proteins was performed with cell lysates derived from HK-2 cells treated with FA(+)HSA and FA(−)HSA at various concentrations (0, 5, 20 or 40 mg/ml) for 48 h.

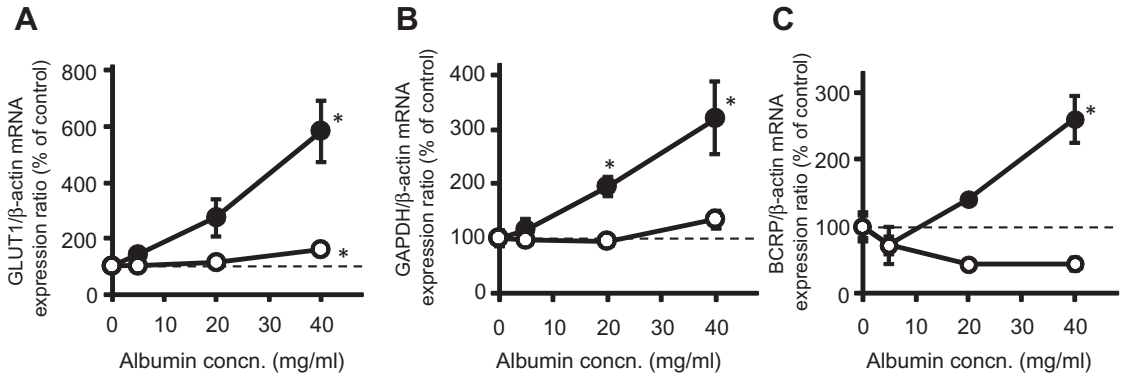

Fig. 2. Expression of mRNAs of HIF-1 target genes GLUT1, GAPDH and BCRP in HK-2 cells treated with FA(+)HSA and FA(−)HSA. The mRNA levels of GLUT1 (A), GAPDH (B), and BCRP (C) in HK-2 cells treated with FA(+)HSA (closed circles) and FA(−)HSA (open circles) at various concentrations (0, 5, 20 or 40 mg/ml) were measured by real-time PCR analysis. The relative level of each target gene mRNA was determined after normalization as to β-actin mRNA. The mRNA levels without albumin (0 mg/ml) were set to 100%. Values are expressed as the means ± SE for three to six monolayers. *$p < 0.05$, significantly different compared with the value for the cells without albumin (0 mg/ml).

did not significantly enhance the mRNA expression of GLUT1 and GAPDH mRNA, while BCRP mRNA expression tended to be decreased by FA(−)HSA treatment in a concentration-dependent manner.

Furthermore, we examined the changes in GLUT1 protein expression in HK-2 cells treated with FA(+)HSA and FA(−)HSA. Western blot analysis showed a much greater increase in the expression of GLUT1 protein in the lysates derived from HK-2 cells treated with FA(+)HSA than with FA(−)HSA (Fig. 3).

3.6. Effects of albumin with and without fatty acids on GLUT transporter activity in HK-2 cells

Next we investigated the effect of FA(+)HSA and FA(−)HSA on GLUT transporter activity. First, to confirm the HIF-1-dependent increase in GLUT function, the effect of a known HIF-1 inducer, $CoCl_2$, on GLUT activity was examined in HK-2 cells. Treatment with $CoCl_2$ enhanced the expression of not only HIF-1 protein but also GLUT1 mRNA (Supplementary Fig. 4A and B). In addition, GLUT inhibitor cytochalasin B-sensitive and sodium-independent uptake of [^{3}H]-D-glucose was enhanced by $CoCl_2$ treatment in a concentration-dependent manner, confirming the HIF-1-mediated increase in GLUT transporter activity (Supplementary Fig. 4C and D). Furthermore, the effects of FA(+)HSA and FA(−)HSA on GLUT function were examined and compared (Fig. 4). In the absence of cytochalasin B, FA(+)HSA treatment increased the uptake of [^{3}H]-D-glucose in a concentration-dependent manner, but FA(−)HSA treatment had no effect on [^{3}H]-D-glucose uptake. Cytochalasin B almost completely inhibited [^{3}H]-D-glucose uptake in cells treated with either FA(+)HSA or FA(−)HSA. Thus, cytochalasin B-sensitive and sodium-independent uptake of [^{3}H]-D-glucose, reflecting GLUT functional activity, was enhanced by treatment with FA(+)HSA but not with FA(−)HSA.

