Phosphorylation of β1-integrin in juxtaglomerular cells helps control blood pressure during the progression of diabetic nephropathy

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Abstract

The induction of a high blood pressure due to diabetic nephropathy depends on the increase in renin secretion from juxtaglomerular cells, but many aspects of how juxtaglomerular cells sense blood pressure changes in the afferent arteriole and consequently react remain unclear. In this study, we detected the juxtaglomerular cell-specific phosphorylation of the threonine-788/789 site of β1-integrin, and its expression was negatively correlated with renin production. This relationship was also observed in a culture system of a juxtaglomerular cell line, suggesting that β1-integrin is deeply involved in the regulation of renin production. The knockdown of β1-integrin in the culture system increased renin production, but the degree of the increase was comparable to the increase in renin production by knockdown of connexin-40, which is considered to be an important molecule that plays a role in the pressure sensing mechanism of juxtaglomerular cells. This suggests that the mechanism underlying the regulation of renin production by β1-integrin in juxtaglomerular cells may contribute to the pressure-sensing function of juxtaglomerular cells themselves. Threonine-788/789 phosphorylation of β1-integrin may be involved in the regulation of this pressoreceptor function.

(176 words)

Introduction

Diabetic nephropathy (DN) and high blood pressure are widely known to be mutually aggravating factors [1–6]. Diabetes mellitus (DM) impairs the myogenic response [7, 8] and TGF responses [9], which are autoregulatory functions of the renal blood flow, thereby dilating afferent arterioles and inducing glomerular hyperfiltration and glomerular capillary hypertension [3].

Hypertension causes oxidative stress and inflammation in the kidney, leading to the development of DN [4]. Activation of RAAS [10], upregulation of endothelin-1 (ET-1) [11, 12], upregulation of reactive oxygen species [13, 14], and downregulation of nitric oxide (NO) [15, 16] induced by a worsening renal function further elevate the blood pressure, leading to a vicious cycle of hypertension and glomerular damage. The rate-limiting enzyme involved in blood pressure regulation by the renin-angiotensin system is renin, and it is key to the renal functional deterioration and the vicious cycle of high blood pressure.

Studies concerning juxtaglomerular cells have revealed the following mechanisms underlying renin secretion: (a) stimulation of renin secretion through β-adrenergic receptors by the excitement of the kidney sympathetic nerves sensing a decrease in the blood flow quantity and blood pressure, (b) control of renin secretion through prostaglandin-E2/adenosine/ATP/NO released from the macula densa, which senses raw urinary NaCl levels; (c) feedback regulation of renin secretion by humoral factors, such as angiotensin II; (d) regulation by pressoreceptors (baroreceptor) of the juxtaglomerular cells, which sense the blood pressure [17, 18, 19]. Many intensive studies have clarified the heteronomy-like mechanisms that receive regulatory factors secreted by organs other than juxtaglomerular cells (a-c); however, the

independent mechanisms underlying how juxtaglomerular cells detect changes in blood pressure of the afferent arterioles by pressoreceptors and control renin production and secretion remains unclear.

Regulation of renin secretion through juxtaglomerular cells sensing the blood pressure or blood flow has been proven by perfusion experiments of isolated glomeruli [20, 21]. The gap junction protein connexin-40 (Cx40) was recently shown to be essential for the pressoreceptor function of juxtaglomerular cells, as knockout of Cx40 specifically in a mouse juxtaglomerular cell resulted in a high blood pressure due to renin hypersecretion congenitally, and no suppression of renin secretion was observed when perfusion pressure was applied to the juxtaglomerular apparatus isolated from the knockout mice [22].

β1-integrin is a molecule involved in cell-cell and cell-substrate attachment by dimerization with various subtypes of α-integrin. For example, α3β1-integrin has been shown to be expressed primarily in podocytes and is suspected of playing a critical role in the attachment of podocytes to the glomerular basement membrane. In addition, podocyte depletion is suspected to occur with the loss of the function of α3β1-integrin [23, 24]. As a regulatory mechanism of the β1-integrin function, phosphorylation/dephosphorylation of the intracellular domain alters the structure of integrin dimers. In particular, phosphorylation of the threonine-788/789 sites is known to regulate cell adhesion and cell motility [25, 26, 27].

We herein report the phosphorylation of β1-integrin specific to juxtaglomerular cells and the relationship between the phosphorylated β1-integrin and renin production. Our findings suggest the regulation of blood pressure by the phosphorylation of β1-integrin in juxtaglomerular cells.

