Changes in the nitric oxide-soluble guanylate cyclase system and natriuretic peptide receptor system in placentas of pregnant Dahl salt-sensitive rats

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Short Title: NO and NPs systems in placenta of Dahl S rat

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Abstracts

Aim Diminished vasodilator activity during pregnancy, which augments vascular responses to vasoconstrictors, is one reason for the onset of preeclampsia and superimposed preeclampsia. It is known that Dahl salt-sensitive (Dahl-S) rats develop salt-sensitive hypertension like African Americans. The present study attempted to assess the changes and the interactions of the NOSs-NO-sGC-cGMP and NPs-NPRs-cGMP systems in the hypertensive placenta using Dahl-S rats as an animal model of superimposed preeclampsia.

Methods Pregnant Dahl-S rats were fed a high-salt diet to induce the development of hypertension and FGR. Using these rats, we investigated the regulation of these two vasodilatation systems, including the kinetics of cGMP, sGC, endothelial NOS (eNOS), inducible NOS (iNOS), NPs (ANP, BNP, CNP), and NPRs (NPR-A, NPR-B, NPR-C).

Results Dahl S rats fed a high-salt diet exhibited hypertension, FGR and thickening of the walls in decidual vessels. The placental cGMP level in the rats fed the high-salt diet was significantly decreased compared with that in controls. The expression levels of eNOS and iNOS mRNA increased significantly, while that of sGC alpha2-sunbnit declined significantly. Messenger RNA levels of NPR-C, a clearance-type receptor of NPs, declined significantly, whereas those of NPs and their functional receptors NPR-A

and NPR-B were unchanged.

Conclusions Since Dahl-S rats with excess salt-loading during pregnancy exhibited pathological changes similar to those observed in human females with preeclampsia/superimposed preeclampsia, this rat could be useful as an animal model of superimposed preeclampsia. In the placentas of hypertensive Dahl-S rats, vasodilatation seemed to be disturbed by the deregulation of both the NO-sGC-cGMP and NPs-NPRs-cGMP systems.

Key words: cGMP, Dahl-S rat, NOSs-NO-sGC system, NPs-NPRs system, superimposed preeclampsia.

Introduction

Genetic factors largely influence the probability and the severity of hypertensive diseases of pregnancy. In fact, black women (African-American ethnicity) have a higher prevalence of this condition than white women (Caucasian ethnicity)[1, 2]. Black women are also more likely (2-3 times) to die from hypertensive disease of pregnancy than white women[3, 4]. Epidemiological studies revealed a higher prevalence of salt-sensitive hypertension in black people than in white people. It is thought that upregulation of the Na-K-2Cl co-transporter at the thick ascending limb of Henle's loop in the kidney is one of the main reasons for the augmented salt-sensitivity in persons of African-American descent[5, 6]. Hyper-function of the Na-K-2Cl co-transporter increased the re-absorption of sodium ion and water from kidney, which augments the tone of systemic blood pressure through the expansion of circulation blood volume. The higher susceptibility to salt-sensitive hypertension frequently seen in blacks, might in part account for their relatively higher prevalence of hypertensive diseases of pregnancy including preeclampsia or superimposed preeclampsia.

Dahl salt-sensitive (Dahl-S) rats are prone to salt-sensitive hypertension. Dahl-S rats exhibit phenotypes of hypertension, proteinuria, arterial wall-thickness, and renal dysfunctions when fed a high-salt diet. Like persons of African-American

ethnicity, Dahl-S rats exhibit an upregulation of the Na-K-2Cl co-transporter at the thick ascending limb of Henle's loop. In this respect, pregnant Dahl-S rats may be very useful as an animal model of hypertensive diseases of pregnancy, especially of superimposed preeclampsia [7].

In normal pregnancy, vascular responses to various vasoconstrictors decline, which produces a tendency toward vasodilatation and a consequent decline of systemic blood pressure[8]. Vasodilator substances of feto-placental and maternal origin bring this vasodilatation-trend seen in normal pregnancy[9]. Whereas in hypoxic placenta of preeclampsia, diminished effects of vasodilator substances lead to an increase in vascular responses to vasoconstrictors. An increased-trend toward vascular constriction emerges not only in placental vasculature but also in maternal systemic circulation[8].

Among the many vasodilators working in pregnancy, nitric oxide (NO) is one of the strongest, but its vasodilatation effect is also decreased in preeclampsia[9]. Nitric oxide is produced by NO synthases (NOS) including endothelial NOS (eNOS) and cytokine-inducible NOS (iNOS)[10-12]. The nitric oxide exerts its effects through functional receptors: a soluble guanylate cyclase (sGC)[13-15], that produces cyclic guanosine monophosphate (cGMP), a second messenger which transduces the post-sGC

signaling within cells[15, 16].

Cyclic guanosine monophosphate (cGMP), which works as a 2nd-messenger in the natriuretic peptides (NPs)-NP receptors (NPRs) system in addition to NOSs-NO-sGC system, induces vasodilatation and brings blood pressure reduction through diminishing tension of vascular smooth muscles[17]. Natriuretic peptides, which include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), exert their effects through NPR functional and/or clearance receptors[18]. ANP and BNP bind to their functional A-type natriuretic receptor (NPR-A), whereas CNP binds to its functional B-type natriuretic receptor (NPR-B) to exert vasodilatation effects through cGMP[18, 19]. The C-type receptor (NPR-C) is bound by all three NPs, and functions as a clearance-type receptor[20, 21].

