AUTHENTICATION OF KEY BIOLOGICAL AND/OR CHEMICAL RESOURCES

Scientific premise forming the basis of the proposed research

Our overall goal is to determine the relationship between the ET-1 system and circadian regulation of sodium excretion. The contribution of high dietary salt toward cardiovascular and related disease goes beyond hypertension alone and now includes susceptibility to auto-immune, metabolic diseases as well as disruption of the circadian clock, further increasing risk for heart failure, stroke, and chronic kidney disease. We know that disruption of the renal ET-1 system leads to abnormalities in renal excretory control and our preliminary data suggest that this regulation is variable according to the time of day. Additional discussion of significance is provided in the Research Strategy.

Rigorous experimental design for robust and unbiased results

All of our experiments have used power analysis to determine the appropriate number of experiments (refer to Vertebrate Animals section for more specific information). Importantly, protocols using a range of genotypes, diets, or drug treatments are designed with individual treatments being assigned in a random fashion. Where possible, treatments will be assigned blindly to the experimenter by another individual in the lab, although some aspects are impossible to hide from the experimenter such as the sex of the animal being studied. Animal experiments will utilize offspring from several different breeding pairs in every experimental group to avoid the potential for a unique genetic bias. Furthermore, groups of animals or series of experiments will be conducted over a period of time such that a given study is not conducted in a single time frame. This avoids any time dependent bias (time of year, temperature or humidity variations, etc.) and accounts for time related variability as a result. In experiments where there is a comparison among groups, samples from each group will be analyzed in concert, thereby preventing any biases that may arise from analyzing individual treatments on different days. Negative and positive controls to determine specificity of antibodies and primers will continue to be used in our studies. Finally, for cell culture experiments, biological replicates of different passages on different days will be performed so that we can minimize any pseudo-replication that is inherent with cell culture experiments.

Consideration of relevant biological variables

All experiments in male and female rats and mice will be conducted in sexually mature adult animals (ages ranging 14-18 weeks of age). Our studies are designed to specifically address whether there are any sex differences, so we will study both male and female rats and mice. Furthermore, variability due to differences in the time of day is being directly addressed in our studies with studies being conducted and biological sampling at different times of day. Animals will be maintained in temperature and humidity controlled space within the animal facilities in the same building as our main laboratories. Lights will be regulated to turn on at 7 am and off at 7pm. To ensure appropriate lighting control, we have developed routine practice of checking on the animals at the lights on/lights off times. Studies with 12 hr urine collections will coincide with this same lights on/lights off timing. Animals will be housed in groups except when in metabolic cages or following recovery from surgical procedures to prevent animals from disturbing incision sites until they are healed.

Authentication of key biological and/or chemical resources

- Cultured IMCD3 cells. Studies in Aim 1 will utilize a well-established, commercially available cell line originally derived from mouse inner medullary collecting ducts. These cells represent principal cells and express all of the components relevant to our studies including, ET-1, ET_B receptors, NOS isoforms, ENaC subunits. Furthermore, the signaling pathways are identical to those identified by freshly isolated inner medullary collecting ducts. All experiments will be derived from cells originally isolated and maintained in liquid nitrogen for the past 20 years in the J. Pollock laboratory and originally purchased from ATCC, mIMCD-3 (ATCC® CRL-2123™). All experiments will be conducted on cells that have been passaged no more than 5 times.
- Antibodies for Western blots (ENaC, NKA, Bmal1). All of the antibodies used in our studies have been characterized in previous studies in other laboratories as well as our own and will be purchased from commercial vendors. Care will be taken to make sure that we always use the exact same antibody including catalog number, source, and type. We will report pertinent details of the antibodies in all publications including lot number, which is especially important for polyclonal antibodies. All Western blots will be conducted using a molecular weight ladder to confirm the appropriate molecular weight. In addition,

