論文の内容の要旨

論文題目：The possible role of RGS2 in the susceptibility to salt hypertensive kidney injury and the mechanism of regulation of RGS2 m RNA expression in vascular smooth muscle cells

和訳：食塩感受性高血圧における易腎障害性へのＲＧＳ２の役割と血管平滑筋細胞を用いたＲＧＳ２ｍ ＲＮＡ産生調節機序に関する研究

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【Purpose】RGS2 (regulator of G-protein signaling-2)-deficient mice exhibit persistent severe hypertension and vascular constriction through enhanced intracellular signal transduction of angiotensin II (Ang II). There is a particularly intriguing relationship between RGS2 and Ang II type 1 receptor (AT_1R), a GPCR (G-protein coupled receptor) that is part of the key pathway leading to progressive organ damage in patients with heart and kidney diseases. However, the mechanism of regulation of RGS2 in vascular system and implications of RGS2 in pathogenesis of hypertensive organ damage remain to be elucidated. In the present study, I examined the regulation of RGS2 mRNA biosynthesis through AT_1R mediated mechanism in vascular smooth muscle cells (VSMCs) and disclosed the role of RGS2 in salt-sensitive Dahl (Dahl S) rats susceptible to hypertensive kidney damage.

【Methods】In vitro study, VSMCs were isolated from the thoracic aorta of 6-week-old male Wistar rats by explant method. Briefly, aortic walls were cut into 2×2 mm strips. Then the strips were placed onto 10×10 cm culture dishes and they were cultured in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

serum (FBS), 100U/ml penicillin, and 100μg/ml streptomycin (Wako, Osaka, Japan). The media were changed with fresh solutions every 3 days. The strips were maintained at 37°C in a humidified, 5% CO_2 atmosphere, and after 10 days, VSMCs outgrowing from the strips were harvested using 0.05% trypsin/0.02% EDTA (Ethylenediaminetetraacetate). The collected cells were cultured in the same media, and for experiments, cells between passages 4 and 10 were used at semi-confluence.

10^6 cells were placed onto a 35×35mm 6-wells plate, and cultured in DMEM with 10% FBS. When the cells reached a semi-confluent growth state, the media were changed to FBS-free media with or without a given concentration of test compounds. The cells were harvested with the trypsin solution, and the collected cells were stored at -80°C until the assay.

Design of in vitro Study

1) Response of RGS2 mRNA to Ang II

Firstly, we investigated RGS2 mRNA response to Ang II. The VSMCs in semiconfluent growth state were incubated in FBS-DMEM including 100nmoles/L Ang II at 37°C for 0 hr to 6 hrs. Then the cells were harvested with the trypsin solution at each time point. The pellets after centrifugation were quickly plunged into liquid nitrogen and the samples were stored under -80°C until RNA extraction.

2) AT_2R and RGS2 mRNA

Ang II exerts its actions through two receptors: AT_1R and AT_2R. We attempted to disclose the role of Ang II AT_1R in the RGS2 mRNA expression. The AT_2R agonist, CGP42112A or the antagonist, PD123, 319, stimulated the cells at 37°C for 2 hrs in FBS-DMEM with or without 100nmoles/L Ang II. After 2 hrs incubation, After 2 hrs incubation, the cells were collected using centrifugation, and pellets were quickly plunged into liquid nitrogen. The samples were stored under -80°C until RNA extraction.

3) ARBs and RGS2 mRNA

Next, we examined whether the effects of Ang II on RGS2 mRNA expression are Ang II AT_1R-mediated events. VSMCs were pre-incubated in media containing 10nmoles/L or 100nmoles/L Ang II for 2 hours. Thereafter, a given concentration of

losartan was added to the media, and the cells were harvested at 0 h to 6 hrs. The cells were centrifuged at room temperature for 5 minutes, and the pellets were quickly frozen in liquid nitrogen. The cells were stored under -80°C until RNA extraction. Then, VSMCs stimulated with losartan, a metabolite of losartan (EXP3174), or olmesartan, with and without Ang II in media. In this experiment, we chose 200nmoles/L EXP3174 and 200nmoles/L olmesartan since the doses are reportedly enough to block AT_1R.

4) Mechanism of direct agonist of losartan for RGS2 mRNA biosynthesis

Since it is reported that the increase of RGS2 mRNA expression in response to Ang II is mediated in part by PKC activation, we examined the role of PKC activity in the alterations of RGS2 mRNA by losartan in Ang II-free media. VSMCs were pre-incubated for 30min in FBS-free DMEM including the PKC inhibitor (GF109203X), and then, a given concentration of losartan was added to the media. After 2 hours, the cells were harvested. The cells were quickly frozen using liquid nitrogen and stored -80°C until the RNA extraction.

Design of in vivo Study

In vivo study, Dahl S rats and spontaneously hypertensive rats (SHR) were fed a high salt diet for 2 weeks from age of 4 weeks. Six-week-old rats fed a low salt diet were subcutaneously given a 4-weeks pressor dose of Ang II 100ng/min/kg BW. Twenty four-hour urine was collected every week using a metabolic cage. At the end of the study, blood samples and the organ of interest were obtained under pentobarbital anesthesia (75 mg/kg BW). The left kidney and aorta were quickly plunged into liquid nitrogen for mRNA determination. The right kidney was used for morphological examination. Blood samples separated and the urine were stored under -80°C until the assay.