Results

Phosphorylation of threonine-788/789 sites of β1-integrin specific to juxtaglomerular cells

To investigate β1-integrin phosphorylation during DN progression, specific antibodies against phosphorylated threonine-788 and threonine-789 of β1-integrin were applied to kidney tissue sections from various stages of streptozotocin-treated rats (STZ rat) to study the distribution and phosphorylation of β1-integrin. Both antibodies showed intensive reactions at the juxtaglomerular apparatus in the kidney of STZ rats and controls (Fig. 1a). Since stained cells seemed to exist around afferent arterioles, double staining with anti-renin antibody was performed, and these cells were confirmed to be renin-secreting juxtaglomerular cells (Fig. 1b).

A negative correlation between the number of cells containing threonine-788/789 phosphorylated β1-integrin and the renin expression

The changes in the phosphorylation of threonine-788/789 of β1-integrin specific to juxtaglomerular cells during DN progression were investigated in the kidneys of STZ rats, along with their relationship with the renin production. The production of renin gradually increased after the onset of DN, peaking in the second

month but then decreasing and returning to the original level in the sixth month. In contrast, the number of juxtaglomerular cells in which threonine-788/789 phosphorylation of β1-integrin was detected gradually decreased after the onset of DN, peaking in the second month and then increasing further before ultimately returning to the original level (Fig. 2). These results showed that there was a negative correlation between the renin production and threonine phosphorylation of β1-integrin during DN progression in STZ rats (correlation coefficient = 0.61).

Activation/inhibition of protein kinase Cε reproduced the negative correlation between threonine-788/789-phosphorylated β1-integrin and the renin expression in vitro

As a negative correlation between the renin production and threonine-788/789 phosphorylation of β1-integrin in juxtaglomerular cells was observed in kidneys of STZ rats, the relationship between them was examined in cultured cells *in vitro*. As4.1 cells, a line of mouse juxtaglomerular cells, were used to examine the changes in renin production after the addition of PMA/BIM-1, an activator/inhibitor of PKCε, which is a subtype of PKC that phosphorylates threonine-788/789 of β1-integrin [27]. As TGF-β1 also reportedly promotes the threonine-788/789 phosphorylation of β1-integrin specifically [26], we also investigated the effect of TGF-β1 addition to the culture.

The results showed that renin production in As4.1 cells after 24 h of drug addition was inhibited by PKC activation, and conversely, renin production was enhanced when PKC was inhibited, confirming a negative relationship between these two, similar to that *in vivo* (Figure. 3a). TGF-β1 also inhibited renin production and supported this relationship. When we monitored the changes in renin production up to 24 h after PMA/BIM-1 treatment, we found that the renin production showed severe changes, including a rapid rise and fall in the early period up to three hours after treatment, but thereafter there was a gradual suppression and steady increase respectively until the end of the experiment (Fig. 3b). Western blotting for phosphorylated threonine-788/789 of β1-integrin showed results compatible with the above findings, wherein the acceleration of phosphorylation by PMA occurred 3 h after drug addition and was sustained for 24 h. BIM-1 suppressed the phosphorylation of the sites, and TGF-β1 promoted the phosphorylation but not as much as PMA (Fig. 3c).

Knockdown of the β1-integrin expression results in out-of-control renin expression, as does knockdown of the connexin-40 expression

Since PMA and BIM are effective across a broad spectrum of PKC subtypes, we cannot ignore the possibility that the changes in renin production seen in Fig. 3 are a combination of effects from other phosphorylation pathways in addition to the phosphorylation of β1-integrin by PKCε. In order to clarify the effect of β1-integrin on renin production, we performed knockdown of β1-integrin in As4.1 cells and examined its effect on renin production. The results showed that knockdown of β1-integrin enhanced renin production in As4.1 cells to about twice that in controls. Knockdown of Cx40, a putative pressoreceptor, also increased the renin production to the same extent, suggesting that these renin production levels represent an uncontrollable state of renin production of juxtaglomerular cells (Fig. 4).

Discussion

In this study, we demonstrated for the first time the phosphorylation of the threonine-788/789 of β1-integrin specific to juxtaglomerular cells and also found a negative correlation between the phosphorylation of this site of β1-integrin and renin production, suggesting that the phosphorylation/dephosphorylation of this site of β1-integrin may be part of the blood pressure regulation mechanism in the process of DN.

