Biochemical and Biophysical Research Communications 408 (2011) 58–64

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of retinol binding protein-4 on vascular endothelial cells

Kohzo Takebayashi [a,*], Ryouichi Sohma [b], Yoshimasa Aso [a], Toshihiko Inukai [a]

[a] Department of Internal Medicine, Dokkyo Medical University Koshigaya Hospital, Koshigaya, Saitama, Japan
[b] Joint Research Center, Dokkyo Medical University Koshigaya Hospital, Koshigaya, Saitama, Japan

ARTICLE INFO

Article history:
Received 23 March 2011
Available online 30 March 2011

Keywords:
RBP4
NO
Endothelial cells

ABSTRACT

The study was designed to investigate the effect of retinol binding protein (RBP)-4 on the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, which mediate the effects of insulin in vascular endothelial cells. The effects of RBP4 on nitric oxide (NO) and insulin-stimulated endothelin-1 (ET-1) secretion and on phosphorylation (p) of Akt, endothelial NO synthetase (eNOS), and extracellular signal-regulated kinase (ERK)1/2 were investigated in bovine vascular aortic endothelial cells (BAECs). RBP4 showed an acute vasodilatatory effect on aortic rings of rats within a few minutes. In BAECs, RBP4-treatment for 5 min significantly increased NO production, but inhibited insulin-stimulated ET-1 secretion. RBP4-induced NO production was not inhibited by tetraacetoxymethylester (BAPTA-AM), an intracellular calcium chelator, but was completely abolished by wortmannin, a PI3K inhibitor. RBP4 significantly increased p-Akt and p-eNOS production, and significantly inhibited p-ERK1/2 production. Triciribine, an Akt inhibitor, and wortmannin significantly inhibited RBP4-induced p-Akt and p-eNOS production. Inhibition of Akt1 by small interfering RNA decreased p-eNOS production enhanced by RBP4 in human umbilical vein endothelial cells. In conclusion, RBP4 has a robust acute effect of enhancement of NO production via stimulation of part of the PI3K/Akt/eNOS pathway and inhibition of ERK1/2 phosphorylation and insulin-induced ET-1 secretion, probably in the MAPK pathway, which results in vasodilatation.

1. Introduction

Retinol binding protein-4 (RBP4) was first identified as a protein that transports retinol to systemic tissues [1]. In a normal state, the liver is the major source of RBP4, with about 20% of the protein produced in the liver, but RBP4 is also produced in adipose tissue [2,3]. Recently, Yang et al. showed that the production of RBP4 in type 2 diabetes increases in the adipose tissue but not in liver, and that elevated circulating RBP4 levels cause insulin resistance by inhibiting phosphatidylinositol 3-kinase (PI3K) activity in the skeletal muscle and increasing phosphoenolpyruvate carboxylase (PEPCK) expression in the liver in a mouse model [4]. However, the association between RBP4 and insulin resistance remains unclear, mainly because of conflicting results from subsequent studies [5–15].

Interestingly, recent clinical studies have shown that circulating RBP4 levels are closely associated with cardiovascular risk factors such as concentrations of serum lipids (including low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG)), systolic blood pressure (SBP) [7–14], sub-clinical inflammation [5] and sub-clinical and clinical cardiovascular disease (CVD) [16]. In patients with type 2 diabetes, we have found a significant negative correlation between RBP4 and flow-mediated vasodilatation (FMD) [12], which reflects endothelial function due to the production of nitric oxide (NO) as a major vasodilatory substance in endothelial cells [17].

The relationship of RBP4 with SBP and FMD could be accounted for by RBP4 inhibition of the insulin-stimulated PI3K/Protein kinase B (Akt) pathway in vascular endothelial cells, since insulin induces NO production via the PI3K/Akt/endothelial NO synthetase (eNOS) pathway in these cells [17]. If this hypothesis is correct, it is likely that RBP4 causes macro- and microvascular impairment independent of its potential deleterious effect on glucose metabolism, since NO also has anti-inflammatory and anti-atherogenic effects [18,19]. However, it remains unclear if RBP4 inhibits the insulin-dependent PI3K/Akt/eNOS pathway in endothelial cells. Therefore, in the current study we investigated the direct effect of RBP4 on vascular endothelial cells.

2. Materials and methods

2.1. Cell culture

Bovine aortic endothelial cells (BAECs) in primary culture (Cell Systems, Kirkland, WA, USA) were grown to 95% confluence

* Corresponding author. Address: Department of Internal Medicine, Dokkyo Medical University Koshigaya Hospital, 2-1-50, Minami-Koshigaya, Koshigaya 343-8555, Japan. Fax: +81 48 965 1127.
E-mail address: takeb@gmail.plala.or.jp (K. Takebayashi).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.116

in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum (FBS) at 37 °C in a humid atmosphere with 5% CO_2. Cells were used between passages 4 and 6. Human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured in endothelial basal medium (EBM) supplemented with 10% FBS.

2.2. Evaluation of cytotoxicity of RBP4

The cytotoxicity of RBP4 was evaluated by a WST-8 assay (Cell Counting Kit 8, Dojindo Laboratories). After overnight starvation in DMEM, RBP4 was added to cells and the absorbance of formazan (produced from WST-8) at 450 nm was measured 3 h after addition of RBP4.