Within normal arteries, these two vasodilatation systems, NOSs-NO-sGC-cGMP and NPs-NPRs-cGMP, have been reported to work in concert with each other. Namely, once deregulation of one side of these vasodilatation systems occurs, the efficacy of the other side increases to compensate for the declined trend of vasodilatation[22, 23]. However, in placenta of hypertensive diseases of pregnancy, there have been no reports concerning the interactions of NOSs-NO-sGC-cGMP system and NPs-NPRs-cGMP system. We have reported previously on the dynamics of placental

ghrelin production using pregnant Dahl-S rats fed a high-salt diet as an animal model of intrauterine growth restriction (IUGR)[7]. The present study investigated whether Dahl S rats fed a high-salt diet during pregnancy could be useful as an animal model for superimposed preeclampsia. Furthermore, we tried to assess the changes and the interactions of the NOSs-NO-sGC-cGMP and NPs-NPRs-cGMP systems in the placentas of salt-loaded Dahl-S rats.

Materials and Methods

Animals

Four-week-old male and female Dahl salt-sensitive (Dahl-S) rats were purchased from Kyudo (Saga, Japan). Rats were maintained at a constant humidity (60 ± 5%), temperature (23 ± 1℃), and light cycle (light period 7 AM to 7 PM) with *ad libitum* access to food and water. Virgin rats were fed a standard laboratory chow containing 0.24% NaCl diet (CE-2, CLEA Co., Ltd., Osaka, Japan). Nine-week-old female rats destined to become pregnant were placed with a fertile male rat. Day 0 of pregnancy was determined by the presence of vaginal plug. Pregnant female rats were divided into two groups: a normal-salt (0.24% NaCl) loading group (NrS-group) fed with CE-2 diet, and a high-salt loading group (HS-group) fed with CE-2 diet

supplemented with 8% (w/w) NaCl. Food and water were available *ad libitum* throughout the study. Systolic blood pressures of pregnant dams were measured using the tail-cuff method on day 0, 7, 14 and 20 of gestation. On day 20 of gestation, dams were anesthetized by fentanyl citrate (10 mg/kg) (Fentanest, Sankyo Co., Ltd., Tokyo, Japan) injection. Placentas were quickly excised, weighed, frozen in liquid nitrogen and stored at -80ºC until the extraction of total RNA or cyclic guanosine monophosphate (cGMP). Every fetus in the sacrificed dams was quickly excised from adherent tissue and weighed.

Preparation of placenta samples for atrial natriuretic peptide and C-type natriuretic peptide assay

To extract the peptide fraction containing atrial natriuretic peptide (ANP) or C-type natriuretic peptide (CNP) from placenta, respective samples stored at -80 ºC were moved to a polypropylene tube (Iwaki ™ centrifuge tube, Tokyo, Japan) containing a 10-fold volume of boiled water, and further boiled for 5 min to inactivate intrinsic proteases. After cooling on ice, acetic acid (AcOH) was applied to the tube to make 1 M AcOH solution. The boiled sample in 1 M AcOH solution was homogenized with a polytron mixer (PT 6100, Kinematica AG, Littan-Luzern,

Switzerland). Homogenized placental samples were centrifuged for 30 min at 12,000 x g and supernatant was recovered. Supernatants from the respective placental samples were passed through a Sep-Pak Plus™ C18 cartridge (Waters Corp., Milford, MA), and the peptide fraction adhered to the cartridge was eluted with 60% acetonitrile (CH3CN) - 0.1% trifluoroacetic acid (TFA), and lyophilized after evaporating the acetonotrile as described previously[24]. Lyophilized samples were stored at -80ºC until the assay for ANP or CNP.

Preparation of placenta samples for cyclic guanosine monophosphate assay

To extract cyclic guanosine monophosphate (cGMP) from placenta, samples stored at -80ºC were homogenized in 6% trichloroacetic acid at 4ºC in the presence of 50 mM 3-isobutyl-1-methylxanthine (IBMX, Nakalai Tesque Inc., Kyoto, Japan) to inhibit phosphodiesterase activity, and centrifuged at 12,000 x g for 15 min. at 4ºC. The supernatant was recovered and washed twice with water-saturated diethyl ether. The upper layer was aspirated and discarded after washing, while the aqueous layer containing cGMP was recovered, lyophilized and stored at -80ºC until assay.

Radioimmunoassay for ANP, CNP and cGMP

ANP, CNP or cGMP contents in placental tissue samples, together with plasma levels of ANP or cGMP in maternal circulation, were measured by radioimmunoassay (RIA) kits for ANP (Peninsula Laboratories, Inc. Belmont, CA), CNP (Phoenix Pharmaceuticals, Inc. Burlingame, CA) or cGMP (Amersham International, Little Chalfont, Bucks., U.K.). Two to three placentas were selected from each dam. These placentas were used for RIA. Briefly, placental tissue samples were dissolved with equipped RIA buffer to a final concentration of 6.67 μg-tissue equivalent /100ml RIA buffer for ANP, 100 mg-tissue equivalent /100 ml RIA buffer for CNP, or 1000 mg-tissue equivalent /100 ml RIA buffer for cGMP. Placental tissue samples, diluted with 100 ml of RIA buffer, were mixed with 100 μl of the equipped antibody solution (rabbit IgG-type) specific for ANP, CNP or cGMP, and incubated overnight at 4°C. Then, 100 μl of ^{125}I-labeled rat ANP, CNP or cGMP solution was added as tracer, and incubated overnight at 4 °C. The antibody-bound tracer was precipitated after mixing with 100μl solution of goat anti-rabbit IgG and 100μl solution of normal rabbit serum, and was then incubated for 90 min at room temperature, following centrifugation at 12,000 x g for 30min at 4°C. After removing the non-bound tracer in supernatant by aspiration, the radioactivity of the remaining pellet was counted in a gamma counter (ARC-600, Aloka, Tokyo Japan). All assays were

performed in duplicate. Plasma concentrations of ANP in dams were also measured by respective RIAs under the same protocol as described above.

