Abstract This study aimed to investigate the role of endogenous angiotensin II (ANGII) in the upregulation of ANG-II AT_{1} receptors in adrenal glands during a low-salt intake. To this end male Sprague-Dawley rats were fed a low-salt diet (0.2 mg/g) for 10 days and were treated with the ANGII-A T_{1} receptor antagonist losartan (40 mg/kg per day) for 2 days, and adrenal mRNA levels for ANGII AT_{1A} and AT_{1B} receptors were determined by RNase protection. The low-salt diet increased AT_{1A} and AT_{1B} receptor mRNA levels by 90% and 220%, respectively. Losartan treatment did not change the basal AT_{1A} mRNA level, but decreased AT_{1B} mRNA by 50%. Treatment of rats on a low-salt diet with losartan did not change the increase of AT_{1A} mRNA but significantly attenuated the increase of AT_{1B} mRNA to 90% of the control value. Stimulation of endogenous ANGII levels by unilateral renal artery clipping for 2 days lowered AT_{1A} mRNA by 25% and increased AT_{1B} mRNA by 30%. Additional treatment with losartan did not affect the decreased AT_{1A} mRNA levels in rats with a unilateral renal artery clip, but significantly attenuated the increase of AT_{1B} mRNA. These findings suggest that sodium deficiency stimulates adrenal AT_{1A} and AT_{1B} receptor mRNA levels primarily via an ANGII-A T_{1}-independent mechanism. The preferential increase of adrenal AT_{1B} mRNA during a low-salt intake could be explained by the elevation of endogenous ANGII levels during sodium deficiency, suggesting that endogenous ANGII acts as an enhancer for adrenal AT_{1B} but not for AT_{1A} receptor gene expression via ANGII-A T_{1} receptors.

Keywords Adrenal gland · Angiotensin II receptor subtypes · Losartan · Low-salt intake · Renal artery clip

Introduction
The renin-angiotensin-aldosterone system (RAAS) plays a central role in salt homeostasis in organisms. Consequently the RAAS is stimulated in states of salt deficiency. The activation of the RAAS by a low-salt intake is initiated by an enhancement of renin secretion from the kidneys, leading to increased levels of circulating angiotensin II (ANGII). ANGII in turn stimulates the production of aldosterone in adrenal glomerulosa cells via ANGII-A T_{1} cell surface receptors. It has been known for a long time that the sensitivity of adrenal aldosterone production to ANGII is increased in states of salt deficiency [12, 13]. A likely reason for this increase of sensitivity is that the number of adrenal AT_{1} receptors increases during a low-salt intake [7, 8, 13, 15, 23]. Further studies have provided consistent evidence that a low-salt intake increases ANGII-A T_{1} mRNA levels in the adrenal gland, suggesting that the upregulation of ANGII-A T_{1} receptor protein is due to an increased expression of the respective ANGII-A T_{1} receptor mRNAs [8, 11, 13, 19]. The AT_{1} receptor family comprises at least two members, termed AT_{1A} and AT_{1B}, which cannot yet be distinguished by pharmacological means, and which are differentially distributed in the different parts of the adrenal gland. AT_{1A} mRNA is expressed in the adrenal cortex as well as in the medulla, whereas AT_{1B} mRNA is predominant in the adrenal cortex [9]. There is preliminary evidence that the functional role of the adrenal ANGII-A T_{1} receptor subtypes may be different with regard to the induction of aldosterone synthase [22]. During a low-salt intake both AT_{1A} and AT_{1B} mRNA were found to be increased in the adrenal gland [8, 14]. Since infusion of exogenous ANGII has been reported to increase adrenal AT_{1} mRNA and AT_{1} receptor protein [10], it has been speculated that the enhancement of adrenal AT_{1} receptors during a low-salt intake is causally related to the increased levels of endogenous ANGII, thus implying a positive feed-back regulation of adrenal AT_{1} receptors by ANGII. This concept is supported by the observations that converting enzyme inhibitors and specific ANGII-
AT<sub>1</sub> receptor antagonists are capable of blunting the increase in adrenal AT<sub>1A</sub> and AT<sub>1B</sub> receptors in response to a low-salt diet [8, 13]. Notably the basal expression of adrenal AT<sub>1</sub> receptors was not found to be changed by converting enzyme inhibition or by ANGII-AT<sub>1</sub> blockade in these studies. Other studies, however, reported a decrease of adrenal basal ANGII-AT<sub>1</sub> mRNA levels by ANGII antagonists, which was probably related to selective downregulation of AT<sub>1B</sub> mRNA [10, 12]. A causal role of endogenous ANGII in the upregulation of adrenal ANGII-AT<sub>1</sub> receptors was contested by the observation that unilateral renal artery clipping, which enhances renin secretion and consequently ANGII formation, in fact lowered adrenal AT<sub>1</sub> mRNA levels [14]. Furthermore studies with isolated bovine adrenocortical cells and human adrenocortical carcinoma cells revealed that ANGII potently downregulates AT<sub>1</sub> mRNA levels in these cells [1, 18]. In ANGII-AT<sub>1B</sub> receptor knockout mice ANGII-AT<sub>1A</sub> receptor mRNA levels are normal, suggesting that the absence of about 50% of the adrenal AT<sub>1</sub> receptors has no influence on the AT<sub>1A</sub> receptor mRNA level [4].