Systolic blood pressure (SBP) was determined every week by the modified tail-cuff method of Friedman and Freed. Plasma and Urinary creatinine concentration were determined with an autoanalyser. Urinary protein excretion was measured using Protein assay kit (Bio-Rad). Half of the each left kidney was fixed in 3.5% formalin solution. Sagittal slices of the kidney were cut and embedded with paraffin, and then two-micrometer sections were excised for the morphological study. These sections were stained with hematoxylin and eosin (HE), and Periodic acid-Schiff (PAS) for histological investigation.

In vitro and vivo studies, RGS2 mRNA expression was determined by Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from VSMCs and tissues (kidney and aorta) using High Pure Isolation / Tissue kit (Roche Diagnostics) according to the manufacturer's instructions. Total RNA concentrations and the purity were determined by a spectrophotometer at wavelength of 260/280 nm. The ratio of OD_{260}/OD_{280} was >1.90 in all extracted RNA samples. 460 μg RNA were used for cDNA synthesis using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. RGS2 and GAPDH mRNA were quantified by a LightCycler 1.5 real-time PCR (Roche Diagnostics) and a LightCycler TaqMan Master (Roche Diagnostics). The primers were designed by software provided on a website of Nihon Gene Research Laboratories Inc., Tokyo, Japan. The probes were selected from Universal Probe Library Probes 1-165 for LightCycler.

【**Results**】In vitro study, RGS2 mRNA expression significantly increased with Ang II stimulation in time-dependent and dose-dependent manners. The agonist (CGP 42114A) and antagonist (PD312, 213) of AT_2R did not influence Ang II-induced up-regulation of RGS2 mRNA expression. These indicated that the AT_2R was not involved in the up-regulation of RGS2 mRNA in response to Ang II stimulation.

In ARBs study, olmesartan and EXP3174 (metabolite of losartan) completely abolished the Ang II-induced increase of RGS2 mRNA expression while losartan in part decreased this elevation of RGS2 mRNA. Surprisingly, however, losartan per se time-dependently and dose-dependently up-regulated RGS2 mRNA expression. This losartan-induced up-regulation of RGS2 mRNA biosynthesis was completely abolished when the cells were pre-treated with olmesartan in prior to the losartan stimulation. These results suggested that losartan up-regulated RGS2 mRNA biosynthesis, through binding to AT_1R.

To determine whether PKC is involved in the enhanced RGS2 mRNA expression by Ang II or losartan, we examined the effects of GF109203X (PKC inhibitor) on the Ang II-induced increase of RGS2 mRNA expression. GF109203X significantly decreased the RGS2 mRNA biosynthesis by 79±1%. Similarly, this inhibitor attenuated the increase of RGS2 mRNA by losartan. However, the decline was much smaller than in case of Ang II stimulation (55±1%). These data indicated that the increase of RGS2 mRNA expression was composed of two components, PKC-dependent and PKC-independent. Particularly, in losartan stimulation, the

PKC-independent component was much greater than the PKC-dependent component.

In vivo study, the blood pressure elevation in Dahl S rats was associated with more than 20 times increase in urinary protein excretions. In contrast, the urinary protein excretions did not change in hypertensive SHR. The difference between the two strains was apparently significant ($p<0.001$). Similarly, glomerular sclerosis was greater in Dahl S rats than in SHR while SHR exhibited higher SBP than Dahl S rats. These data strongly suggested that Dahl S rats strain was prone to hypertensive renal damage, as compared with SHR strain. The two strains have different mechanisms for hypertension. In order to examine whether Dahl S rats are susceptibility to hypertensive kidney damage besides organ damage due to volume expansion, we minimized the influence of volume expansion using Dahl S and Dahl salt-resistant (Dahl R) rats fed a low salt diet, and investigated the susceptibility to the kidney damage following Ang II induced hypertension.

Ang II infusion for 4 weeks significantly increased SBP in both Dahl S and Dahl R rats fed a low salt diet. In Dahl S rats, the increase in SBP was associated with kidney injury with greater glomerular sclerosis and much more urinary protein excretion than in Dahl R rats. Moreover, RGS2 mRNA expression following Ang II infusion was significantly increased in the kidney or aorta of Dahl R rats whereas in Dahl S rats the normal responses were apparently blunted.

【Conclusions】We demonstrated that there is a negative feedback loop between Ang II mediated signal transduction and RGS2 biosynthesis. Ang II antagonism interrupts the feedback mechanism, thereby increasing the signaling through RGS2 down-regulation. However, some ARB, losartan, behaves as a direct stimulant to RGS2 mRNA. The changes of RGS2 mRNA are influenced by PKC-dependent and -independent mechanisms. The PKC-independent mechanism is mainly due to the direct action of losartan on RGS2 mRNA biosynthesis. PKC-independent mechanism was not clear; however, some structural moiety of losartan might be required to stimulate RGS2 mRNA biosynthesis. In this context, we found that EXP3174 did not increase RGS2 mRNA expression. Since EXP3179 is reported to work through intracrine mechanism, the direct effects of losartan may be due to EXP3179. We have not data on EXP3179, and this is our next step to do.

The in vivo study demonstrated that Dahl S rats are prone to hypertensive renal injury through hypersensitivity to mechanical stress and/or possibly, through

up-regulation of Ang II signal transduction by blunted response of RGS2 mRNA expression. This difference of alteration of RGS2 mRNA response to Ang II might be due to difference of transcriptional regulation between Dahl S and Dahl R rats. We hypothesized that some alterations occur in the promoter area of RGS2 gene in Dahl S rats. This is our future research direction.

Considering the data from my in vitro and in vivo studies, characterization of RGS2 action and the mechanisms of RGS2 regulation may lead to a promising strategy for the development of novel antihypertensive treatment.