RT-PCR profiling for the expression levels of responsible molecules controlling the vascular tone of feto-placental circulation

We adopted a semi-quantitative RT-PCR system to estimate the placental expression levels of mRNAs for ANP (Accession Number; M27498), BNP (Accession Number; M25297), CNP (Accession Number; D90219), natriuretic peptide receptor type A (NPR-A: Accession Number; NM012613), natriuretic peptide receptor type B (NPR-B: Accession Number; NM053838), natriuretic peptide clearance receptor (NPR-C: Accession Number; L27339), alpha 1-subunit of soluble guanylate cyclase (sGCα1: Accession Number; U60835), alpha 2-subunit of soluble guanylate cyclase (sGCα2: Accession Number; NM023956), constitutive endothelial-type nitric oxide synthase (eNOS: Accession Number; NM021838) [12] and cytokine-inducible nitric oxide synthase (iNOS: Accession Number; NM12611)[12] as described previously[25]. In brief, whole placental tissue (including both basal and labyrinth zone) was homogenized, and total RNA was extracted by an acid guanidium thiocyanate-phenol chloroform method[26] using a commercially available RNA isolation reagent (RNA-Bee, TEL-TEST, Inc.,

USA). Messenger RNA was prepared from placental total RNA by oligo(dT)-cellulose chromatography using a commercially available messenger RNA purification kit (Oligotex™-dT30 <Super> mRNA Purification Kit, Takara Bio, Inc., Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 0.1 mg of messenger RNA using a commercially available reverse transcription kit (Super Script-III™, Life Technologies Japan, Tokyo, Japan). Polymerase-chain reaction (PCR) was performed in a final volume of 50 μl containing 0.1 μg of cDNA, 10 pmol each of primer sets, 200 μM each of dNTP mixture, 1.25 unit of taq DNA Polymerase (Go taq, Promega Corporation, WI, USA). The primers used are listed in Table 2. G3PDH (Rat G3PDH Control Amplimer Set**,** Takara Bio, Inc., Tokyo, Japan) transcript was co-amplified from each cDNA template as an internal control for each PCR reaction. Every PCR product was loaded onto a 2.0% agarose gel, and size-fractionated by electrophoresis. Electrophoresed products were visualized under ultraviolet light system (Printgraph™ system, Atto-Corp., Tokyo) with ethidium bromide staining. The quantification of each PCR product from ANP, BNP, CNP, NPR-A, NPR-B, NPR-C, eNOS, iNOS, sGCα1 and sGCα2 transcripts was performed using the free soft ware system NIH-image (http://rsb. info.nih.gov/nih-image), and the respective amounts were normalized using that of G3PDH amplified simultaneously.

Eight to twelve pregnant rats were used for the preparation of placentas for the RT-PCR study. One to two placentas were selected from each dam. The expression levels of mRNAs for NOSs, NO-receptor subunits, NPs and NPRs in the placentas obtained from the pregnant rats were examined by RT-PCR. The RT-PCR study was repeated two or three times, with duplicate samples.

Histological examination

Harvested placentas from pregnant rats (NrS- or HS-group) were placed in Zamboni's fixative solution, dehydrated, paraffin embedded, sectioned, and stained with Hematoxylin & Eosin staining (HE) or Mallory-Azan staining (Azan)[27, 28].

We performed a semiquantification study to evaluate the wall-thickening of the decidual artery as described previously[29]. In brief, short-axis images of decidual arteries (internal diameters: 30-100 μm) were obtained by light microscopy (H&E staining at a magnification of x 400), and the inner border of the lumen and the outer border of the arterial wall were traced in each arterial image. Thereafter, the respective area encircled by the tracing was measured. Finally, the wall-to-lumen ratio of the respective decidual artery was calculated. One to two placentas were examined from each dam, and two to three arteries in each stained section of placenta were evaluated.

Statistical Analysis

Data were expressed as means ± standard deviations (S.D.). Statistical significance was determined by one-way ANOVA followed by post hoc test (Scheffe's F-test). A p value < 0.05 was considered to be statistically significant.

Results

Maternal blood pressure and fetal and placental weights in pregnant Dahl-S rats

Pregnant Dahl-S rats fed a high-salt diet (dams in the HS-group, n=15) exhibited significantly higher blood pressure on day 7 (p<0.01), day 14 (p<0.01) and day 20 (p<0.01) of gestation compared to control Dahl-S rats fed a normal-salt diet (dams in the NrS-group, n=10). NrS-group rats remained in normotensive range throughout the gestational period (Table 1). On day 20 of gestation, fetal body weight within dams in the HS-group (dams, n=15, fetuses, n=152) was significantly lower than that in the NrS-group (dams, n=10, fetuses, n=127, p<0.05) (Table 1). There was no significant difference in the placental weights between the two groups (NrS; 555± 90mg, dams, n=10, placentas, n=127, HS; 555±80mg, dams, n=15, placentas, n=152) on day 20 of gestation.

Histopathological findings in placenta of Dahl-S rats

Light microscopy of placenta revealed thickening of the walls of decidual arteries with hyaline degeneration in the high-salt loading group (HS-group) (Fig. 1). After staining with azan, perivascular fibrosis (reflecting the hypertrophied vascular wall containing elastic fibers) was observed around the decidual arteries in the HS-group (Inset in Fig. 1). The index of the "wall-to-lumen ratios" of decidual arteries and arterioles in the HS-group (2.914±0.694, dams, n=8, $P<0.001$) was larger than that seen in the Nrs-group (1.572±0.275, dams, n=5). These findings indicated the development of arteriosclerosis in the placental arteries and arterioles.

