Chronic activation of vasopressin V2 receptor signalling lowers renal medullary oxygen levels in rats


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Abstract

Aim: In the present study, we aimed to elucidate the effects of chronic vasopressin administration on renal medullary oxygen levels.

Methods: Adult Sprague Dawley or vasopressin-deficient Brattleboro rats were treated with the vasopressin V2 receptor agonist, desmopressin (5 ng/h; 3d), or its vehicle via osmotic minipumps. Immunostaining for pimonidazole and the transcription factor HIF-1α (hypoxia-inducible factor-1α) were used to identify hypoxic areas. Activation of HIF-target gene expression following desmopressin treatment was studied by microarray analysis.

Results: Pimonidazole staining was detected in the outer and inner medulla of desmopressin-treated rats, whereas staining in control animals was weak or absent. HIF-1α immunostaining demonstrated nuclear accumulation in the papilla of desmopressin-treated animals, whereas no staining was observed in the controls. Gene expression analysis revealed significant enrichment of HIF-target genes in the group of desmopressin-regulated gene products ($P = 2.6 \times 10^{-21}$). Regulated products included insulin-like growth factor binding proteins 1 and 3, angiopoietin 2, fibronectin, cathepsin D, hexokinase 2 and cyclooxygenase 2.

Conclusion: Our results demonstrate that an activation of the renal urine concentrating mechanism by desmopressin causes renal medullary hypoxia and an upregulation of hypoxia-inducible gene expression.

Keywords: gene expression analysis, gene ontology analysis, hypoxia-inducible factor, pimonidazole, urine concentrating.

Modulation of renal free water excretion by arginine-vasopressin (AVP) is an essential mechanism for the maintenance of extracellular fluid osmotic homoeostasis. Dysregulation of this process causes distinct diseases like diabetes insipidus and the syndrome of inadequate antidiuresis (Ball 2007, Ellison & Berl 2007) but has also been implicated in the pathogenesis of renal diseases like the cardiorenal syndrome, contrast nephropathy or diabetic nephropathy (Bardoux et al. 1999, Persson et al. 2005, Perico et al. 2009, Schrier et al. 2009).

An essential structure for the generation of both, concentrated and diluted urine is the thick ascending limb of the loop of Henle (TAL) with its type 2 Na+, K⁺,2Cl⁻-cotransporter (NKCC2). Characteristically, the epithelium of the TAL is water impermeable, and NaCl reabsorption in this nephron segment therefore causes dilution of the tubular fluid. At the same time, reabsorbed NaCl accumulates in the renal medullary interstitium and provides the driving force for counter-current multiplication and the subsequent water reabsorption by the collecting duct. The antidiuretic
effects of AVP are mainly mediated by its V₂ receptor, which is abundantly expressed along the TAL and the collecting duct (Mutig et al. 2007). Here, acute stimulation with AVP causes phosphorylation and membrane insertion of NKCC2 and aquaporin 2 (AQP2) and thus increases transepithelial NaCl and water transport in TAL and collecting duct, respectively (Hoffert et al. 2008, Welker et al. 2008). In addition, AVP stimulates sodium reabsorption by the collecting duct by a mechanism that includes the action of the epithelial sodium channel ENaC (Blanchard et al. 2011). Chronic activation of AVP V₂ receptor signalling induces de-novo synthesis of NKCC2, AQP2 and various other transporters and channels (Ecelberger et al. 2001). In addition, chronic exposure to AVP causes growth processes in the TAL, which lead to a marked hypertrophy of the outer medulla (Bouby et al. 1985). Indirect effects of AVP result from the interstitial hyperosmolality, which induces the synthesis of organic osmolytes and prostaglandins in epithelia and interstitial cells to facilitate adaptation to the hyperosmotic environment (Burger-Kentischer et al. 1999, Yang et al. 1999, Moeckel et al. 2003). The principal determinant of medullary oxygen consumption is the rate of active reabsorption along the medullary thick ascending limb (Breizis et al. 1994). Additional energy is necessary for protein synthesis as well as for adaptive processes including growth (Rolfe & Brown 1997).