Various reports have described the effects of phosphorylation of threonine-788/789 of β1-integrin on cell adhesion and motility [25, 26, 27], and a recent report noted that this site of β1-integrin functions as a phospho-switch to activate/deactivate integrin dimers and regulate integrin functions, such as the strength of cell adhesion [30]. In juxtaglomerular cells, this mechanism may contribute to the production and secretion of renin, which is a specific function of this type of cell.

Everett et al. reported that renin production is reduced in advanced diabetes [31]. They counted the number of renin-positive cells per juxtaglomerular apparatus in a strain of rats that spontaneously develop diabetes and reported that the index peaked at two months after the onset of diabetes, which is consistent with our findings in this study. In our observation of STZ rats, the blood pressure continued to increase after the onset of diabetes, but the renin production began to decrease at two months after the onset of diabetes, and the blood pressure gradually decreased accordingly. During this process, the level of threonine-788/789 phosphorylation of β1-integrin was negatively correlated with the change in renin production, but the causal relationship between the phosphorylation of β1-integrin and renin production was unknown. To confirm this, studies using a juxtaglomerular cell line showed that the activation of integrin dimers by phospho-switching is required to suppress renin production. In STZ rats, the phospho-switch is inactivated after the onset of DN, which may prevent the suppression of renin production in response to elevated blood pressure. Thereafter, some unknown mechanism reactivates the phospho-switch, thereby restoring the suppression of renin production. There may be a temporal change in the predominance of PKCε and phosphatase activities during DN.

In addition, the mechanism by which activated integrin dimers suppress renin production may involve regulating the sensing of pressure by juxtaglomerular cells. This is because renin production in cultured cells is not regulated by a heteronomous mechanism that receives regulatory factors secreted from other organs and cells but is controlled by the juxtaglomerular cells themselves and thus may be involved in the pressoreceptor function, the only independent regulatory mechanism of renin production in juxtaglomerular cells.

Cx40 is essential for the pressoreceptor function in juxtaglomerular cells, and knockout mice with this molecule are known to be unable to suppress renin secretion [22]. Cx is a component of the intercellular adhesion apparatus and is expected to be well related to integrins, which are also cell adhesion molecules on the cell membrane. In fact, direct/indirect interaction between β1-integrin and Cx has been reported in several studies [32, 33, 34]. Knockdown of Cx40 in the present study resulted in an unregulated increase in renin production, as in Cx40 knockout mice, while the knockdown of β1-integrin

also increased it to the same extent, strongly suggesting that these molecules may be involved in the same renin production mechanism. This suggests that β1-integrin may be involved in the pressure sensing of Cx40 in juxtaglomerular cells. For example, there may be a mechanism that controls the sensitivity of Cx40 pressure sensing on the cell membrane by altering the cell-substrate adhesion strength. These results strongly suggest that the fine regulation of pressure sensing by the synergistic interaction of integrins and Cx40 controls the renin production of juxtaglomerular cells.

A further analysis of the mechanism of blood pressure sensing by Cx40 and its regulation by integrins including mechanism of phosphorylation/dephosphorylation of β1-integrin during DN may lead to the elucidation of the pressoreceptor-mediated regulation of blood pressure and the development of new DN therapies targeting this mechanism.

Materials And Methods

Animal experimental protocols were approved by the Institutional Animal Care and Use Committee (Permission number: 194019) and carried out according to the Tokai University Animal Experimentation Regulations. All animal studies were also carried out in compliance with the ARRIVE guidelines [35]. All methods were carried out in accordance with relevant guidelines and regulations.

Animals

Five-week-old male SD-rats (CLEA Japan Inc., Tokyo, Japan) underwent intravenous injection of streptozotocin (STZ; 60 mg/kg). After one week, individuals with more than 400 mg/dl blood glucose were used subsequent experiments as an animal model of DN.

Immunohistochemistry

Kidneys were recovered from rats at various stages of diabetes. Specimens were fixed and embedded in paraffin, and 4-μm thick sections were prepared. Sections were deparaffinized, dehydrated and soaked in 1% blocking reagent (Invitrogen, Carlsbad, CA, USA) in phosphate-buffered saline (PBS). Mouse monoclonal anti-renin antibody (MAA889Ra21; Cloud-Clone Corp., Houston, TX, USA) and rabbit polyclonal antibodies for Thr788-phospho-integrin β1 (PA5-38256; ThermoFisher, Waltham, MA, USA) and Thr789-phospho-integrin β1 (PA5-36779; ThermoFisher), respectively, were used as the primary antibodies. After washing with PBS containing 0.05% Tween 20, horseradish peroxidase-conjugated anti-mouse/rabbit IgG antibody was applied. After washing as above, the bound antibodies were visualized with 3,3-diaminobenzidine (DAB). Goat anti-mouse IgG antibody conjugated with Alexa Fluor 594 and goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 were used as secondary antibodies for detection by fluorescence microscopy.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was recovered from kidney tissues or cultured cells with an RNaqueous RNA purification kit (ThermoFisher) and converted to cDNA using a SuperScript III First-Strand Synthesis System