Taken together, the information available clearly indicates that states of sodium deficiency are associated with increased mRNA levels for adrenal AT<sub>1A</sub> and AT<sub>1B</sub> receptors. However, the mechanisms leading to the increase of adrenal ANGII-AT<sub>1</sub> mRNA levels, in particular the role of endogenous ANGII, are not yet clear. Since this uncertainty may have resulted from the different experimental models used, from different durations of treatment regimen and from different methods used to semiquantify AT<sub>1</sub> receptor gene expression, it appeared of interest to us to investigate more systematically adrenal AT<sub>1A</sub> and AT<sub>1A</sub> receptor mRNA expression during a low-salt intake using RNase protection assays as specific and robust methods for the semiquantification of the respective mRNA levels. In particular, we were interested to assess the role of endogenous ANGII in the influence of salt intake on adrenal AT<sub>1</sub> receptor mRNA levels.

We found that a low-salt intake led to a sustained and preferential increase of AT<sub>1B</sub> receptor mRNA in the adrenal gland of rats. AT<sub>1B</sub> receptor mRNA expression was selectively regulated via ANGII-AT<sub>1</sub> receptors in a positive fashion. Endogenous ANGII appears to be an enhancer of adrenal AT<sub>1B</sub> receptor gene expression, which, however, also requires an additional ANGII-independent mechanism to upregulate adrenal ANGII-AT<sub>1</sub> receptor mRNA during sodium deficiency.

### Materials and methods

**Animal experiments**

Male Sprague-Dawley rats (220–250 g) kept in the local animal house were used for the studies. Six different groups were examined in our experiments.

1. **Control animals** received normal chow (6 mg NaCl/g) and remained untreated.
2. **Animals kept on a low-salt diet**. Rats were maintained for 10 days on rat chow balanced in all respects except for a low sodium (0.2 mg/g) content (Altromin).
3. **Animals with losartan treatment**. Blockade of the ANGII receptors was achieved by treatment of the animals with the receptor antagonist losartan (40 mg/kg) which was applied by gavage every 24 h for 2 days.
4. **Animals kept on a low-salt diet and given losartan treatment**. Animals kept on a low-salt diet for 10 days were treated with losartan for the last 2 days of the diet.
5. **Animals with a unilateral renal artery clip**. Rats were anaesthetized by methohexitol (75 mg/kg i.p.), and the left kidney was exposed by abdominal incision. Sterile silver clips (0.2 mm ID, Degussa) were then placed on the left renal arteries for 48 h.
6. **Animals with a unilateral renal artery clip and losartan treatment**. Rats with a unilateral artery clip were treated with losartan for 2 days, starting on the day of surgery.