2.3. Measurement of NO and evaluation of the effect of RBP4 on NO production in BAECs

Production of NO was assessed using diaminofluorescein-2 (DAF-2; Daiichi Pure Chemicals, Tokyo, Japan), an NO-specific fluorescent dye. After overnight starvation, cells were washed in phosphate-buffered saline (PBS). RBP4 refined from human urine (Phoenix Pharmaceuticals, Burlingame, CA) and dissolved in PBS was used in these experiments. The effect of RBP4 on NO production was investigated through simultaneous addition of RBP4 with PBS, DAF-2. Supernatant liquid was collected 5 min after addition of DAF-2, and NO production was measured as fluorescence intensity (FI) using an F-2500 Spectrophotometer (Hitachi, Tokyo, Japan). The assay wavelengths were 495 nm (excitation maximum) and 515 nm (emission maximum). The effect of RBP4 was examined based on the effects of *O*,*O*'-bis(2-aminophenyl) ethylene glycol-*N*,*N*,*N*',*N*'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), an intracellular calcium chelator (Dojindo Laboratories, Kumamoto, Japan) and wortmannin, a PI3K inhibitor (Sigma–Aldrich Japan), on NO production.

2.4. Endothelin-1 (ET-1) assay

ET-1 in the supernatant was assayed using an Endothelin-1 IBL Kit (Immune-Biological Laboratories, Gunma, Japan). Each sample for ET-1 measurement was collected under the same conditions as those used for NO measurement.

2.5. Western blotting

Cell lysates were prepared using 300 μg of lysis buffer [100 mM NaCl, 20 mM HEPES (pH 7.9), 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail, and phosphatase inhibitor cocktail] (Roche Applied Sciences, Indianapolis, IN, USA). Samples (50 μg total protein) were separated by 10% SDS–PAGE and then immunoblotted on polyvinylidene difluoride membranes. The blots were incubated with the primary antibody at 4 °C overnight and probed with a peroxidase-conjugated secondary antibody using standard methods. Immunoreactive proteins were visualized on X-ray films by enhanced chemiluminescence. Blots were quantified by scanning densitometry (GS-800 Calibrated Densitometer, Bio-Rad Laboratories, Hercules, CA, USA). Anti-Akt antibody, anti-phospho-Ser473-Akt antibody, anti-eNOS antibody, anti-phospho-Ser1177 (Ser1179 in bovine) eNOS antibody, anti-extracellular signal-regulated kinase (ERK)1/2 antibody, and anti-phospho-ERK1/2 antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). As additional study, the effects of inhibition of Akt and PI3K on RBP4-induced p-Akt and p-eNOS production were evaluated using triciribine (Merck Chemicals, Darmstadt, Germany) and wortmannin (Sigma–Aldrich), respectively.

2.6. Transfection of small interfering (si)RNA in HUVECs

Due to technical problems with transfection of siRNAs in BAECs, HUVECs were used in this experiment, with an siRNA transfection reagent (DharmaFECT) and control siRNA and siRNA for human Akt1 (Dharmacon, Lafayette, CO, USA). HUVECs were transfected with 100 nM control or Akt1 siRNA according to the manufacturer's recommended protocols. Seventy-two hours after transfection, the culture medium was replaced with fresh DMEM without FBS for 24 h, and subsequently cells were treated with RBP4 for 2 min. eNOS and p-eNOS protein expression in the siRNA-transfected cells was analyzed using Western blotting. The efficacy of knockdown of Akt by the Akt1 siRNA was confirmed by real time PCR. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) and converted to cDNA using an ExScript RT Reagent Kit (Takarabio, Shiga, Japan) with random oligonucleotide primers. Real time PCR was performed on an Applied Biosystems 7500 system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takarabio) with gene-specific primers (Takarabio). The PCR conditions were 95 °C for 10 s, followed by 40 cycles at 95 °C for 30 s. GADPH mRNA (Takarabio) was used as a control for RNA template normalization.

2.7. Vasodilatatory effects of RBP4 in Wistar and Goto-Kakizaki rats

Animal studies were conducted according to the "Guiding principles for the Care and Use of Laboratory Animals" of the Japanese Pharmacological Society. Animal studies were performed by specialists in experimental laboratory in Hamamatsu Pharma Research Co. Ltd. (Hamamatsu, Japan).

Fifteen-week-old male Wistar (WT) rats (non-diabetic control rats; $n = 3$) and Goto-Kakizaki (GK) rats (type 2 diabetes model rats; $n = 3$) were purchased from Japan SLC, Shizuoka, Japan. The body weight and fasting plasma glucose level on the day before the experiment were 264.0 ± 2.1 g and 88.7 ± 2.8 mg/dL, respectively, in WT rats and 301.0 ± 2.7 g and 145.3 ± 4.4 mg/dL, respectively, in GK rats. Rats were anesthetized with an overdose of pentobarbital and sacrificed by phlebotomy from the carotid artery. The chest of each rat was opened and the entire descending aorta was immediately dissected. Rings of the thoracic aorta of 5 mm in length were then prepared under a microscope. Two rings with endothelial cells were made from each rat (one for exposure to RBP4 and the other as a control; total 12 rings). As a RBP4 regent, human recombinant RBP4 (Abcam, Cambridge, UK) was used. An endotoxin test of the recombinant RBP4 indicated that it contained relatively low endotoxin levels (0.5 EU/mg) [20].