At the same time, medullary perfusion is decreased by an incompletely understood mechanism that involves both V₁ and V₂ AVP receptor pathways (Thurau et al. 1960, Zimmerhackl et al. 1985, Kiberd et al. 1987, Harrison-Bernard & Carmines 1994). We hypothesized that increased transport activity, adaptive growth processes and reduced medullary perfusion in the context of enhanced renal urine concentration leads to a markedly decreased oxygen availability in the renal medulla.

To test this, we have made use of established histochemical and immunohistochemical methodology for the detection of hypoxia and the transcription factor, hypoxia-inducible factor-1α (HIF-1α). The results of a microarray-based gene expression analysis of AVP-regulated gene products (Paliege et al. 2012) were evaluated regarding an enrichment of known HIF-target genes. Using these techniques, we were able to show that chronic treatment with the AVP V₂ receptor analogue 1-desamino-8-D-Arg vasopressin (dDAVP) causes widespread medullary hypoxia and induces HIF-dependent gene expression. We speculate that medullary hypoxia during antidiuresis increases the susceptibility to insults and may thereby contribute to the development of renal disease (Nangaku et al. 2013).

Materials and methods

This study is conformed with Good Publishing Practice in Physiology (Persson & Henriksson 2011).

Animal studies and tissue preservation

For immunohistochemistry studies, adult Sprague Dawley and Brattleboro rats (n = 5 per group) were treated with vehicle or dDAVP, as previously described (Paliege et al. 2012). At the end of the treatment period, all animals received an intraperitoneal injection of pimonidazole (60 mg/kg body wt; Natural Pharmacia International, Belmont, USA). After 30 min, rats were anesthetized by short isoflurane inhalation (Abbott, Wiesbaden, Germany) followed by an injection of pentobarbital sodium (60 mg/kg body wt ip, FAGRON, Barsbüttel, Germany). The abdominal cavity was opened, and kidneys were flushed for 30 s with a solution containing sucrose dissolved in PBS via retrograde perfusion of the abdominal aorta to remove blood and unbound pimonidazole. Kidneys were subsequently perfusion-fixed for 5 min with 3% paraformaldehyde (PFA, Merck, Darmstadt, Germany) in PBS. After perfusion, kidneys were carefully removed and processed for paraffin embedding or cryostat sectioning using routine methodology.

Microarray studies

Gene expression profiling was performed in the microarray facility of the Zentrum für Medizinische Forschung of the University Mannheim (Mannheim, Germany), as previously described (Paliege et al. 2012). Raw and normalized data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; GEO accession number: GSE34225).

Immunohistochemistry

Pimonidazole staining was performed using the Hypoxyprobe plus kit (Natural Pharmacia International, Belmont, USA). This technique has been widely used to detect tissue areas with a partial pressure of oxygen below 10 mmHg and is based on the immunohistochemical detection of pimonidazole adducts, which are formed in an oxygen-sensitive reaction (Supplemental Figure 1) (Varia et al. 1998, Rosenberger et al. 2009). Staining was performed in a standardized fashion following a previously established protocol without antigen retrieval or signal amplification techniques (Rosenberger et al. 2009). In modification of the protocol, we used 12-μm cryostat sections to increase antigen abundance. Sections were rinsed briefly in
TBS containing 1% Tween 20 (TBST; Merck, Darmstadt, Germany). Endogenous peroxidases were blocked for 5 min using 3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany). After rinsing in TBST, sections were incubated with a blocking solution containing 1% bovine serum albumin in TBST to saturate unspecific protein-binding sites. A mouse monoclonal anti-pimonidazole antibody labelled with fluoresceinisothiocyanat (FITC) was applied in a dilution of 1 : 100 in blocking solution. Sections were incubated for 2 hours to increase antibody penetration. After multiple short rinses in TBST, mouse monoclonal anti FITC antibody labelled with horseradish peroxidase was applied for 2 hours. Signal was generated using diaminobenzidine.