we will use positive controls and loading controls. The full blot will be scanned and data will be backed up and cataloged in laboratory notebooks as well as computer files. The antibodies to be used in our experiments have been characterized by Dr. Mark Knepper (NIH) and are commercially available from StressMarq (Victoria, BC). These antibodies include: anti-ENaC (alpha, beta and gamma specific antibodies), anti-NKCC2, and anti-NCC. Anti-Na^+-K^+ATPase antibodies will be purchased from the Iowa Hybridoma bank (α5), and anti-BMAL1 will be purchased from Santa Cruz (sc-8850). We will use transfected cells overexpressing the protein of interest, or if possible, cells (or samples from knockout animals) where we have knocked out the protein of interest as controls for specificity of the antibodies.

- PCR primers. The clock gene primers for use in qRT-PCR have all been successfully characterized in previous studies. Controls will include qPCR without cDNA to determine primer/dimer, and no reverse transcriptase (no RT) controls will be run on each plate to control for any potential genomic contamination.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Probe (56-Fam-5'→3'-TAMTSp)
Bmal1	TCCGATGACGAACTGAAACAC	CTCGGTCACATCCTACGACAA	CAAAAATCCATCTGCTGCCCTGAGAAT
Cry1	CATCAACAGGTGGCGATTTT	CCCGAATCACAAACAGACGA	TTTAATTTTCGTAGATTGGCATCAAGATCCTCAAGA
Cry2	GTGTGAATGCAGGCAGCTG	ACAGGGCAGTAGCAGTGGAA	ATGTGGCTGTCCTGCAGTGCTTTCTTC
Per1	GGTTCAGGATCCCACGAAG	AAGAGTCGATGCTGCCAAAG	AGCACCTCAGCCAGCATCACCC
Per2	GCAGCCTTTCGATTATTCTTC	GCTCCACGGGTTGATGAAG	ATTCGATTCCGCACACGCAACG
Clock	GAACTTGGCGTTGAGGAGTCT	GTGATCGAACCTTTCCAGTGC	AGACAGCTGCTGACAAAAGCCAAGATTCTG

- ELISAs (ET-1, aldosterone, renin). The commercially available ELISA kits used in our studies have been extensively characterized through the years. There are numerous commercially available assays for ET-1 and so a number of years ago, we compared ELISAs from several companies. Unfortunately, most of the kits were developed for in vitro model systems and plasma and urine concentrations of ET-1 were often below the lowest standard available for the kit. We discovered, however, that the QuantiGlo® chemiluminescent sandwich ELISA from R&D Systems had the most sensitivity. Nonetheless, we routinely add a lower concentration to the standard curve to ensure that the assay is linear in the lower concentration range. Aldosterone and renin measurements will be conducted using well-established and well-characterized commercially available assays.
    - ET-1 (urine and plasma), QuantiGlo® Colorimetric Sandwich ELISA R&D Systems, #QET00B
    - Aldosterone (urine and plasma), EIA Cayman Chemical, #501090
    - Plasma renin concentration, ANGI kit, Bachem, # S-1188, following manufacturer's protocol III and according to published methods.

- Animal diets. Recent studies have demonstrated that the source and composition of rodent chow can have a significant effect on salt sensitivity in some strains of rat. This raises the possibility that variations in animal diet could affect the phenotype. Our animal facility provides a consistent diet purchased from Harlan Teklad for both rats and mice (7917-070115M NIH-31 Irradiated Open Formula Mouse/Rat Diet 7917). Our studies will consistently use the exact same diet for all of our studies to ensure that there are no diet-dependent variations in our experiments. This standard chow will be used when manipulating the NaCl content in the diets such that there are no differences aside from NaCl.

- ET_A and ET_B antagonists. We will only be using antagonists that have been extensively characterized in terms of selectivity and potency. We will use the ET_A selective antagonist, ABT-627, also known as atrasentan. ABT-627 is the active enantiomer of the racemic compound, A-127222. The ET_B selective antagonist, A-192621, has also been fully characterized. Doses given to animals have been demonstrated to be at or near maximal receptor antagonism while still maintaining selectivity.