Each ring was mounted vertically between two hooks in organ chamber myographs filled with 5 ml of Krebs–Henseleit solution of composition 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose aerated with 95% O_2 and 5% CO_2.

Isometric tension was measured using a carrier amplifier (AP-621G, Nihon Kohden, Tokyo, Japan) and recorded with a multiple pen recorder (R-64, Rikadenki, Tokyo, Japan). After equilibration for 60 min, the rings were precontracted with a submaximal concentration of phenylephrine (10^{-6} M). Then, to evaluate whether the endothelial function of each ring was maintained, the rings were exposed to cumulative concentrations of acetylcholine (Ach). Subsequently, the rings were again contracted with phenylephrine, and RBP4 (25 μg to give a concentration of 5 μg/mL) or the same volume of saline as control was added to the organ chamber. An additional study in three more 15-week-old male GK rats (body weight and fasting plasma glucose on the day before the experiment of 304.3 ± 10.3 g and 151.0 ± 5.3 mg/dL, respectively) was performed to assess the effects of 5 μg/mL RBP4 on rings with an

endothelium that were treated with 100 μM N^{w}-nitro-L-arginine methyl ester (L-NAME) ($n = 3$), which inhibits eNOS production, and on rings without an endothelium ($n = 3$).

2.8. Statistical methods

All data are presented as means ± standard error (SE). Multiple comparisons were performed using an analysis of variance (ANOVA). If the ANOVA was significant, a Bonferroni test was used as a post hoc test. Comparison between two variables was performed using an unpaired t test. A P value of less than 0.05 was considered to indicate statistical significance in all analyses.

3. Results

3.1. Evaluation of cytotoxicity of RBP4

Pretreatment with RBP4 did not show cytotoxicity in a WST-8 assay in concentration of 0.5–8 μg/mL.

3.2. RBP4-induced stimulation of NO production and an inhibitory effect of RBP4 on insulin-induced ET-1 secretion in BAECs

Treatment with RBP4 for 5 min significantly increased NO production by about 3-fold at 4 μg/mL RBP4 (Fig. 1A). RBP4-induced