Further characterization of pimonidazole immunoreactive structures was achieved by double labelling of pimonidazole-stained sections with antibodies against NKCC2 for TAL and AQP2 for collecting ducts using well-characterized antibodies and established methodology (Mutig et al. 2007, Paliege et al. 2012). HIF-1α immunohistochemistry was performed using a well-characterized antibody and high-amplification technique, as described previously (Rosenberger et al. 2003, Paliege et al. 2010).

All sections were examined using a Leica DMRB microscope equipped with fluorescence and interference contrast modules (Leica, Wetzlar, Germany). All images were acquired using a SPOT RT 2.3.0 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and the MetaView imaging System (Molecular Devices, Downingtown, PA USA).

Statistical analysis

Quantification of immunohistochemistry signals was performed in a blinded manner using a semi-quantitative scoring system for stained area, as previously described (Raleigh et al. 2001). Inner and outer medullas were evaluated separately. A score of 1 was assigned to sections with labelling of up to 15%, a score of 2 to sections with labelling of up to 30% and a score of 3 to sections with labelling of more than 30% of the observed area. Sections without staining were scored as zero. Based on this score, animals were ranked, and rank sums were calculated for controls and dDAVP-treated animals. Statistical analysis was performed using Mann–Whitney U test with $P < 0.05$ considered as statistically significant.

For evaluation of microarray results, Hochberg multi-test was applied and a cut-off of greater than 1.3-fold induction, and $P < 0.01$ was considered as statistically significant. Gene ontology enrichment analysis was performed using the web-based GOSSIP Gene Ontology biological profiling Toolkit (Bluthgen et al. 2005). All upregulated genes were included into the analysis. To test for an enrichment of HIF-target genes in the group of dDAVP-regulated gene products,
we made use of a published list of HIF targets (Semenza 2003b, Rosenberger et al. 2005). In addition, we performed a PubMed/MEDLINE search for the years 2006 to 2011 using the search terms: hypoxia-inducible factor and target gene expression. Potential candidates were screened for the presence of a hypoxia response element in the promoter sequence. Using these different sources, we identified 173 HIF-target genes 109 of which were also present in the microarray. The number of regulated products was compared with the number expected during random distribution. Statistical significance of enrichment was determined by chi-squared test.

**Results**

Effect of dDAVP on renal accumulation of pimonidazole adducts

Analysis of pimonidazole abundance in kidneys of Sprague Dawley rats showed significant staining in the inner stripe of the outer medulla with maximal signal intensity in the initial portions of the TAL on the junction to the inner medulla. Vascular bundles, collecting ducts and thin limbs were devoid of staining. Treatment with dDAVP resulted in an overall increased size of the stained area as well as an increased intensity of the staining as compared with the controls (Fig. 1a,b). In the inner medulla of untreated Sprague Dawley rats, staining was restricted to scattered interstitial cells in the centre of the papilla (Fig. 1c). In contrast, the inner medulla of the dDAVP-treated animals showed homogenous, intense staining of all structures in the centre of the papilla, whereas its periphery adjacent to the renal pelvis did not show pimonidazole immunoreactivity (Fig. 1d).

**Figure 2** Chronic vasopressin V2 receptor activation causes medullary accumulation of pimonidazole adducts in Brattleboro rats. Low-power micrographs showing increased amounts of immunoreactive pimonidazole adducts in the outer (OM; a,b) and inner (IM; c,d) medulla of Brattleboro rats treated for three days with 5 ng/h dDAVP (dDAVP; b,d) as compared with vehicle-treated controls (control; a,c). Distribution and intensity of pimonidazole staining in the untreated Brattleboro rats is similar to the untreated Sprague Dawley rats. However, accumulation of pimonidazole adducts following dDAVP treatment was less pronounced in the Brattleboro rats. Immunoperoxidase staining; original magnification 100x.