(ThermoFisher). qRT-PCR was performed with the cDNA as previously described [28]. The reaction mixture was prepared as described in the kit's instruction manual (TaqMan Gene Expression Assays; ThermoFisher), containing primers and probes for rat or mouse renin (Assay ID: Rn00561847_m1, Mm02342887_mH) and endogenous controls (18S ribosomal RNA or β-actin). PCR was performed on an ABI StepOne Plus (ThermoFisher). Data were analyzed by the comparative Ct method, and the amounts of renin mRNA were expressed relative to that of endogenous controls.

Cell culture

The immortalized mouse juxtaglomerular cell-line As4.1 [29] was cultured with DMEM medium (ATCC, Manassas, VA, USA) containing 10% fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin in 5% CO_2 atmosphere at 37°C. Phorbol 12-myristate 13-acetate (50 ng/ml; PMA; R&D Systems, Minneapolis, MN, USA), 2 μM bisindolylmaleimide (BIM-1; R&D Systems), and 1 ng/ml transforming growth factor-β1 (TGF-β1; R&D Systems) were added to the cultures. Stealth RNAi siRNAs for β1-integrin (MSS205553; ThermoFisher) and for connexin-40 (MSS236624; ThermoFisher) were mixed with RNAiMax transfection reagent (ThermoFisher) for knockdown experiments.

Western blotting

Cells were lysed by xTractor buffer (Takara Bio, Shiga, Japan) with ProteoGuard protease inhibitor (Takara Bio) and Cryonase nuclease (Takara Bio). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with NuPAGE 4–12% Bis-Tris gel (ThermoFisher) and proteins were transferred to a PVDF membrane with an iBlot dry blotting system (ThermoFisher). After blocking with 5% cow-albumin in Tris-buffered saline, the PVDF membrane was treated with the rabbit polyclonal antibodies for Thr788/789-phospho-integrin β1 mentioned above, washed with TBS containing 0.05% Tween 20 and treated with horseradish peroxidase-conjugated anti-rabbit IgG antibody. After washing, the reactions were visualized with DAB.

Statistical analyses

Results are represented as the mean (± standard deviation) of at least four samples. Comparisons between two groups were done using Student's *t*-test. Spearman's correlation coefficient analysis was used to examine the relationship between parameters. $P < 0.05$ was considered statistically significant.

Declarations

Acknowledgments

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Author contributions

N.S. designed the study and was involved in all experiments and data analysis; M.Kondo performed the immunohistochemistry; M.O. performed the qPCR; K.S. performed the cell culture; H.M. performed the animal experiments; M.Kimura performed the statistical analysis; M.T. supervised the research team and managed the research progress; N.K., M.A. and M.F. contributed to the interpretation of the results and reviewed the manuscript.

Competing interests

M.T. discloses the following relationships: honoraria from Kyowa Hakko Kirin, Chugai Pharmaceutical, Eli Lilly Japan K.K., MSD Corporation, Sumitomo Dainippon Pharma Co., Ltd.; grants from Sumitomo Dainippon Pharma Co., Ltd., Eli Lilly Japan K.K., LifeScan Japan, Kowa Company, Ltd., MSD Corporation, Sanwa Kagaku Kenkyusho. M.F. has acted as a consultant for Kyowa Hakko Kirin, Bayer Japan, and Novartis; has received honoraria from Kyowa Hakko Kirin, Chugai Pharmaceutical, Bayer Yakuhin, Novartis, Genzyme, and Abbot Japan; and has received grants/research support from Kyowa Hakko Kirin, Chugai Pharmaceutical, and Bayer Yakuhin. The other authors declare that they have no relevant financial or non-financial interests.