Plasma levels of ANP, cGMP and auricle contents of ANP in dams of NrS- and HS-group

On day 20 of gestation, plasma level of ANP in the HS-group (538.0 ± 154.5 pg/ml, n=6) was significantly higher ($p<0.001$) than that in the NrS-group (171.4 ± 32.6 pg/ml, n=6). Also, plasma level of cGMP on gestational day 20 in the HS-group (870.4 ± 362.4 fmol/ml, n=5) was significantly ($p<0.05$) higher than that in the NrS-group (377.4 ± 222.7 fmol/ml, n=7). Right auricle content of ANP in HS-group dams (114.4 ± 24.7 μg/mg tissue, n=5) on the same gestational day was significantly

lower ($p<0.001$) than that in the NrS-group (279.6 ± 54.2 μg/mg tissue, n=6).

Placental contents of ANP, CNP and cGMP in NrS- and HS-group

On day 20 of gestation, there was no significant difference in placental ANP contents between the HS-group (1.12 ± 0.12 pg/mg tissue, dams, n=8) and NrS-group (1.11 ± 0.17 pg/mg tissue, dams, n=9). There was also no significant difference in placental CNP contents between the two groups (HS; 6.28 ± 1.27 fg/mg tissue, dams, n=9, NrS; 6.54 ± 1.20 fg/mg tissue, dams, n=9) on gestational day 20. In contrast, placental cGMP was significantly lower in the HS-group (1.93 ± 0.76 fmol/mg tissue, dams, n=8) than in the NrS-group (4.15 ± 2.62 fmol/mg tissue, dams, n=8) ($P<0.05$) (Fig. 2). Since the placental production of BNP was far smaller than that of ANP or CNP, we only checked the relative expression level of BNP (as shown in Fig. 4), and did not check the peptide content of BNP in placenta.

Gene expressions of NO-synthases (eNOS, iNOS) and NO-receptor subunits (sGC-α1, sGC-α2) in NrS- or HS-group

The expression levels of mRNA for eNOS and iNOS were significantly higher in placentas of the HS-group on day 20 of gestation than in the NrS (eNOS; NrS,

100.0 ± 15.8 arbitrary unit, dams, n=8, HS, 141.1 ± 26.0 arbitrary unit, dams, n=10, $p<0.01$), (iNOS; NrS, 100.6 ± 15.2 arbitrary unit, dams, n=6, HS, 132.9 ± 13.5 arbitrary unit, dams, n=9, $p<0.001$) (Fig. 3A-B). There was no significant difference in placental expression of sGCα1 between the HS-group (101.6 ± 11.1 arbitrary unit, dams, n=6) and NrS-group (108.5 ± 13.4 arbitrary unit, dams, n=5), although the expression of mRNA for sGCα2 was significantly lower in the HS-group (54.7 ± 8.4 arbitrary unit, dams, n=7) compared to the NrS-group (100.3 ± 32.2 arbitrary unit, dams, n=6) ($p<0.01$) (Fig. 3C-D).

Gene expressions of natriuretic peptides and natriuretic peptide receptors in NrS- or HS-group

There were no significant differences in the expression levels of respective mRNAs for ANP, BNP or CNP in placenta between the NrS- and HS-group (ANP; NrS, 101.2 ± 19.5 arbitrary unit, dams, n=7 vs. HS, 117.2 ± 21.3 arbitrary unit, dams, n=10) (BNP; NrS, 101.2 ± 10.6 arbitrary unit, dams, n=6 vs. HS 112.1 ± 12.7, arbitrary unit, dams, n=7) (CNP; NrS, 101.6 ± 44.8 arbitrary unit, dams, n=6 vs. HS, 107.6 ± 46.5 arbitrary unit, dams, n=8) on day 20 of gestation (Fig. 4A-C). The placental expression of NPR-A and NPR-B mRNAs did not differ between the two groups on

gestational day 20 (NPR-A; NrS-group, 100.4 ± 14.4 arbitrary unit, dams, n=6, HS-group, 114.2 ± 17.2, arbitrary unit, dams, n=5, NPR-B; NrS-group, 100.8 ± 17.4 arbitrary unit, dams, n=5, HS-group, 103.5 ± 17.1 arbitrary unit, dams, n=5) (Fig. 4D, 4E). However, placental expression of NPR-C mRNA in the HS-group (53.1 ± 16.5 arbitrary unit, dams, n=6) was significantly lower than that in the NrS-group (100.5 ± 23.4 arbitrary unit, dams, n=6) (P<0.001) (Fig. 4F) on day 20 of gestation.

Discussion

In this study, we fed Dahl S rats an 8%-salt diet after confirming their pregnancy. This manipulation resulted in a hyaline degeneration and vascular wall hypertrophy in the blood vessels of decidual tissue on day 20 of gestation. This change in utero-placental circulation may cause a secondary ischemic condition of placenta in "salt-sensitive" pregnancy of Dahl-S rats. A similar condition occurs in humans, with higher prevalence in African-American ethnicity than Caucasian ethnicity[1,2].

In pregnant Dahl-S rats fed an 8% salt-diet (HS-group) on gestational day 20, we detected a significantly lower level of cGMP in placenta. In the same placental tissue of HS-group, a significant increase was noted in the expression level of mRNAs

for both eNOS and iNOS, which seems to be a compensatory regulation to stimulate the vasodilatation-response in placental vasculature through the augmented production of nitric oxide (NO). Meanwhile, in the same placental tissue, the expression level of mRNA for α1-subunit of soluble guanylate cyclase (sGCα1), one of the functional receptors for NO, did not change, and the expression level of mRNA for the α2-subunit of soluble guanylate cyclase (sGCα2), another functional receptor for NO, declined significantly. From these findings, it is possible that a dysfunction within the NO-sGC-cGMP pathway, especially at the level of sGC-production, caused a reduction in the production-rate of cGMP, which led to deregulation of the placental vasodilatation system in the HS-group. Expression of sGCα1 is seen ubiquitously in many tissues and organs, but that of sGCα2 is observed in limited organs including uterus and placenta[30]. Since the expression site of sGCα2 in placenta matches the site of NO-production[31], it seems likely that the change in the expression levels of sGCα2 in placenta affects the utero-placental circulation by altering the vascular tone of placental vessels, including decidual arterioles.

