Expression of adrenomedullin in hypoxic and ischemic rat kidneys and human kidneys with arterial stenosis

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Sandner, Peter, Karl Heinz Hofbauer, Hanna Tinel, Armin Kurtz, Helle C. Thiesson, Peter D. Ottosen, Steen Walter, Ole Skott, and Boye L. Jensen. Expression of adrenomedullin in hypoxic and ischemic rat kidneys and human kidneys with arterial stenosis. Am J Physiol Regul Integr Comp Physiol 286: R942–R951, 2004. First published January 8, 2004; 10.1152/ajpregu.00274.2003.—To investigate regional aspects of hypoxic regulation of adrenomedullin (AM) in kidneys, we mapped the distribution of AM in the rat kidney after hypoxia (normobaric hypoxic hypoxia, carbon monoxide, and COCl2 for 6 h), anemia (hematocrit lowered by bleeding) and after global transient ischemia for 1 h (unilateral renal artery occlusion and reperfusion for 6 and 24 h) and segmental infarct (6 and 24 h). AM expression and localization was determined in normal human kidneys and in kidneys with arterial stenosis. Hypoxia stimulated AM mRNA expression significantly in rat inner medulla (CO 13 times, 8% O2 6 times, and COCl2 8 times), followed by the outer medulla and cortex. AM mRNA level was significantly elevated in response to anemia and occlusion-reperfusion. Immunoreactive AM was associated with the thin limbs of Henle’s loop, distal convoluted tubule, collecting ducts, papilla surface epithelium, and urothelium. AM labeling was prominent in the inner medulla after CO and in the outer medulla after occlusion-reperfusion. The infarct border zone was strongly labeled for AM. In cultured inner medullary collecting duct cells, AM mRNA was significantly increased by hypoxia. AM mRNA was equally distributed in human kidney and AM was localized as in the rat kidney. In human kidneys with artery stenosis, AM mRNA was not significantly enhanced compared with controls, but AM immunoreactivity was observed in tubules, vessels, and glomerular cells. In summary, AM expression was increased in the rat kidney in response to hypoxic and ischemic hypoxia in keeping with oxygen gradients. AM was widely distributed in the human kidney with arterial stenosis. AM may play a significant role to counteract hypoxia in the kidney. Anemia; hypertension; infarct; oxygen

The vasodilator peptide adrenomedullin (AM) was originally discovered in human pheochromocytoma cell extracts by its ability to initiate cAMP production in target cells (18, 19, 31). AM was shown to have a wide tissue distribution under physiological conditions (32) and no single glandular source of systemic AM was identified. Hence, AM is thought primarily to act in a paracrine fashion. The kidneys transcribe and release systemic AM was identified. Hence, AM is thought primarily physiological conditions (32) and no single glandular source of AM was shown to have a wide tissue distribution under experimental maneuvers: hypoxic hypoxia, functional and anemic anemia, transient global ischemia, and permanent, localized ischemia in the rat kidney. These findings were compared with data obtained from normal human kidney tissue and in human kidney tissue from patients with established renal artery stenosis and hypertension.

MATERIALS AND METHODS

In vivo rat experiments. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German and Danish legislation on animal welfare. Male Sprague-Dawley rats (200–250 g) that had free access to standard rat chow and tap water were used for the experiments and treated in the following manner. Control rats were placed in gas-tight boxes that were supplied with room air (n = 6). Normobaric normocapnic hypoxia rats were placed in a cage that was continuously supplied with a gas mixture of 8% O2-92% N2 for 6 h.
(n = 6). Functional anemia was induced by carbon monoxide (CO) admixture. These rats were placed in a gas-tight cage, continuously supplied with room air plus 0.1% CO for 6 h (n = 6). Rats were subcutaneously injected with CoCl₂ (60 mg/kg), and these animals were euthanized 6 h later (n = 6). Fifth, anemia was induced. The hemacrit was lowered by blood volume reduction (n = 6). On day 1, rats were bled retroorbitally (8.4 ± 0.9 ml), followed by a second bleeding 24 h later, where 5.7 ± 0.7 ml was collected. Five hours after the second bleeding, the rats, with controls, were euthanized. Sixth, occlusion-reperfusion was performed. Rats were anesthetized with hypnorm (0.25 mg/kg fentanyl citrate and 8 mg/kg fluanisone) and 4 mg/kg diazepam, and placed on a heating table. The abdomen was opened. The left renal artery was occluded for 1 h, reopened, and the rats regained consciousness. For analgesia, the rats were treated with buprenorphine (0.1 mg/kg sc). The rats were observed for 6 h (n = 4) and for 24 h (n = 4). Finally, kidney infarct was induced. The rats were anesthetized as above and a segment artery branch from the renal artery was dissected free and ligated. The rats were euthanized after 6 h (n = 4) and after 24 h (n = 4).