**Figure 3** Immunofluorescence localization of pimonidazole staining in outer medullary TAL. Double labelling of kidney sections of Brattleboro rats with pimonidazole (a,c) and type 2 Na+, K+,2Cl−-cotransporter (NKCC2) revealed preferential accumulation of immunoreactive pimonidazole adducts in profiles of the TAL of dDAVP-treated animals (c,d) as compared with controls (a,b). No staining was observed in profiles of the collecting duct or the outer medullary interstitium. Double labelling immunostaining; original magnification 400x.
Analysis of pimonidazole staining in Brattleboro rats revealed a similar distribution with less pronounced effects of dDAVP (Fig. 2a-d). Double labelling of pimonidazole-stained sections with antibodies against NKCC2 and AQP2 confirmed accumulation of pimonidazole adducts in the TAL (Fig. 3) and medullary collecting duct (Fig. 4). Immunoreactive pimonidazole adducts were distributed homogeneously throughout the cytosol of the respective target structures. A synopsis of pimonidazole accumulation in dDAVP treated rats is provided in Figure 5.

**Effect of dDAVP on abundance and nuclear accumulation of HIF-1α**

As expected, HIF-1α staining was rarely observed in the kidneys of vehicle-treated Brattleboro rats (Fig. 4a), whereas it was readily detectable in the papilla of the dDAVP-treated animals (Fig. 4b). High-power micrographs (Fig. 4c and d) demonstrated HIF-1α accumulation in the nuclei of cells of the collecting ducts and thin limbs as well as endothelial and medullary interstitial cells of the dDAVP-treated animals, thus mirroring the widespread distribution of papillary pimonidazole staining (Fig. 6). No HIF-1α staining was found in the cortical or medullary parts of the TAL (data not shown).

**Effect of dDAVP on medullary gene expression**

Affymetrix GeneChip® Rat Genome 230 2.0 Array was used to study dDAVP-dependent gene expression in the outer medulla of Brattleboro rats (Paliege et al. 2012). This array contains 16950 individual gene products 75 (0.44%) of which were induced greater than twofold and 513 (3%) greater than 1.3-fold by dDAVP treatment. Gene ontology (GO) analysis showed significant regulation of gene products related to extracellular matrix generation, insulin-like growth factor signalling, stress response, cell growth and morphogenesis, probably reflecting growth...
processes of the outer medulla (Table 1). Several of these GO-terms also include HIF-target genes but until now, no individual GO-term for hypoxia response or, more specifically, for HIF-target genes has been compiled.

**Effect of dDAVP on expression of HIF-target genes**

To test the microarray results for an enrichment of HIF-target products, we generated a comprehensive list of previously identified HIF targets containing a total of 173 gene products, 109 of which were also present in the microarray (Supplemental Table 1). Of those 109 products, 21 (19%) were significantly induced by the dDAVP treatment, which relates to the 3% that would be expected during random distribution. The enrichment of HIF targets in the group of dDAVP-regulated gene products was highly significant ($P = 2.6 \times 10^{-21}$), as determined by chi-squared test. Regulated products included insulin-like growth factor binding proteins 1 and 3, angiopoietin 2, fibronectin, cathepsin D, hexokinase 2 and cyclooxygenase 2. (Table 2) whereas other typical HIF-target genes like vascular endothelial cell growth factor (VEGF) or erythropoietin were not regulated.

**Discussion**

Sustained antidiuresis is achieved by a complex mechanism that involves the activation of multiple energy-consuming transport processes, de-novo synthesis of required transporters and channels, and a hypertrophic growth response of medullary structures. At the same time, medullary blood flow becomes decreased. The question, whether the combination of these processes causes an aggravation of medullary hypoxia, has not been answered so far. Here, we show that chronic treatment with the antidiuretic AVP V$_2$ receptor analogue dDAVP causes severe hypoxia of key structures involved in the urine concentration mechanism and stimulates hypoxia-inducible gene expression.