All three types of NPs (ANP, BNP and CNP) together with their receptors (NPRs: NPR-A, NPR-B and NPR-C) have been reported to exist in placenta of rats, mice and humans[20, 32-35]. In placentas of HS-group, the decline of mRNA level for

NPR-C (clearance-type receptor) was more significant than in the NrS-group. From this finding, one may speculate that this decline of NPR-C expression is a compensatory process to enhance the activity of the NPs-NPRs system by increasing the chance for ANP, BNP and CNP to bind to their functional receptors, NPR-A and NPR-B. However, we could not detect any significant increase in the placental contents of ANP or CNP. Similarly, in comparison to the NrS-group, no significant changes were detected in HS-group placentas with regard to expression of mRNAs for ANP, BNP and CNP together with their functional receptors. These changes in the production and/or the clearance of NPs within the HS-group placentas, especially in the production of ANP, were far different from those seen within the auricles (a source of ANP secreted into the systemic circulation) of dams in the HS-group. In the HS-group, the secretion of ANP from dam's auricle into the systemic circulation increased in order to correct the maternal hypertension, which was partly reflected by the increased content of plasma cGMP in the HS-group[36]. In contrast, in placentas of HS-group, no effective responses of NPs-NPRs system occurred to ameliorate damage to the utero-placental circulation as reflected in part by the decrease of placental cGMP.

These findings may imply neither of the two main pathways of vasodilatation worked effectively to protect the vascular construct against excess blood pressure or

excess blood volume within placentas of the HS-group.

This study had a major limitation. We did not check the precise location of the NPs, NPRs, NOSs, sGC or cGMP within the placental tissue of the Dahl-S rats. We checked only the expression levels (mRNA levels) of NPRs, NOSs and sGCs, and could not confirm the tissue content of the proteins (receptors and enzymes) due to limitations of our experimental technique. From our data, it was difficult to clarify the source of the cGMP in the placenta, and it might have been expressed by trophoblasts, vessels and the abundant blood supply, etc. Further studies using immunohistochemistry will be needed to elucidate the precise origin of the decreased placental cGMP within the feto-placental system in this animal model.

A certain proportion of female humans with hypersensitivity to salt develop preeclampsia[4-6]. In this study, the condition of pregnant Dahl-S rats with high salt intake (HS-group) resembled that of these women. These rats can develop pathological changes similar to those observed in women with preeclampsia/superimposed preeclampsia with a mild to severe condition by changing the salt intake and/or salt loading period. Elucidating the pathological conditions in these rats may lead to the development of effective strategies to prevent or modify the severity of preeclampsia or superimposed preeclampsia in women with hypersensitivity to salt. It may also be

possible to confirm the effects of prevention or treatment for preeclampsia/superimposed preeclampsia in Dahl S rats, using them as a model of preeclampsia.

For women with hypersensitivity to salt, it is desirable to reduce sodium intake in order to prevent hypertension from salt-sensitivity, as well as to prevent secondary conditions such as dysfunction of utero-placental and/or feto-placental circulation. In this study, placental cGMP declined in pregnant Dahl-S rats in the HS-group. In placentas of this model, there was a deregulation at the level of sGCα2 expression within the NO-sGC signaling pathway. Therefore, treatments to increase sGC activity and improve cGMP remodeling (production and degradation) might help to increase the utero-placental circulation in such cases of disturbed pregnancy. Until now, the outcomes of therapeutic trials on pregnancy-induced hypertension, preeclampsia or FGR focusing on the feto- and utero-placental circulation, have been limited and inconsistent. In contrast, there have been several trials using chemical compounds such as L-arginine[37, 38], YC-1[39, 40] or PDE5-inhibitor[41-43] that modulate the tissue contents of NO, the sensitivity of sGC, or the degradation process of cGMP, respectively. It would be intriguing to study the effect of these compounds on the utero- and feto-placental circulation and/or fetal growth in this Dahl-S rat model of

maternal hypertension.

In conclusion, since Dahl-S rats with excess salt loading during pregnancy exhibited pathological changes, such as hypertension, FGR and thickening of the walls of decidual arteries, similar to those observed in human females with preeclampsia/superimposed preeclampsia, this rat could be useful as an animal model of superimposed preeclampsia. In this animal model, we found a significant decline in the placental content of cGMP, which seems to be due to the deregulation of both NOSs-sGC-cGMP system and NPs-NPRs-cGMP system. The Dahl-S rat may be an interesting animal model to study disorders of placental circulation associated with hypertensive diseases of pregnancy, especially those caused by maternal hypersensitivity to salt.

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Disclosure

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Table 1. The maternal blood pressure and fetal weights

	Gestational age			
	Day 0	Day 7	Day 14	Day 20
BP (mmHg)				
NrS-group	124 ± 9	130 ± 8	127 ± 7	122 ± 8
HS-group	120 ± 9	146 ± 11 [b)]	154 ± 17 [b)]	160 ± 21 [b)]
Fetal weight (g)				
NrS-group	N.D.	N.D.	N.D.	4.34 ± 0.76
HS-group	N.D.	N.D.	N.D.	3.93 ± 0.66 [a)]

The systolic blood pressure (BP) and fetal weight (Fetal weight) in the pregnant Dahl-S rats fed a high-salt diet containing 8% NaCl (HS-group) or those fed a normal salt diet containing 0.24% NaCl (NrS-group). The BP values were obtained from three consecutive measurements and were averaged and recorded at each time point. Each value represents the mean ± S.D. (dams in the NrS-group, n=10; dams in the HS-group, n=15). Fetal weights were measured on day 20 of gestation. Each value represents the mean ± S.D. (fetuses in the NrS-group, n=127; fetuses in the HS-group, n=152). [a)], $p<0.05$; [b)], $p<0.01$ significantly different from NrS-group; N.D., not determined.

