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Short communication

Detection of ethanolamine altering in fetuses of pregnancy-associated hypertensive mice treated with vasodepressors by using UPLC and MALDI-TOF/MS

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ABSTRACT

Since serotonin, homocysteine and oxytocin are known to fluctuate during mammalian gestation, we screened amines altered in pregnant-associated hypertensive (PAH) mice by tagging their amino groups with 6-aminoquinoline carbamoyl (AQC) group in concert with ultra high-performance liquid chromatography (UPLC). Interestingly, a candidate amine significantly increased in PAH mice was recovered to the basal level, when treated with antihypertensive drugs. Mass spectrometric analyses indicated that the molecular mass of this amine was 61.2, which was identified as ethanolamine.

1. Introduction

Maternal body fluid volume and endocrine systems alter dramatically during gestations, in which some of amines, such as serotonin [1], homocysteine [2] and oxytocin [3], are known to fluctuate in blood and/or urine of normal pregnant woman. On the other hand, inappropriate changes of these levels have been reported in diseases complicating pregnancy, such as pregnancy-induced hypertension [4,5], which globally affects up to 10% of expectant mothers and gives transient hypertension damaging their kidney, liver, and heart.

We have previously developed pregnancy-associated hypertensive (PAH) mice, which show hypertension in late pregnancy, due to the excessive production of angiotensin II [6]. Since amines described above are commonly equipped with amino-groups, we characterized PAH-related amines based on amino group-specific fluorescent derivatization technique [7]. In the present study, we identified ethanolamine, which is increased in fetal extracts from PAH mice and decreased in response to anti-hypertensive drugs.

2. Materials and methods

2.1. Reagents

Ethanolamine chloride, L-norvaline (Nva), urea were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Hydralazine (Hdz) was obtained from Wako Pure Chemicals (Osaka, Japan). Olmesartan (Olm) was gratefully gifted from Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). All of solvents and reagents used in this study were HPLC-grade.

2.2. Animals

PAH mice were obtained by crossbreeding females expressing the human angiotensinogen gene with males carrying the human renin gene as described previously [6]. Age-matched normal pregnant mice (WT mice) were acquired by mating female C57BL/6J mice (Clea Japan, Inc., Tokyo, Japan) with male mice of the same strain. For experiments, primiparous mice (2–5 months old) were

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used. The day of mating was defined as day 0 of the pregnancy (P0). All mice were housed in a 12 h light–12 h dark cycle with food and water available *ad libitum*. All animal experiments were carried out in a humane manner according to the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2.1. Administrations of drugs

Olm was dissolved in sterile water containing 0.01% $NaHCO_3$ and 0.01% $KHCO_3$, and administered at a dose of 15 mg/L in drinking water to maintain the systolic blood pressure (SBP) of PAH mice within 90–120 mmHg from P13 to P19 as previously described [8]. In order to keep the SBP of PAH mice within above range, Hdz was administered in drinking water, starting at 62.5 mg/L from P13 to P15, increased at 250 mg/L from P15 to P17, and boosted at 375 mg/L from P17 to P19.

2.3. Sample preparations

Pregnant mice (P19) were anesthetized with pentobarbital sodium, and removed blood from postcaval veins. Immediately, uteruses were excised and put on ice. After fetal viscera were enucleated from isolated fetuses, tissues were flash-frozen in liquid nitrogen. Frozen samples were ground into a fine powder by using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). A subset of frozen tissue powders was homogenized in ice-cold PBS and cell debris and insoluble matters were removed by centrifugation at 14,000 × *g* for 10 min at 4°C. Protein concentrations in the lysates were determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Visceral lysates were divided into each aliquot and spiked with the same amount of L-Nva as an internal control. The extracts were processed as follows: acetonitrile was added into the extracts to obtain deproteinization (final concentration 85%, v/v), and the mixtures were incubated for 10 min. on ice. After centrifugation (14,000 × *g* for 10 min. at 4°C), the solvent was evaporated from the supernatant, and the dried samples were dissolved in Milli-Q water to derivatize with AccQ-Tag Ultra-Fluor™ derivatisation kit (Waters, Milford, MA, USA). Briefly, samples (50 μl) and 50 μl borate buffer (0.2 M, pH8.8) were combined with 10 μl of fluorescent derivatizing reagent (AccQ-Fluor™) in a tube under vigorous vortexing for 10 s, followed by the incubation at room temperature for 1 min.