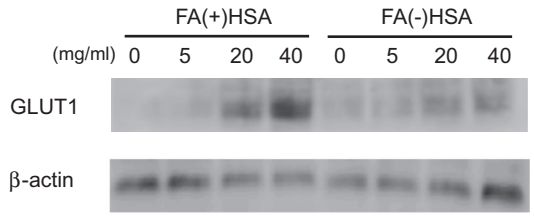

Fig. 3. Expression of GLUT1 protein in HK-2 cells treated with FA(+)HSA and FA(−)HSA. Western blot analysis of GLUT1 and β-actin proteins was performed with cell lysates derived from HK-2 cells treated with FA(+)HSA and FA(−)HSA at various concentrations (0, 5, 20 or 40 mg/ml).

3.7. Effects of albumin with and without fatty acids on PPARγ mRNA expression in HK-2 cells

Fatty acids and oleic acid are reported to increase the mRNA expression of PPARγ in chicken preadipocytes [24,25]. Consistent with the reports, treatment with FA(+)HSA but not FA(−)HSA induced the mRNA expression of PPARγ in HK-2 cells in a concentration dependent manner (Supplementary Fig. 5).

4. Discussion

In our previous study, we found that albumin loading induces HIF-1α protein in HK-2 cells [17]. To determine whether or not fatty acids bound to albumin are involved in the up-regulation of expression of HIF-1α protein and its target genes, the effects of fatty acid-bearing albumin and fatty acid-depleted albumin on the expression of HIF-1α protein and its target genes were compared. This study showed that fatty acids bound to albumin, rather than the albumin molecules themselves, are responsible for the increase in HIF-1α protein in HK-2 cells treated with albumin.

It has been proposed that the proteinuria-induced progression of nephrotoxicity is not directly due to the protein molecule itself but fatty acids bound to albumin. Erkan et al. [26] revealed that albumin overload-induced cytotoxicity and apoptosis were closely related to the fatty acid content of the albumin that was loaded to cultured proximal tubular cells. We also observed that treatment with fatty acid-bearing albumin significantly decreased cell viability and increased the leakage of LDH from the cells, while no significant changes in cell viability and LDH leakage were observed in HK-2 cells treated with fatty acid-depleted albumin. Furthermore, oil red O staining revealed more cellular lipid deposits in cells treated with fatty acid-bearing albumin, compared to in ones treated with fatty acid-depleted albumin. Thus, it is likely that the extent of the cytotoxicity is due to the intracellular content of fatty acids in albumin-loaded cells.

NF-κB, a nuclear transcription factor, is reported to be activated in renal tubules in various experimental models of renal injury [27] and proteinuric patients [28]. In addition, albumin overload activated NF-κB in cultured renal tubular cells [4,6,29], which was dependent on the generation of reactive oxygen species (ROS) [6,30]. Recently, Baek et al. [7] reported that fatty acid-bearing albumin, but not fatty acid-free albumin, induced activation of NF-κB in HK-2 cells. Furthermore, Bonello et al. [31] found that the reactive oxygen species-mediated induction of HIF-1α mRNA was dependent on the activation of NF-κB in pulmonary artery smooth muscle cells. Our previous paper [17] reported that fatty acid-bearing albumin overload significantly increased the expres-