At the end of the experiments the animals were killed by decapitation and blood was collected from the carotid arteries, ethylenediaminetetraacetic acid (EDTA) was added to the blood and plasma renin activity (PRA) was determined.

The adrenals were rapidly removed and frozen in liquid nitrogen. The organs were stored at –80°C until isolation of total RNA, which was extracted from the frozen adrenals as described by Chomczynski and Sacchi [6].

**Determination of AT<sub>1A</sub> and AT<sub>1B</sub> mRNA**

AT<sub>1A</sub> and AT<sub>1B</sub> mRNAs were measured by RNase protection assay. To generate an AT<sub>1A</sub> and an AT<sub>1B</sub> cRNA probe for specific AT<sub>1</sub> receptor mRNA or AT<sub>1</sub> mRNA detection without cross-hybridization signals, we chose cDNA sequences from AT<sub>1A</sub> and AT<sub>1B</sub> regions where no sequence homology exists between the receptor subtypes.

For detection of AT<sub>1A</sub> mRNA, an antisense RNA probe was generated by in vitro transcription of the plasmid vector pSP73 (Promega-Serva, Heidelberg, Germany) containing a PCR-derived fragment of rat AT<sub>1A</sub>cDNA [16]. The PCR fragment, amplified by the upstream primer (5'-GGC CGA GCC GGC ACC AGC-3', binding at 73–91 bp in the 5'-noncoding region) and the downstream primer (5'-CTC AAC CCA GAA AAA AAA A-3', binding at 241–260 bp in the 5'-noncoding region), was cloned in a BamHI/EcoRI-digested pSP73 vector, using standard protocols. After linearization with HindIII and in vitro transcription with SP6 RNA polymerase, the resulting 241-bp fragment was used to detect AT<sub>1A</sub> mRNA in the protection assay. Performing an RNase protection assay with this complementary RNA produces a 187-bp protected fragment of the AT<sub>1A</sub> gene transcript.

The upstream primer (5'-AGT CTG CAT CTT TTT TTT GG-3'; binding at 1249–1276 bp in the 3'-noncoding region) and downstream primer (5'-ACC TCC AGC CCT TCT AG-3'; binding at 1374–1392 bp in the 3'-noncoding region) were used to amplify a 146-bp fragment of rat AT<sub>1B</sub>cDNA [20]. Cloning in a BamHI/EcoRI-digested pSP73 vector, using standard protocols. After linearization with HindIII and in vitro transcription with SP6 RNA polymerase yielded a 208-bp antisense RNA transcript that was used to detect AT<sub>1B</sub> mRNA. Sequencing of the inserts confirmed the identity with the published sequence of rat AT<sub>1A</sub> and AT<sub>1B</sub> mRNAs. Transcripts were continuously labelled with [*γ-32P]*guanosine triphosphate (14.8 TBq/mmol or 400 Ci/mmol, Amersham) and purified on a Sephadex G-50 spin column. For hybridization, total RNA was dissolved in a buffer containing 50% formamide, 40 mmol/l sodium-N,N,N'-triethylenetetramine-tetraacetic acid (EDTA) (pH 8) and the radiolabelled RNA transcript was added to 20 µg RNA in a total volume of 35 µl. For hybridization in a humid chamber at 65°C for 18 h, the RNA was denatured in the presence of 50% formamide and 0.1% sodium dodecyl sulphate. After autoradiography of the dried gel at –80°C for 1 day, AT<sub>1A</sub> and AT<sub>1B</sub> were excised from the gel, and radioactivity was counted with a liquid scintillation counter (1500 Tri-Carb, Packard Instrument Company, Downers Grove, Ill., USA).