2.4. Chromatographic conditions

Acquity ultra high-performance liquid chromatography (UPLC) system™ (Waters) and HPLC were performed as previously described [9,10]. Briefly, 6-aminoquinolyl carbamyl (AQC) derivatized amines were separated on an AccQ-Tag™ amino acid analyzer C_{18} column (Waters, 1.7 μm, 2.1 × 100 mm) with a VanGuard™ cartridge (Waters) at 40 °C with an acetonitrile (solvent B) gradient in AccQ™ ultra eluent A concentrate (5%, v/v) and water (95%, v/v) (solvent A) at a flow rate of 0.25 ml/min. The following gradient elution was used: initial isocratic elution with 0.1% solvent B for 0.54 min, followed by linear gradient elution from 0.1 to 3.7% B until 8 min, jumping to 11.0% B within 0.5 min and keeping same concentrate for 1 min, running up to 13.0% B within 0.5 min, increasing to 20.0% B by 13.5 min, followed by gradient to 80% B until 18.5 min and finally holding at 80% B until 22 min. The column was then subsequently returned to the initial conditions within 0.5 min and equilibrated for 4.5 min before the next sample injection.

The fractionated peak was re-chromatographed by HPLC for the estimation of purity. This analysis was achieved on a Cosmosil πNAP column (5 μm, 4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan) and Milli-Q water and methanol were used as solvent A and B, respectively. After sample injection, separation was performed under isocratic conditions with 44%B, and maintained for 8 min. In order to elute tightly bound substances, the column was flushed with 100% methanol for 10 min and re-equilibrated under isocratic conditions for 10 min prior to the next injection [9]. In both the UPLC and HPLC systems, fluorescences from analytes were detected at λex_{250nm} and λem_{395nm}. Data were collected using Empower™ 2HPLC software for Windows (Waters).

2.5. Mass spectrometry

MALDI-QIT-TOF/MS was carried out with AXIMA Resonance (Shimadzu, Kyoto, Japan), in which a three-dimensional quadrupole ion trap is employed with a time-of-flight mass measurement stage. MALDI was produced using pulsed laser light (337 nm) generated by a nitrogen laser with a maximum pulse repetition rate of 10 Hz. All data were processed using AXIMA launchpad 2.9™ software (Shimadzu, Kyoto, Japan). Tandem mass experiments of MS^n ($n = 1$ or 2) were performed using argon gas as the collision gas, and the collision induced dissociation (CID) spectra were acquired by setting collision energies at 160 (arbitrary unit, range of energy is 0–1000). In both the MS and MS2 modes, ions were extracted by applying a potential between the two endcaps and pulsed into the TOF system with an accelerating potential. Mass spectra from a sum of 200 laser shots were recorded. The ion source pressure was approximately 1.5×10^{-6} Pa for the reflectron part and 6.5×10^{-5} Pa inside the QIT. For the post-source decay (PSD) experiments, fragment ion spectra were acquired in reflectron positive ion mode with AXIMA Performance MALDI-TOF/MS instruments (Shimadzu) with ion accelerating voltage of 20 kV. In both analyses, the analyte solution was mixed with a 0.5 μl solution of 10 mg/ml recrystallized 2,5-dihydroxybenzoic acid (DHB, matrix for MALDI-MS grade, Shimadzu GLC (Tokyo, Japan)), dissolved in a 0.05% (v/v) TFA and 50% (v/v) acetonitrile solution on a MALDI target plate and dried. External calibrations were performed daily using standards of DHB ($[M+H]^+ = 155.03$, monoisotopic ion), and bradykinin 1–7 ($[M+H]^+ = 757.40$, monoisotopic ion).

2.6. Standard curves and recovery

Calibration standards for the ethanolamine (0, 1.25, 2.5, 5, 10 pmol) were prepared from stock solutions diluted in Milli-Q water and stored at −20 °C. For preparing the calibration curves, the tissue lysates were spiked with standards for ethanolamine and an L-Nva and subjected to derivatization and chromatography as described above. Calibration curves were calculated by plotting the ratio of the peak area of analytes to the area of the internal standard *vs.* analyte quantity. For determining the recovery rate, standard solutions were spiked into the samples and subjected to derivatization and chromatographic separation.

2.7. Statistical analysis

For two independent samples, statistical comparison was performed using Student's *t*-test and Welch's *t*-test with GraphPad Prism version 5 for Macintosh (GraphPad Software, San Diego, CA, USA), as appropriate. *P* value <0.01 was considered statistically significant.

3. Results and discussion

Maternal failures of amine metabolism, such as thyroid hormone [11] and homocysteine [12], affecting the fetal growth were reported, however, fetal metabolic alterations in the pregnant complications have not been known. PAH mice display transient