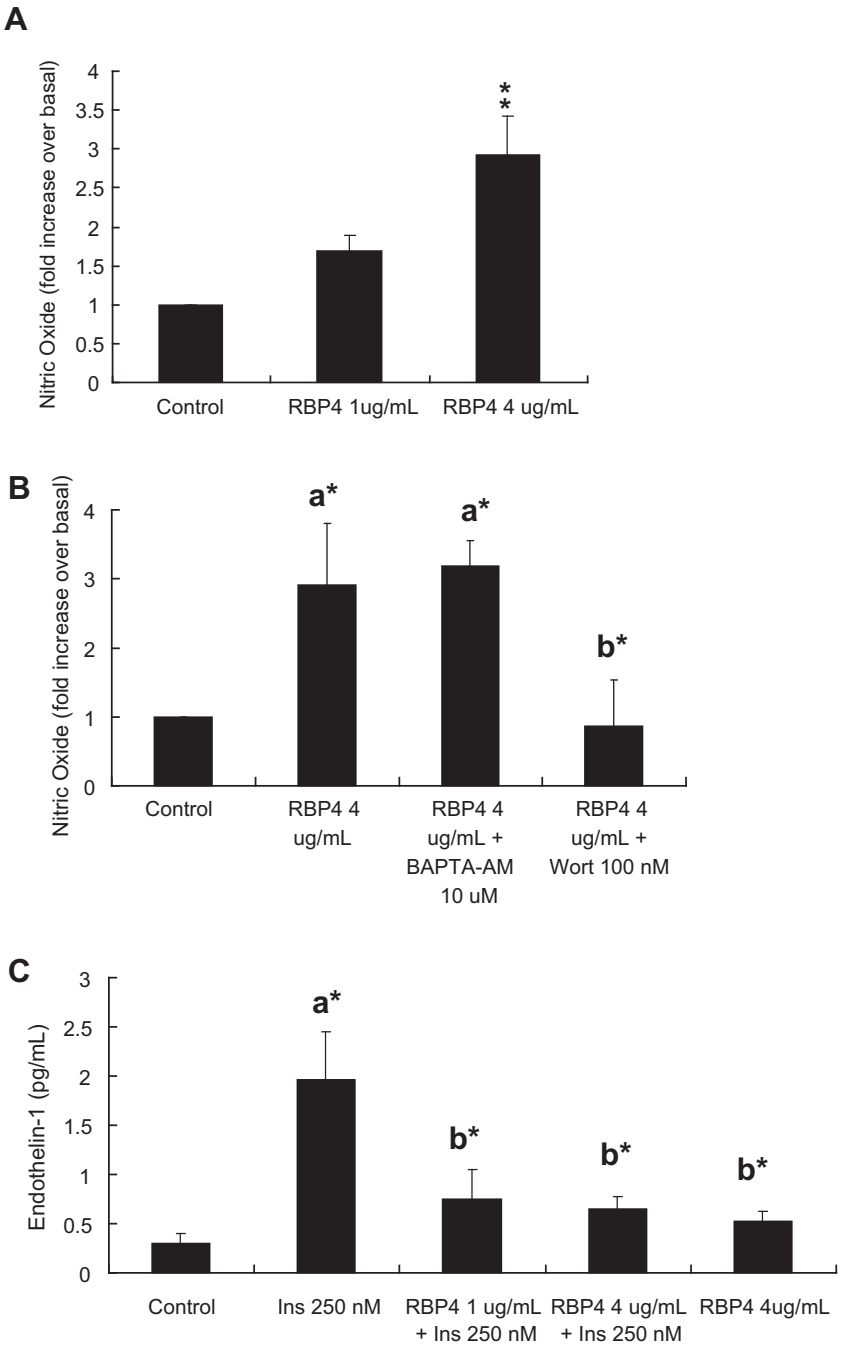

Fig. 1. Effect of RBP4 on NO production and ET-1 secretion in BAECs. (A) The effect of 5 min treatment with RBP4 (1, 4 μg/mL) on NO production. *Significant difference vs. control ($n = 5$, $P < 0.05$). (B) The effect of pretreatment of 10 μM BAPTA-AM for 20 min or 100 nM wortmannin (Wort) for 1 h on NO production stimulated by 4 μg/mL RBP4 for 5 min; a^* and b^* indicate significant differences vs. control and RBP4 (4 μg/mL) alone, respectively ($n = 4$, $P < 0.05$). (C) The effect of RBP4 (1, 4 μg/mL) for 5 min on 250 nM insulin-induced ET-1 secretion. a^* and b^* indicate significant differences vs. control and insulin (250 nM), respectively ($n = 4$, $P < 0.05$).