Table 2. PCR primers and conditions used to amplify genes of interest in placenta

Gene	Primer Segeuence (5' – 3') sense (S) antisense (AS)	PCR condition				Size of products (bp)
		Dena*	Anne*	Elon*	cycles	
ANP	ATGAGCTCCTTCACCACC (S)	95 °C	58 °C	72 °C	30	451
	GTACCGGAAGCTGTTACA (AS)	90 s	90 s	120 s		
BNP	GAGAGAGCAGGACACCAT (S)	95 °C	60 °C	72 °C	32	468
	AAAGAAGAGCCGCAGGCA (AS)	90 s	90 s	120 s		
CNP	CACAGCAGTAGGACCCGTG (S)	95 °C	55 °C	72 °C	32	175
	GAGGGCCGGAGTGAGAGTA (AS)	30 s	60 s	60 s		
NPR-A	AAGAGCCTGATAATCCTGAGTACT (S)	95 °C	55 °C	72 °C	30	451
	TTGCAGGCTGGGTCCTCATTGTCA (AS)	30 s	60 s	60 s		
NPR-B	TCAAACACATGAGAGATGTTC (S)	95 °C	58 °C	72 °C	30	729
	TATTGGCATACTGTTCCATGC (AS)	90 s	90 s	120 s		
NPR-C	ATCGTGCGCCACATCCAGGCCAGT (S)	95 °C	55 °C	72 °C	30	573
	TCCAAAGTAATCACCAATAACCTC CTGGGTACCCGC (AS)	30 s	60 s	60 s		
sGCα1	AGTGTGCCTCGGAAAATCAATGT (S)	95 °C	55 °C	72 °C	30	237
	CCCTGATGCTTTGCCTAAGAAGTT (S)	30 s	60 s	30 s		
sGCα2	TGACTCCTGATGGAAGACCC (S)	95 °C	55 °C	72 °C	34	429
	GCTTGTGCTTTTTGGAGGAG (AS)	30 s	60 s	60 s		
eNOS	GCTTCAGGAAGTGGAAGCTG (S)	95 °C	60 °C	72 °C	32	226
	AAGATTGCCTCGGTTTGTTG (AS)	60 s	60 s	60 s		
iNOS	CACCTTGGAGTTCACCCA (S)	95 °C	58 °C	72 °C	30	170
	ACCACTCGTACTTGGGATGC (AS)	30s	60s	60s		
G3PDH	ACCACAGTCCATGCCATCAC (S)	95 °C	58 °C	72 °C	20	452
	TCCACCACCCTGTTGCTGTA (AS)	30 s	60 s	60 s		

*Dena; Denaturation, Anne; Annealing, Elon; Elongation, s; seconds

Cycles, PCR -cycle; bp, base pair

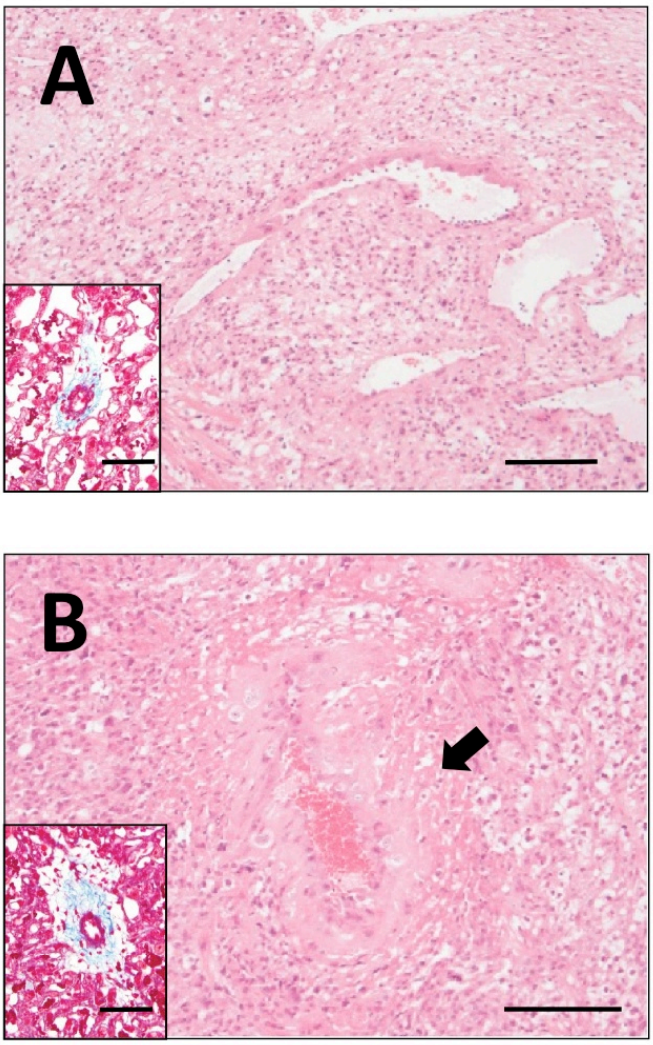

Fig. 1 Takushima S. et al.

Figure 1. Histological findings in placentas of pregnant Dahl-S rats fed a normal-salt diet containing 0.24% NaCl (NrS-group) (Fig.1A) or those fed a high-salt diet containing 8% NaCl (HS-group) (Fig.1B) on day 20 of gestation. In H&E staining, decidual arteries in placentas of the HS-group (Fig.1B, large panel) exhibited wall thickening with hyaline degeneration (arrow head). The degree of perivascular fibrosis detected by Mallory-Azan staining (blue fibers) (Fig.1B, inset) was increased in the HS-group (Fig.1A, inset) compared with NrS-group, on day 20 of gestation. Original magnification x100 (Scale bar=50 μm).