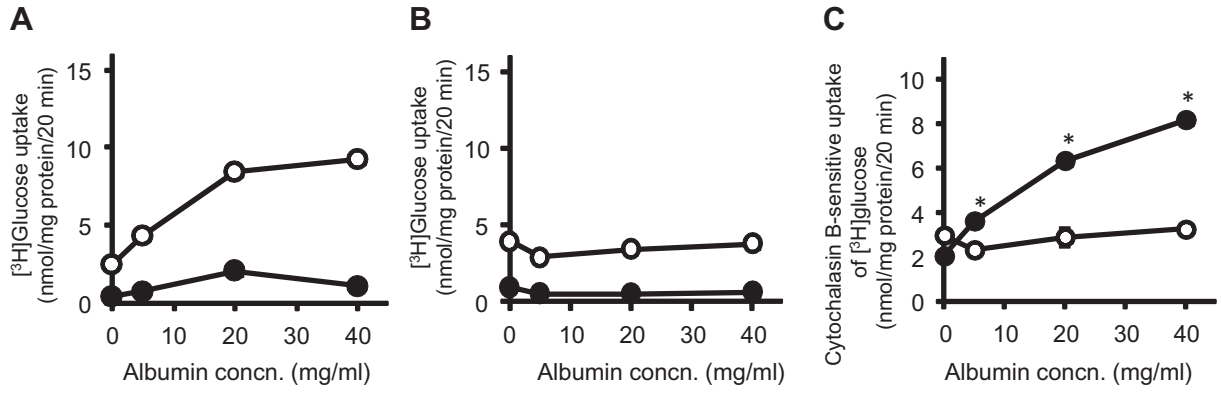

Fig. 4. Effects of albumin with and without fatty acids on D-[^{3}H]glucose uptake by HK-2 cells. Cells were treated with FA(+)HSA (A) and FA(−)HSA (B) at various concentrations (0, 5, 20 or 40 mg/ml), and then the uptake of D-[^{3}H]glucose (1 mM) in 20 min at 37 °C was measured in the sodium-free buffer in the absence (open circles) or presence (closed circles) of 10 μM cytochalasin B. (C) Cytochalasin B-sensitive D-[^{3}H]glucose uptake was evaluated by subtracting the uptake activity in the presence of cytochalasin B from that in the absence of cytochalasin B in cells treated with albumin at the same concentration. Each symbol represents the mean ± S.E. for three monolayers. $^*p < 0.05$, significantly different from the value for each control.

sion level of mRNA as well as protein of HIF-1α, but fatty acid-depleted albumin had no significant effect on the level of HIF-1α mRNA in HK-2 cells. Overall, it is likely that NF-κB activation, at least in part, is involved in the increases in HIF-1α mRNA and protein in HK-2 cells treated with fatty acid-bearing albumin. However, further studies are needed to clarify the molecular mechanism(s) underlying albumin-overload mediated induction of HIF-1α protein.

In accordance with the expression of HIF-1α protein, HIF-1 target mRNA genes such as those of GLUT1, GAPDH and BCRP were induced by the treatment with fatty acid-bearing albumin, but not with fatty acid-depleted albumin. These observations suggest that HIF-1α is directly involved in the albumin overload-induced expression of these mRNA genes. Furthermore, in order to examine changes in protein and functional levels, we focused on GLUT1 protein. Western blot analysis showed a greater increase in GLUT1 protein expression in the HK-2 cells treated with fatty acid-bearing albumin than that with fatty acid-depleted albumin. In addition, GLUT activity, which was evaluated as sodium-independent and cytochalasin B-sensitive uptake of [^{3}H]-D-glucose, was enhanced by fatty acid-bearing albumin treatment in a concentration-dependent manner, while fatty acid-depleted albumin treatment had no significant effect on the uptake activity. Thus, our results indicate that fatty acids bound to albumin modulate not only HIF-1 target gene mRNA expression but also the expression and function of the gene products. Further studies are required to determine the physiological and pathophysiological meanings of the modulation of the functional activity of HIF-1 target gene products by fatty acid-bearing albumin overload.

In conclusion, we found that fatty acids bound to albumin play an important role in albumin overload-induced expression of HIF-1α protein and HIF-1 target genes such as GLUT1 mRNA. Furthermore, the expression level of GLUT1 protein and GLUT-mediated uptake activity were enhanced by the presence of fatty acids bound to albumin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.146.

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