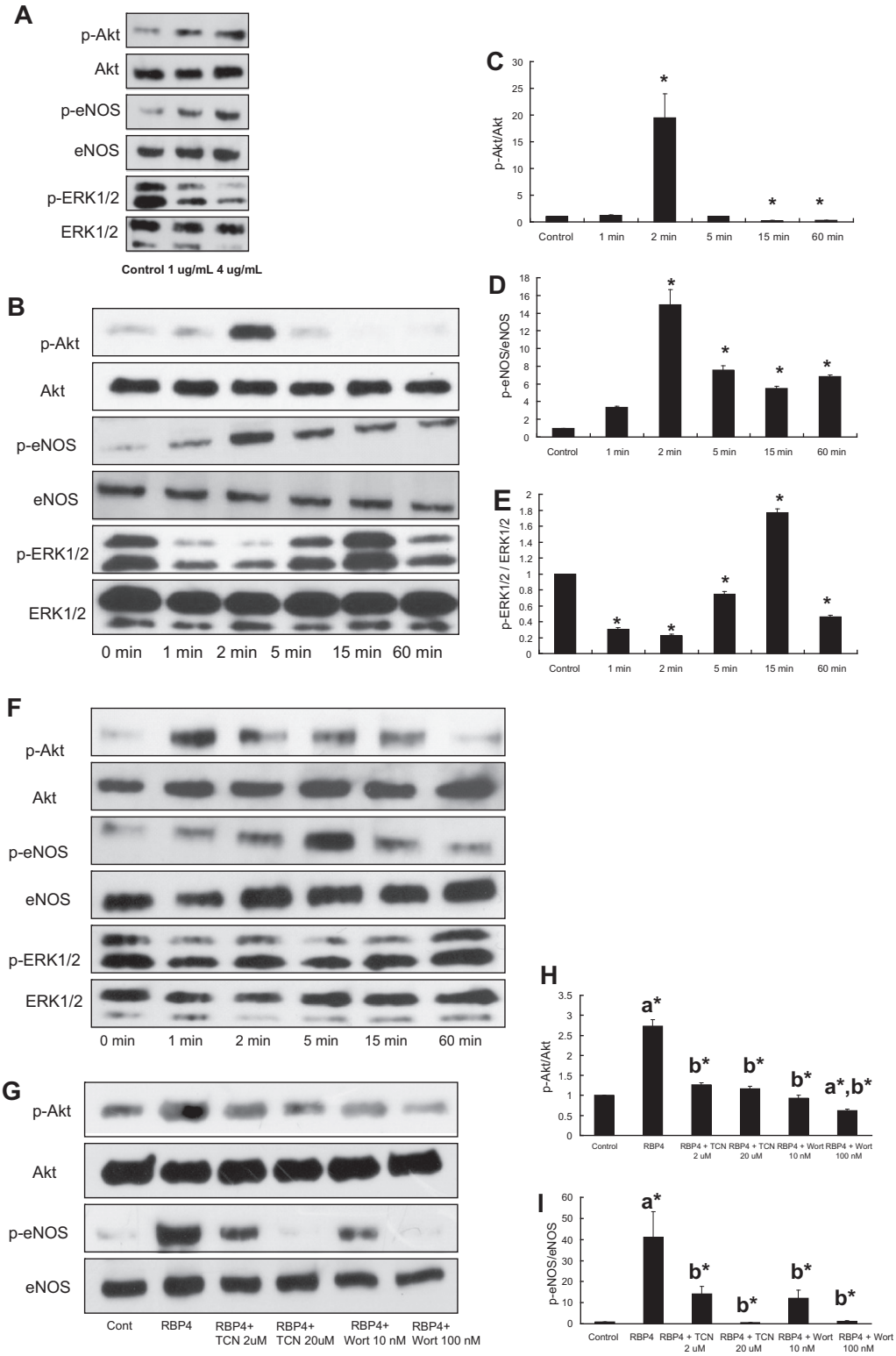

Fig. 2. (A) The effect of RBP4 (1, 4 μg/mL for 2 min) on phosphorylation of Akt, eNOS, and ERK1/2 in BAECs. Representative samples of Western blotting in three experiments are shown. (B–E) Time series analysis of the effect of RBP4 on phosphorylation of Akt, eNOS, and ERK1/2 in BAECs. Representative samples of Western blotting (B) and data from densitometry scans (C–E) are shown. Data are shown as the mean ± SE of three independent experiments and are expressed as the fold increase over the basal level. *Significant difference vs. control ($P < 0.05$). (F) Time series analysis of the effect of RBP4 on phosphorylation of Akt, eNOS, and ERK1/2 in BAECs, using human recombinant RBP4. Representative samples of Western blotting in three experiments are shown. (G–I) The effect of triciribine (TCN) (2, 20 μM for 1 h) or wortmannin (Wort) (10, 100 nM for 1 h) on RBP4 (4 μg/mL for 2 min)-induced p-Akt and p-eNOS production in BAECs. Representative samples of Western blotting (G) and data from densitometry scans (H, I) are shown. Data are shown as the mean ± SE of three independent experiments and are expressed as the fold increase over the basal level. a* and b* indicate significant differences vs. control and RBP4 (4 μg/mL) alone, respectively ($P < 0.05$).

NO production was not inhibited by BAPTA-AM, but was completely abolished by wortmannin (Fig. 1B). RBP4-treatment significantly inhibited insulin-induced ET-1 secretion in BAECs, and there was no significant difference in ET-1 secretion between control treatment and RBP4 alone (Fig. 1C).

3.3. RBP4-induced phosphorylation of Akt and eNOS and inhibition of phosphorylation of ERK1/2

RBP4 (for 2 min) increased p-Akt and p-eNOS in concentration-dependent manner, and decreased p-ERK1/2 in concentration-dependent manner (Fig. 2A). A time series analysis showed that RBP4 significantly increased p-Akt and p-eNOS levels and significantly decreased p-ERK1/2 after 2 min (Fig. 2B–E). The increased p-Akt and p-eNOS production caused by RBP4 treatment for 2 min was significantly inhibited by triciribine and wortmannin (Fig. 2G–I). Furthermore, we also confirmed whether the effects of human recombinant RBP4 and human urine RBP4 are similar because human recombinant RBP4 was used in animal studies. RBP4 increased p-Akt and p-eNOS at 1–5 min, and decreased p-ERK1/2 from 1 min. Therefore, the effects of 2 RBP4 products on p-Akt, p-eNOS and p-ERK1/2 were basically similar (Fig. 2F).

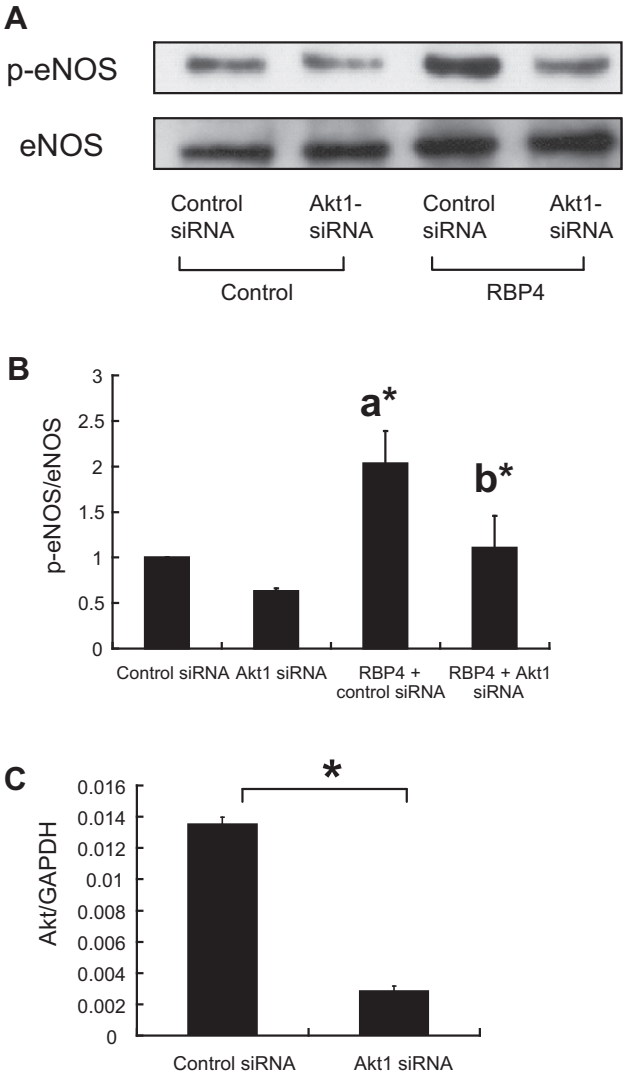

Fig. 3. Effects of Akt1 siRNA on RBP4 (4 μg/mL for 2 min)-stimulated p-eNOS production in HUVECs. Representative samples of Western blotting (A) and data from a densitometry scan (B) are shown. Data are shown as the mean ± SE of three independent experiments and are expressed as the fold increase over the basal level. a^* and b^* indicate significant differences vs. control siRNA and control siRNA with RBP4, respectively ($n = 3$, $P < 0.05$). (C) Knockdown efficacy of Akt mRNA by Akt1-siRNA shown by real time PCR. Data are shown as the mean ± SE of three independent experiments. * indicates a significance difference ($P < 0.05$).