Renal oxygen levels have been intensely studied using oxygen electrode measurements, anatomical analysis and pimonidazole histochemical staining. The results of these studies are summarized in a recent review (Rosenberger et al. 2009). Using a protocol adapted for low antigen abundance, we were able to demonstrate intense pimonidazole staining in the medullary TAL as well as in epithelial, vascular and interstitial cells in the dDAVP-treated animals. High-amplification immunoperoxidase staining; original magnification 100× for a and b and 400× for c and d.
published data regarding the distribution of pimonidazole adducts in rodent kidneys (Manotham et al. 2004, Rosenberger et al. 2009, Prasad et al. 2010). Accumulation of pimonidazole adducts in the initial portions of the TAL is likely to reflect increased oxygen consumption due to a stimulated transcellular ion transport in response to dDAVP treatment. In line with this concept, previous studies have demonstrated an increased abundance and transport activity of NKCC2 and Na/K-ATPase in the mTAL following an increased abundance and transport activity of NKCC2 and Na/K-ATPase in the mTAL following chronic dDAVP stimulation (Molony et al. 1987, Ecelbarger et al. 2001, Sakuma et al. 2005, Mutig et al. 2007, Welker et al. 2007, 2008).

Accumulation of pimonidazole adducts in the core of the inner medulla may result from low oxygen availability secondary to reduced medullary perfusion and increased ENaC-mediated sodium reabsorption by the inner medullary collecting duct (Ecelbarger et al. 2001, Blanchard et al. 2011). Lack of pimonidazole staining in the borders of the papilla may reflect increased oxygen partial pressure due to diffusion from the renal pelvis, which receives its arterial blood supply from multiple sources independent of the medullary perfusion (Sporer & Seebode 1981). In aggregate, the results from our pimonidazole studies thus support the hypothesis of an aggravated medullary hypoxia in response to dDAVP treatment.

This concept was further strengthened by our observation of increased levels of HIF-1α in the inner medulla following dDAVP treatment. This finding corroborates the results from a previous study showing accumulation of HIF in various medullary structures in dehydrated animals (Manotham et al. 2005). Interestingly, neither dehydration nor dDAVP treatment had caused HIF-1α accumulation in the TAL. In fact, a low capacity for HIF-activation of the TAL as compared with the collecting duct appears to be a general phenomenon, which may explain the high susceptibility of the TAL for hypoxic damage (Heyman et al. 1988, 1991, 2005, Rosenberger et al. 2002, 2006, 2008).

Analysis of microarray data revealed significant overrepresentation of known HIF-target genes in the group of dDAVP-regulated gene products. These included proteins involved in insulin-like growth factor signalling and the regulation of cell metabolism and extracellular matrix synthesis. In addition, we observed a marked induction of cyclooxygenase 2, the main enzyme involved in medullary prostaglandin production. This finding confirms previous data showing increased levels of cyclooxygenase 2 and augmented synthesis of prostaglandin E2 in the medulla of dehydrated rats or in Brattleboro rats treated with AVP (Yang et al. 1999, Zhang et al. 2004). As prostaglandin E2 increases medullary perfusion and inhibits TAL transport activity, this response is considered to function as a negative feedback loop aimed at preventing medullary ischaemia. Accordingly, interference with medullary prostaglandin synthesis pathways has been shown to cause renal damage (Sabatini 1996, Harris