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Figures

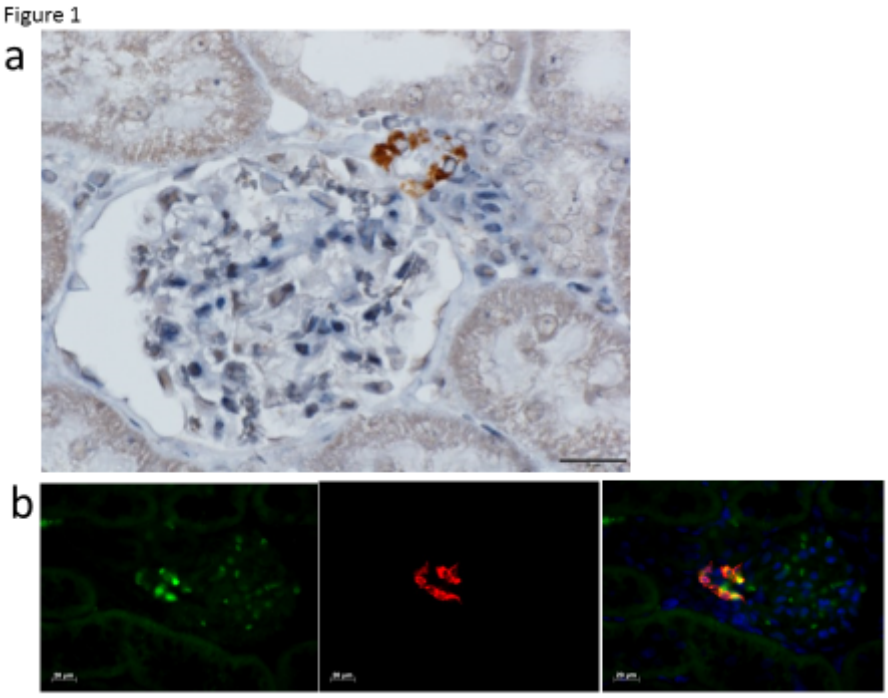

Figure 1

Detection of threonine-788/789 phosphorylation of β1-integrin in juxtaglomerular cells. a. The reaction of antibodies for Thr788/789-phospho-integrin β1 on sections of normal rat kidney was visualized by DAB and counterstained by hematoxylin. Bar: 20 μm. b. Double staining with fluorescent antibodies for renin (green) and Thr788/789-phospho-integrin β1 (red). Autofluorescence of red blood cells was detected as weak green signals. The merged figure with DAPI nuclear staining (blue) showed cells co-expressing renin and phosphorylated integrin β1. Bars: 20 μm.

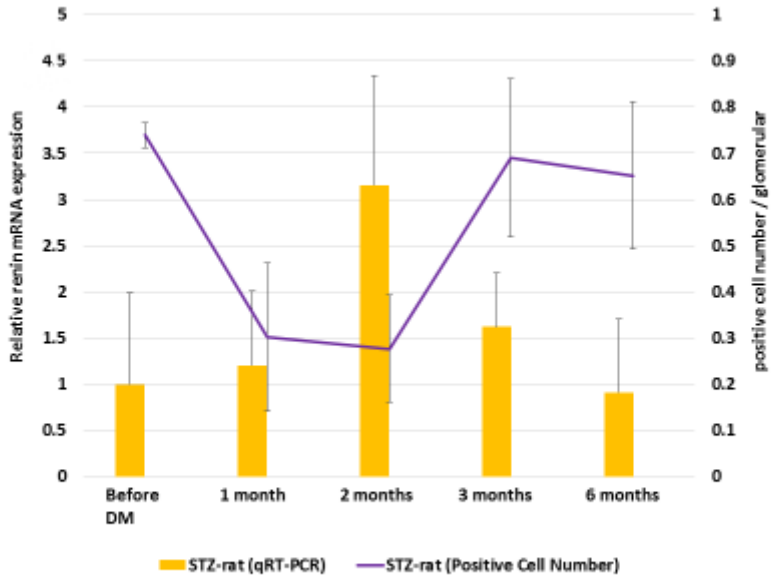

Figure 2

Negative relationship between the renin expression and number of cells positive for Thr788/789-phophorylation of integrin β1. The renal renin expression at various points in STZ rats was determined by qRT-PCR and represented as bars (left axis). The number of cells positive for Thr788/789-phospho-integrin β1 and number of glomeruli were counted in sections of kidneys of STZ rats, and their ratios were represented as a line (right axis).