3.4. Inhibition of RBP4-enhanced p-eNOS production in HUVECs by Akt1 siRNA

siRNA for Akt1 significantly inhibited the mRNA levels of Akt (Fig. 3C). Addition of RBP4 significantly increased phosphorylation of eNOS in HUVECs compared with controls, and siRNA for Akt1 significantly inhibited RBP4-stimulated p-eNOS expression (Fig. 3A, B).

3.5. The effect of RBP4 on rat thoracic aorta rings by RBP4

Significant dilatation was observed in aortic rings from WT rats (about 20%) and GK rats (about 50%) just after addition of RBP4. Dilatation reached a maximum after 1–5 min and then gradually decreased from 20% to 15% in rings from WT rats and from 50% to 30% in rings from GK rats after 1 h (Fig. 4A, B). The effect of RBP4 was also investigated for GK rat aortic rings with an endothelium that were treated with L-NAME and for rings without an endothelium. Similar significant dilatation with RBP4 alone was found for rings with an endothelium in the absence of L-NAME, compared with those treated with saline. However, there were no significant differences in dilatation among rings treated with saline, those with an endothelium treated with RBP4 and L-NAME, and those without an endothelium treated with RBP4 (Fig. 4C).

4. Discussion

In the current study, we first examined the effect of RBP4 on NO production and ET-1 secretion in BAECs. Contrary to our expectation, RBP4 at 4 μg/mL increased NO production by approximately 3-fold within 5 min in a concentration-dependent manner. This effect was not influenced by BAPTA-AM (an intracellular calcium chelator), but wortmannin (a PI3K inhibitor) completely abolished the effect of RBP4. On the other hand, RBP4 treatment for 5 min significantly inhibited insulin-induced ET-1 secretion in BAECs. Furthermore, phosphorylation of Akt and eNOS was increased and that of ERK1/2 was decreased in a concentration-dependent manner. A time series analysis of Akt, eNOS and ERK1/2 in BAECs showed that RBP4 significantly increased the levels of p-Akt and p-eNOS and significantly reduced p-ERK1/2, with maximum effects at 2 min. A basically similar result was obtained in an additional experiment using human recombinant RBP4, although the time for phosphorylation was somewhat variable (1–5 min). Furthermore, both wortmannin and triciribine (an Akt inhibitor) inhibited phosphorylation of Akt and eNOS in a concentration-dependent manner. In HUVECs, siRNA inhibition of expression of mRNA for Akt1 (the predominant isoform of Akt in the vasculature and in endothelial cells [17]) significantly decreased the level of RBP4-stimulated p-eNOS, suggesting that eNOS phosphorylation by RBP4 is Akt1-dependent. Taken together, these results suggest that RBP4 has an acute effect on NO production that probably occurs via the PI3K/Akt/eNOS pathway (but not the calcium/calmodulin pathway), similarly to insulin, and that RBP4 also has an inhibitory effect on insulin-induced secretion of ET-1 (a vasoconstriction factor) that is probably associated with a decrease in ERK1/2 phosphorylation, which has an effect opposite to that of insulin.

Rapid and robust dilatation of aortic rings from WT and GK rats caused by treatment with 5 μg/mL RBP4 was observed *ex vivo*. The degree of dilatation reached a maximum after 1–5 min and then gradually decreased for up to 1 h after addition of RBP4. Importantly,