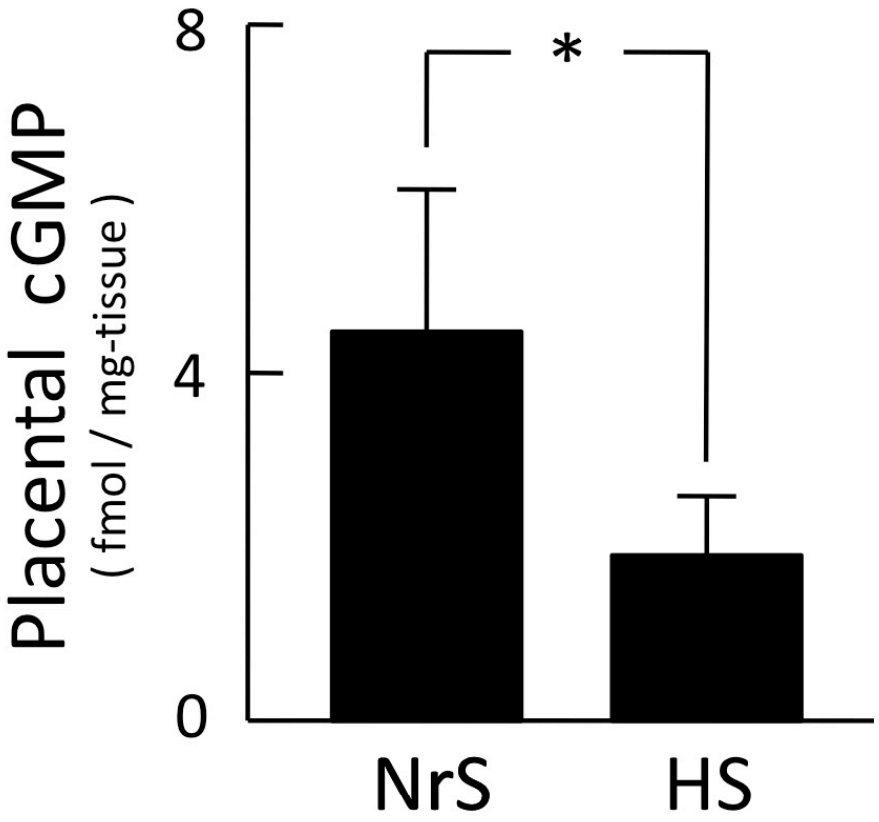

Fig.2 Takushima S et al

Figure 2. Placental cGMP levels in Dahl-S rats fed normal salt diet containing 0.24% NaCl (NrS) or those fed high salt diet containing 8% NaCl (HS) on day 20 of gestation. cGMP levels were measured by radioimmunoassay. Each value represents mean ± S.D. (n=8 dams in each group). *, $p<0.05$ significantly different from indicated values.