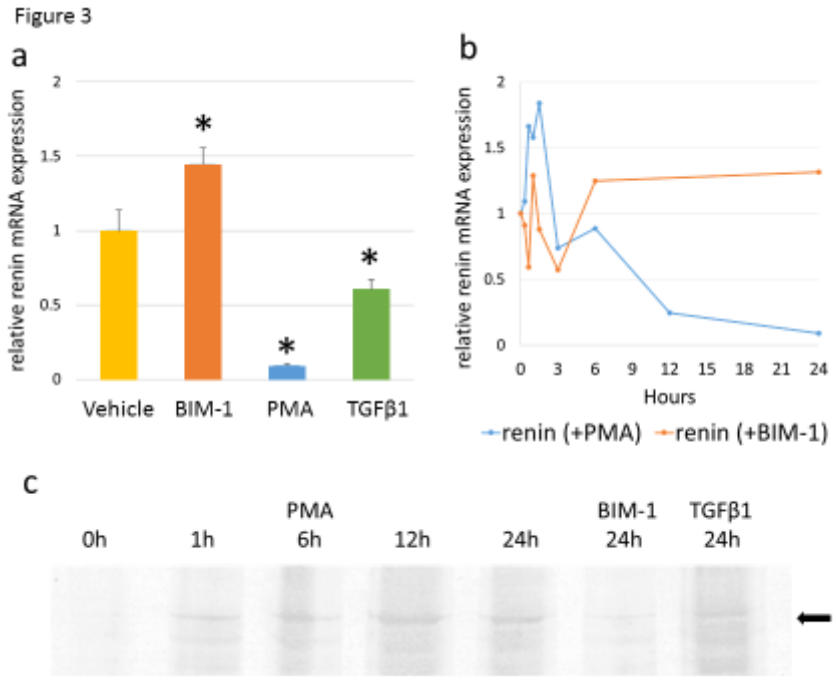

Figure 3

Effects of phosphorylation/dephosphorylation of Thr788/789 of integrin β1 on the renin expression in the juxtaglomerular cell line As4.1. a. As4.1 cells were cultured with 2 μM BIM-1, 50 ng/ml PMA and 1 ng ml TGF-β1 for 24 h. The renin expression was measured by qRT-PCR, and the expression relative to control (vehicle) was represented. Asterisks indicate significant differences ($p<0.05$) from the control. b. Time-course representation of the renin mRNA expression in As4.1 cells cultured with 2 μM BIM-1 (orange) or 50 ng/ml PMA (blue) for 24 h, measured by qRT-PCR. c. Phosphorylation/dephosphorylation of Thr788/789 of integrin β1 in As4.1 cells cultured with 50 ng/ml PMA (1, 6, 12, 24 h), 2 μM BIM-1 (24 h) or 1 ng ml TGF-β1 (24 h). Western blotting detected by anti-phospho-Thr788/789 integrin β1. The arrow indicates the band position of phosphor-Thr788/789 integrin β1.

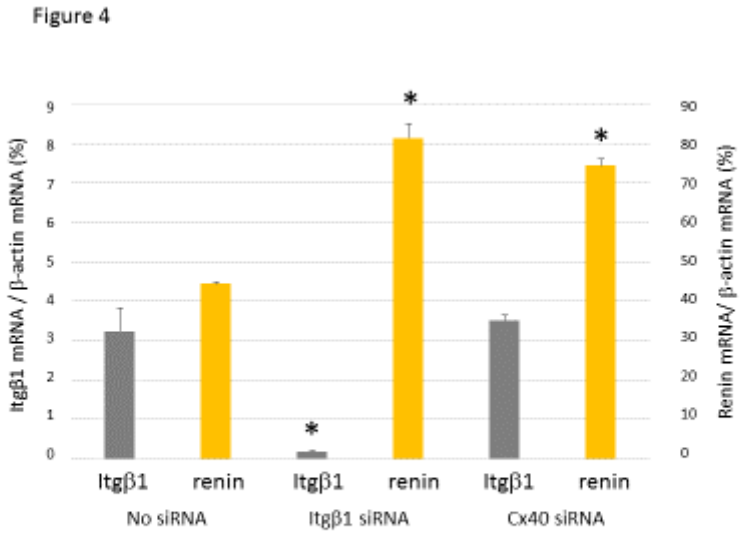

Figure 4

Effects of knockdown of integrin β1 or Cx40 on the renin expression in the juxtaglomerular cell line As4.1. Gene knockdown was performed by culturing As4.1 cells with siRNA for integrin β1 (Itgβ1 siRNA) or Cx40 (Cx40 siRNA) for 24 h. Vehicle was added to the control culture (no siRNA). The expression of integrin β1 (Itgβ1, left axis) or renin (renin, right axis) was quantitated by qRT-PCR. Asterisks indicate significant differences ($p<0.05$) from the control.

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