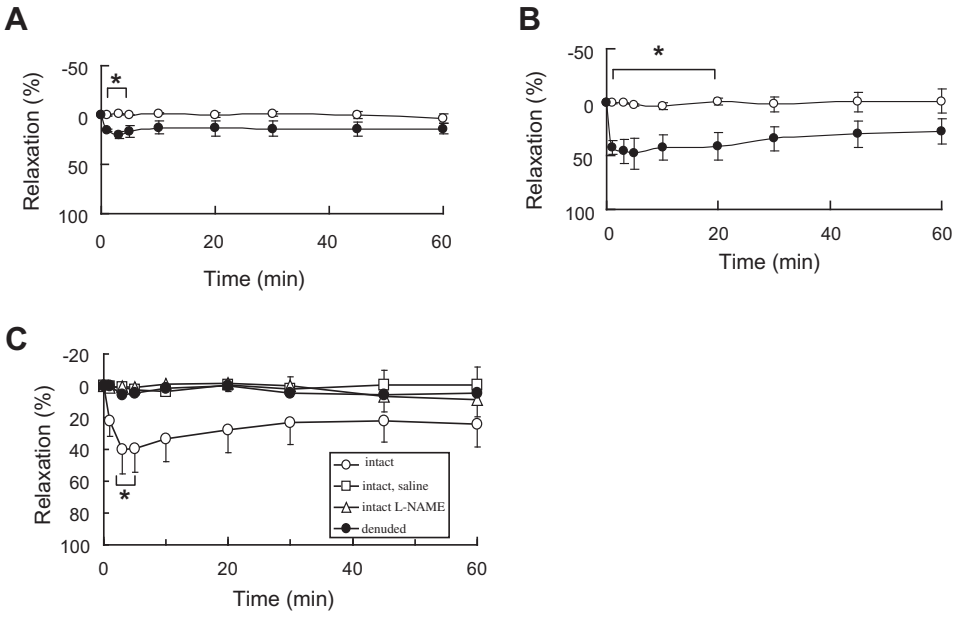

Fig. 4. Vascular effects of RBP4 on aortic rings isolated from Wistar (WT) and Goto-Kakizaki (GK) rats. (A, B) Time series analysis of relaxation of isolated rings treated with RBP4 or saline from WT (A) and GK rats (B). In (A, B), data are shown for incubations in Krebs-Henseleit solution containing physiological saline (○) and RBP4 (5 μg/mL) (●) (n = 3, respectively). * indicates a significant difference vs. control (P < 0.05). (C) Additional study of aortic rings from three more GK rats: () rings with an endothelium with RBP4 in the absence of L-NAME, (□) rings with an endothelium treated with saline without RBP4, (△): rings with an endothelium with RBP4 (5 μg/mL) treated with 100 μM L-NAME, () rings with RBP4 (5 μg/mL) without an endothelium. *Significant difference vs. saline-treated rings (P < 0.05).

addition of L-NAME, which inhibits eNOS production, to rings with endothelial cells abolished the dilatation effect of RBP4, and RBP4 had no effect on aortic rings without endothelial cells. These results support those found *in vitro* and show that RBP4 has a vasodilatory effect that is dependent on NO production in endothelial cells. As previously described, insulin increases NO production via the PI3K/Akt/eNOS pathway in vascular endothelial cells [17]. However, insulin has only a weak vasodilatory effect in aortic rings (about 10%) [21] because of simultaneous ET-1 production in vascular endothelial cells by insulin [17]. We also confirmed that insulin had hardly any vasodilatory effect on aortic rings of rats in a previous study [22]. Therefore, we speculate that the robust vasodilatory effect of RBP4 shown in the current study depends not only on effects on the PI3K/Akt/eNOS pathway but also on an effect on the ERK1/2-ET-1 pathway that is probably opposite to that of insulin.

The serum concentration of RBP4 is approximately 40–50 μg/mL in humans [11,23], which is approximately 10-fold the concentration used in the *in vitro* and *ex vivo* experiments in this study. However, most RBP4 is bound to retinol in the circulation, with a reported binding ratio of approximately 85% [24]. Therefore, free (unbound) RBP4 (apo-RBP4) levels in the circulation are similar to the RBP4 concentration used in this study. This suggests that RBP4 may have an acute vasodilatory effect at its physiological concentration. However, we note that there is no evidence that circulating levels of RBP4 change acutely under physiological conditions in humans. Therefore, from a clinical perspective, the chronic effect of RBP4 appears to be more important than the acute effect found in this study. Nonetheless, because the acute effect of RBP4 appears to be potentially beneficial for the vascular endothelium due to the increase in NO, we believe that this property of RBP4 may be of interest pharmacologically.

We did not investigate the chronic effect of RBP4 on vascular endothelial function. However, RBP4 is unlikely to have a direct clinical influence on vascular tonus, because the effects of RBP4 on endothelial cells and aortic rings were transient. Therefore, the possible correlation between serum RBP4 levels and SBP or FMD in type 2 diabetes [7–14] might be based on indirect mechanisms, and not on an effect of RBP4 on the vascular endothelium.

Several aspects of this study should be considered. First, we note that NO production in endothelial cells was measured by DAF2 fluorescence in the supernatant, which may explain the requirement for a high dose of insulin to obtain full NO production. Therefore, a more sensitive quantitative assessment of intracellular NO production may have been achieved using fluorescent microscopy with a cell permeable form of DAF2, such as DAF2 diacetate. Second, in the study of RBP4 vasodilatation of aortic rings, commercial recombinant RBP4 was used that may include endotoxin. However, the endotoxin levels in the recombinant RBP4 were relatively low (0.5 EU/mg) and a previous report [25] showed that it is unlikely that this level of endotoxin affects aortic vasodilatation. Finally, studies of the chronic effects of RBP4 require performance of a study with a longer-term observation period.

In conclusion, we found that RBP4 has a unique and robust acute effect in vascular endothelial cells that involves enhancement of NO production via stimulation of the PI3K/Akt/eNOS pathway and inhibition of insulin-induced ET-1 secretion, resulting in vasodilation of isolated aortic rings.

References

[1] M. Kanai, A. Raz, D.S. Goodman, Retinol-binding protein: the transport protein for vitamin A in human plasma, J. Clin. Invest. 47 (1968) 2025–2044.
[2] D.S. Goodman, Plasma retinol-binding protein, Ann. NY Acad. Sci. 348 (1980) 378–390.
[3] C. Tsutsumi, M. Okuno, L. Tannous, R. Piantedosi, M. Allan, D.S. Goodman, W.S. Blaner, Retinoids and retinoid-binding protein expression in rat adipocytes, J. Biol. Chem. 267 (1992) 1805–1810.
[4] Q. Yang, T.E. Graham, N. Mody, F. Preitner, O.D. Peroni, J.M. Zabolotny, K. Kotani, L. Quadro, B.B. Kahn, Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes, Nature 436 (2005) 356–362.
[5] P. Balagopal, T.E. Graham, B.B. Kahn, A. Altomare, V. Funanage, D. George, Reduction of elevated serum retinol binding protein in obese children by lifestyle intervention: association with subclinical inflammation, J. Clin. Endocrinol. Metab. 92 (2007) 1971–1974.
[6] M. Broch, J. Vendrell, W. Ricart, C. Richart, J.M. Fernandez-Real, Circulating retinol binding protein-4, insulin sensitivity, insulin secretion, and insulin