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>X fold of control</th>
<th>P</th>
<th>Reference</th>
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<td>(Semenza 2003b)</td>
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Local modulation of AVP signalling is further exerted by the antagonistic free radicals nitric oxide and superoxide anion. Similar to prostaglandin E2, nitric oxide causes vasodilatation of the descending vasa recta and thus increases medullary perfusion (Cao et al. 2010). In addition, it inhibits NaCl transport by the thick ascending limb (Edwards & Layton 2011). Medullary levels of nitric oxide have been shown to increase upon AVP treatment (Shin et al. 1999, Martin et al. 2002). However, the cellular source and the specific AVP receptor involved in this regulation are not univocally agreed upon (Hirata et al. 1998, Martin et al. 1999, Martin et al. 2002). In accordance with this notion, it has been shown that AVP may alter the cellular redox status of medullary interstitial fluid (Thomas et al. 2002). However, it is not clear whether AVP increases or decreases nitric oxide bioavailability to cause vasoconstriction of the descending vasa recta and an increase in TAL transport activity (Edwards & Layton 2011, Ahmeda & Johns 2012). The main source for ROS in the medulla is the TAL where ROS synthesis has been shown to depend on specific oxidases and mitochondrial respiratory chain enzymes (Zou et al. 2001, Li et al. 2002). The effects of AVP on medullary ROS synthesis have not been studied so far. However, as ROS synthesis rate has been shown to correlate with NaCl transport activity (Zou et al. 2001, Ohsaki et al. 2012), activation of TAL transport by AVP is likely to increase ROS synthesis. Other vasoactive mediators that have been implicated in the regulation of AVP signalling in the renal medulla include angiotensin 2, endothelin, ATP, adenosin and eicosanoids such as 20-HETE and EETs (Sarkis et al. 2005, Stricklett et al. 2006, Dobrowolski et al. 2007, Rieg et al. 2008, Evans et al. 2010, Kuczeriszka et al. 2011, Novak 2011, Storkebaum & Carmeliet 2011, Li et al. 2012, Kennedy-Lydon et al. 2013). Thus, it evolves the picture of a complex network of endo-, auto- and paracrine signalling pathways involved in the regulation of the urine concentration mechanism. However, further studies are needed to fully comprehend the contributions of these individual products to the modulation of AVP signalling.

Among the classical effects of the HIF-mediated hypoxia response is the induction of pro-angiogenic cytokines like VEGF and the subsequent formation of novel blood vessels (Semenza 2003a). However, microarray data showed unaltered VEGF expression in the dDAVP-treated animals despite increased levels of HIF-1α in the collecting duct as the predominant site for medullary VEGF synthesis (Maharaj et al. 2006). Because VEGF expression has been shown to be regulated by other factors in addition to HIF, this finding may be explained by an activation of inhibitory modulators. In agreement with this notion, analysis of microarray data revealed a strong induction of insulin-like growth factor binding protein (IGFBP) 3, which has been shown to effectively inhibit VEGF expression in various malignant and non-malignant tissues (Mathur & Mathur 2003, Kim et al. 2012, Oh et al. 2012). Function, cellular source and the mechanisms governing IGFBP3 synthesis in the renal medulla are currently unknown. We speculate that an inhibition of vasulogenesis during the pronounced hypoxia associated with the activation of renal urine concentration may be an important mechanism for the maintenance of the structure of the medullary vasculature. In line with this notion, we observed an upregulation of several other antiangiogenic factors including angiopoietin 2, IGFBP 5 and 6, fibulin 1 and 5 and the extracellular matrix protein decorin following dDAVP treatment (Rho et al. 2008, Xie et al. 2008, Neill et al. 2012, Zhang et al. 2012, Bach et al. 2013). Further studies are necessary to validate this concept.

In conclusion, we have shown that chronic activation of the renal urine concentrating mechanism leads to a marked decline in oxygen levels in the inner and in the outer medulla. We speculate that these low oxygen levels may render the kidney vulnerable to insults such as the application of radiocontrast agents or a reduction in renal perfusion during medical procedures. Our results may provide a mechanism-based rational for the clinical practice of volume substitution prior to the intervention.

Conflict of interest
No conflicts of interest are declared by the authors.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Scheme for the activation of pimonidazole under hypoxic conditions. Activation of pimonidazole requires its reduction to a nitro radical anion. This step is inhibited at oxygen concentrations above 10 mmHg. The activated intermediate that binds to hypoxic cells is believed to be a hydroxylamine derivative resulting from the sequential addition of four electrons to pimonidazole. The hydroxylamine intermediate reacts with thiol-containing peptides and proteins to form stable adducts that can be detected by immunochemical assays (adapted from Varia *et al.* 1998).

**Table S1** Expression data for HIF-target genes following 3-day treatment of Brattleboro rats with 5 ng/h dDAVP.