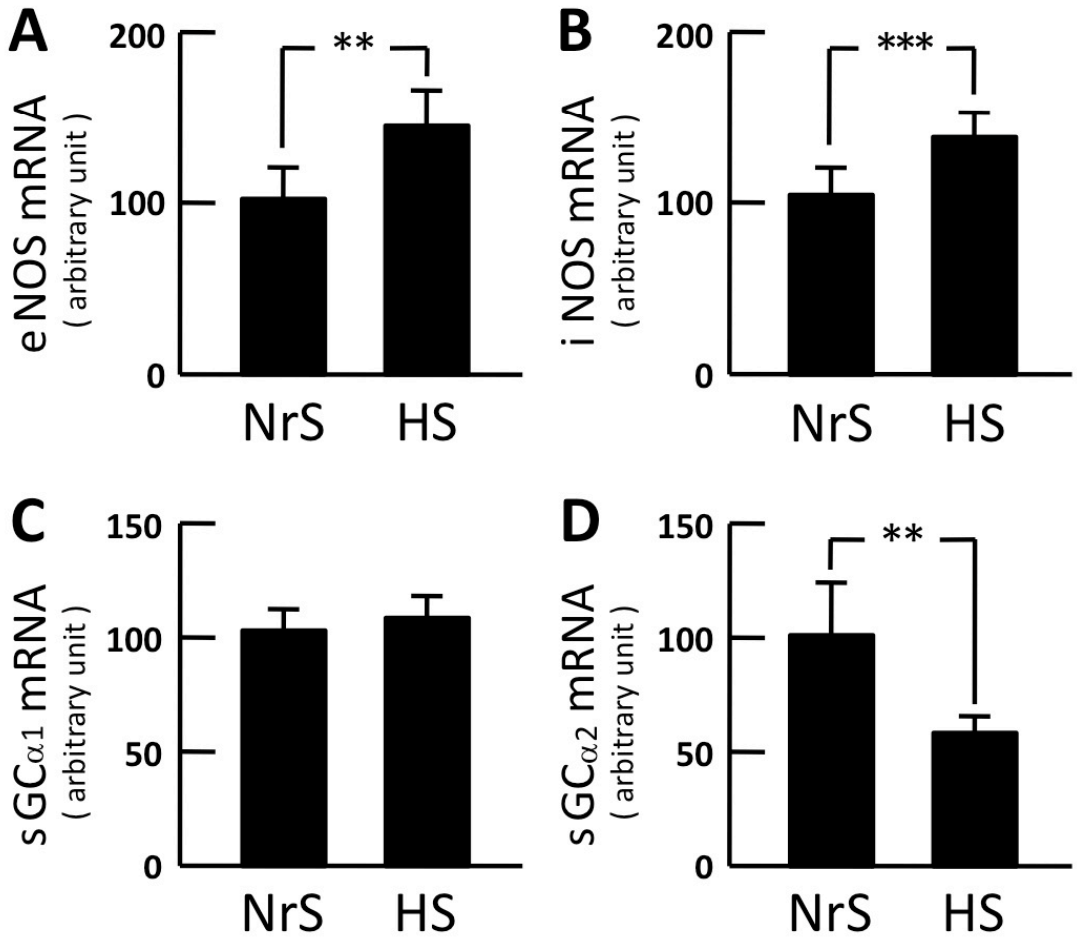

Fig. 3 Takushima S et al

Figure 3. Relative expression levels of mRNAs for eNOS (A), iNOS (B), sGCα1 (C) and sGCα2 (D) in placentas of pregnant Dahl-S rats fed a normal-salt diet (NrS) or high-salt diet (HS). Each value represents the mean ± SD (n=5-10, dams in each group). **, $p<0.01$ and ***, $p<0.001$ significantly different from indicated values. Eight to twelve pregnant rats in each group were used to prepare the placentas for this procedure (dams in the NrS-group, n=8~10; dams in the HS-group, n=10~12).

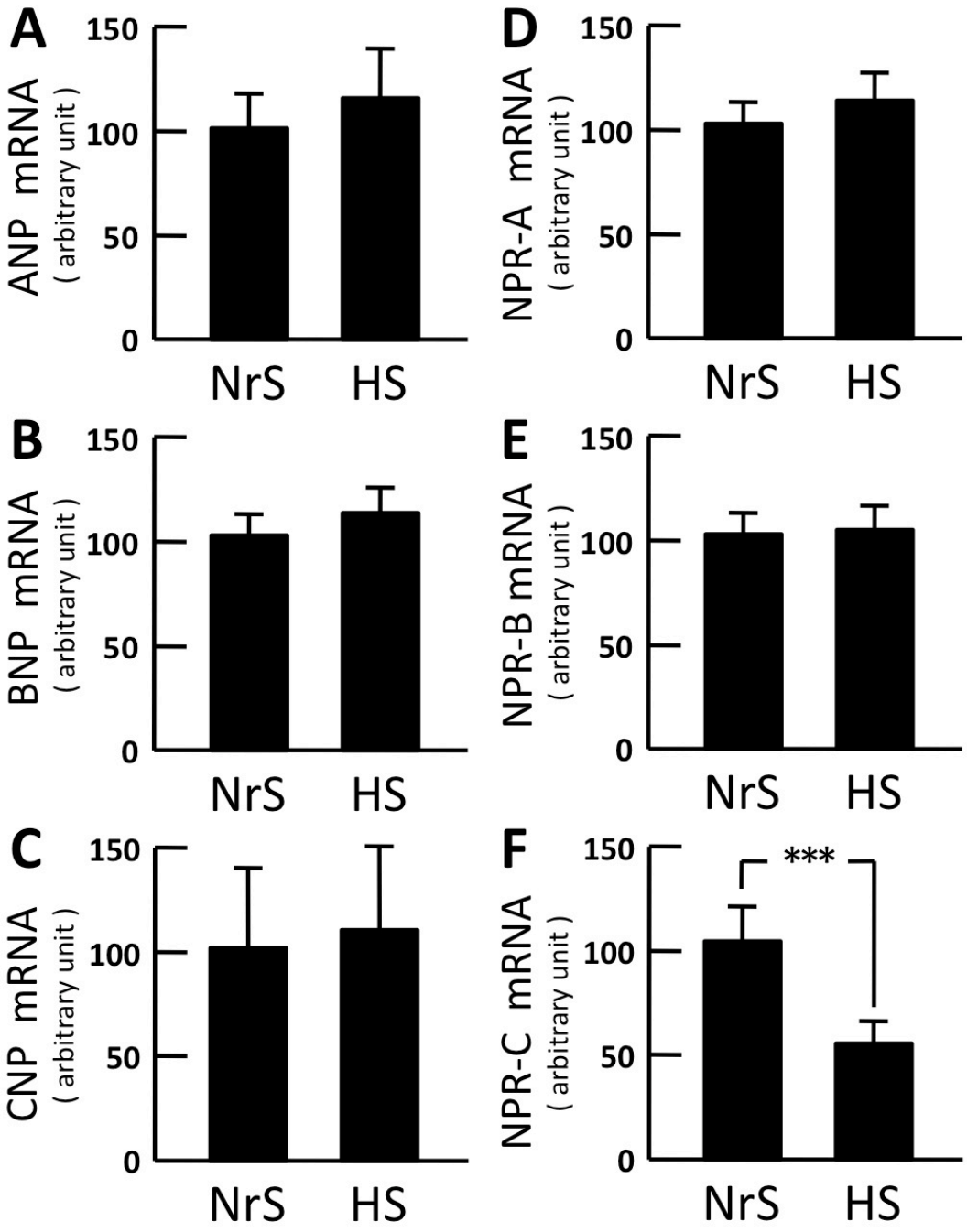

Fig. 4 Takushima S et al

Figure 4. Relative expression levels of mRNAs for natriuretic peptides: ANP (A), BNP (B) and CNP (C), together with their receptors: NPR-A (D), NPR-B (E) and NPR-C (F) in placentas of pregnant Dahl-S rats fed a normal-salt diet (NrS) or high-salt diet (HS). Each value represents the mean ± SD (n=5-10 dams each in the NrS and HS groups). ***, $p<0.001$ significantly different from indicated values. Eight to ten_pregnant rats were used from each group to prepare the placentas for this procedure (dams in the NrS-group, n=8~10; dams in the HS-group, n =10).