disposition index in obese and nonobese subjects, Diabetes Care 30 (2007) 1802–1806.
[7] C. Erikstrup, O. Mortensen, B.K. Pedersen, Retinol-binding protein 4 and insulin resistance (letter to the editor), N. Engl. J. Med. 355 (2006) 1393–1394.
[8] T.E. Graham, Q. Yang, M. Bluher, A. Hammarstedt, T.P. Ciaraldi, R.R. Henry, C.J. Wason, A. Oberbach, P.A. Jansson, U. Smith, B.B. Kahn, Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects, N. Engl. J. Med. 354 (2006) 2552–2563.
[9] N. Kloting, T.E. Graham, J. Berndt, S. Kralsch, P. Kovas, C.J. Wason, M. Fasshauer, M.R. Schon, M. Stumvoll, M. Bluher, B.B. Kahn, Serum retiniol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass, Cell Metab. 6 (2007) 79–87.
[10] D.C. Lee, J.W. Lee, J.A. Im, Association of serum retinol binding protein 4 and insulin resistance in apparently healthy adolescents, Metabolism 56 (2007) 327–331.
[11] Q. Qi, Z. Yu, X. Ye, F. Zhao, P. Huang, F.B. Hu, O.H. Frano, J. Wang, H. Li, Y. Liu, X. Lin, Elevated retinol-binding protein 4 are associated with metabolic syndrome in Chinese People, J. Clin. Endocrinol. Metab. 92 (2007) 4827–4834.
[12] K. Takebayashi, M. Suetsugu, S. Wakabayashi, Y. Aso, T. Inukai, Retinol binding protein-4 levels and clinical features of type 2 diabetes patients, J. Clin. Endocrinol. Metab. 92 (2007) 2712–2719.
[13] K. Takebayashi, Y. Aso, T. Inukai, Role of retinol-binding protein 4 in the pathogenesis of type 2 diabetes, Expert Rev. Endocrinol. Metab. 3 (2008) 161–173.
[14] M. von Eynatten, P.M. Lepper, D. Liu, K. Lang, M. Baumann, P.P. Nawroth, A. Bierhaus, K.A. Dugi, U. Heemann, B. Allolio, P.M. Humpert, Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease, Diabetologia 50 (2007) 1930–1937.
[15] A. Yao-Borengasser, V. Varma, A.M. Bodles, N. Rasouli, B. Phanavanh, M.J. Lee, T. Starks, L.M. Kern, H.J. Spencer, A.A. Rashidi, R.E. McGehee, S.K. Fried, P.A. Kern, Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone, J. Clin. Endocrinol. Metab. 92 (2007) 2590–2597.
[16] E. Ingelsson, L. Lind, Circulating retinol-binding protein 4 and subclinical cardiovascular disease in elderly, Diabetes Care 32 (2009) 733–735.
[17] R. Muniyappa, M. Montagnani, K.K. Koh, M.J. Quon, Cardiovascular actions of insulin, Endocr. Rev. 28 (2007) 463–491.
[18] D. Berendji-Grun, V. Kolb-Bachofen, K.D. Kronke, Nitric oxide inhibits endothelial IL-1[β]-induced ICAM-1 gene expression at the transcriptional level decreasing Sp-1 and AP-1 activity, Mol. Med. 7 (2001) 748–754.
[19] C. Rask-Madsen, G.I. King, Mechanisms of disease: endothelial dysfunction in insulin resistance and diabetes, Nat. Clin. Pract. Endocrinol. Metab. 3 (2007) 46–56.
[20] Product Insert, Abcam Ltd, Cambridge, UK. RBP4 Protein (catalog no. ab59967).
[21] J.D. Symons, S.L. McMillin, C. Riehle, J. Tanner, M. Palionyte, E. Hillas, D. Jones, R.C. Cooksey, M.J. Birnbaum, D.A. McClain, Q.J. Zhang, D. Gale, L.J. Wilson, E.D. Abel, Contribution of insulin and Akt1 signaling to endothelial dysfunction and blood pressure, Cir. Res. 104 (2009) 1085–1094.
[22] K. Takebayashi, R. Sohma, R. Naruse, Y. Aso, T. Inukai, Effect of insulin on aortic rings isolated from Wister rats, Dokkyo J. Med. Sci. 37 (2010) 9–14.
[23] N. Takashima, H. Tomoike, N. Iwai, Retinol-binding protein 4 and insulin resistance, N. Engl. J. Med. 355 (2006) 1392.
[24] S.K. Frey, J. Spranger, A. Henze, F.H. Pfeiffer, F.J. Schweigert, J. Raila, Factors that influence retinol-binding protein 4-tansthyretin interaction are not altered in overweight subjects with type 2 diabetes mellitus, Metabolism 58 (2009) 1386–1392.
[25] P.R. Myers, T.F. Wright, Tanner MA & Adams HR 1992 EDRF and nitric oxide production in cultured endothelial cells: direct inhibition by *E coli* endotoxin, Am. J. Physiol. 262 (1992) H710–H718.