In all experimental series, the animals were euthanized by decapitation and kidneys were quickly removed. In some of the series (changes in ambient atmosphere, anemia), one kidney was macroscopically dissected in the cortex, outer medulla, and inner medulla and subsequently frozen in liquid N₂, and stored at −80°C. For immunohistochemistry the following rat kidneys were fixed: control (n = 4), 0.1% CO 6 h (n = 2), 6 h and 24 h infarct (n = 2 each), 6 and 24 h occlusion-reperfusion (n = 2 each), and anemia (n = 4). The kidneys were fixed by retrograde perfusion through the aorta with isotonic NaCl for 2 min at a pressure of 100 mmHg and then with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, for 5 min. The kidney blocks were postfixd overnight, dehydrated, and embedded in paraffin.

Patient tissue samples. Tumor-adjacent unaffected kidney tissue and kidneys removed because of arterial stenosis and systemic hypertension (n = 4) were obtained from patients undergoing radical nephrectomy at the Department of Urology, Odense University Hospital, Odense, Denmark. All patients gave informed written consent to the use of tissue. The study protocol was approved by the local ethical committee. None of the patients had received chemotherapy or radiation therapy before nephrectomy. The four patients with renovascular hypertension were two men and two women, aged 35–62 yr, all with the diagnoses of macroscopic kidney atrophy and arterial stenosis. The four patients whose kidneys were used for controls were one woman and three men, aged 47–69 yr, with clear cell carcinomas. Each kidney, with or without tumor, was divided into several fractions. Tumor-adjacent normal kidney tissue (control) and “stenotic” tissue was separated in cortex and medulla and rapidly frozen in liquid N₂. Because of marked atrophy of the kidneys with arterial stenosis, it was not possible to discriminate between the outer and inner medulla during dissection, so the kidneys were divided in the cortex and total medulla. For histological analysis, tissue samples comprising all regions of the kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and frozen in N₂-cooled isopentane for cryosectioning.

Cloning of human AM cDNA. Total RNA was extracted from rat kidneys according to the protocol of Chomczynski and Sacchi (3), as previously described. Total RNA was isolated from human kidney tissue with the RNeasy kit from QiaGen by following the instructions provided by the manufacturer. PCR was used to amplify sequences specific for human AM and β-actin. Primer sequences were as below and primers were synthesized (Invitrogen) with restriction sites for BamHI (sense) and EcoRI (antisense) in the 5’ direction (+15 bp) to allow for directional cloning in vector pSP73 (Promega, Rødovre, Denmark) as described previously (1). Plasmid was sequenced by T7 polymerase (Promega) on an ABI Prism genetic analyzer with ready-reaction mix from ABI.
Semi-quantitative RT-PCR analysis of AM mRNA was done by TaqMan-PCR: 1 μg of total RNA was digested with DNase I and reverse transcribed with the use of Superscript-II RT-PCR kit (Invitrogen). cDNA was amplified with TaqMan Universal Master Mix (Applied Biosystems); 94°C-15 s and 60°C-1 min repeated for 40 cycles. The concentration of primers was 300 nmol/l and labeled probe 150 nmol/l. The parameter Ct is the PCR cycle number, defined as the point at which the fluorescence generated by cleavage of the probe passed a fixed threshold above baseline. Ct values were detected for AM and for β-actin mRNA as housekeeping gene. Since we detected no alteration of β-actin mRNA level by the present experimental protocols, mRNA expression data were normalized with respect to β-actin. The expression was calculated using the formula expression \( = 2^{(Ct_{actin} - Ct_{AM})} \), where dCt is the difference in Ct value of AM and β-actin. Data are given as relative expression (re) in arbitrary units.

**Primer sequences.** Six primer sequences were used in this study. 1) Rat AM, sense: 5'-GGC AGC ATT GAA CAG ACC-3'; antisense 5'-AAG GCA GTG GCT CAG ACC-3', spans bases 738–945, 208 bp and 15 bp linker sequence, altogether 223 bp (GenBank accession no. 012715). 2) Rat AM for quantitative PCR: sense 5'-GCA GTT CCG AAA GAA GTG GAA-3', antisense 5'-GCT GCT GGA CGC TTG TA-3', spans bases 255–324, 70 bp (GenBank accession no. 012715) and nested probe 5'-AAG TGG GCG CTA AGT GGT GGG AAG A-3'. 3) Human AM: sense 5'-ATG AA GCC GTG TCG CGT C-3', antisense 5'-AAG TGG TTT TCC ATG CTC TGG-3' (GenBank accession no. d43639). Primers were designed to anneal to exon 2 (bases 2356–2373) and in exon 4 (3026–3043), and thereby discriminate between amplification of genomic DNA (688 bp) and cDNA (310 bp). 4) Rat AQP-2: sense: 5'-AGG AGC ATG TGG GAA-3', antisense: 5'-AGC TCA GGC CTT GCT-3', spans 811 bp (GenBank accession no. D13906). 5) Rat β-actin (2): this primer set spans an intron to distinguish between amplification of cDNA and genomic DNA. The expected size of a genomic amplification product is 313 bp. 6) Human β-actin: sense 5'-CCA AGG TTC TAC ACA ATG TGG-3', antisense 5'-CACCAG AGC ATG CCT GTC-3', covering bases 1,392–1,579, 188 bp (GenBank accession no. xm004814).

**Statistics.** Levels of significance between groups were calculated with the use of unpaired Student’s t-test and by ANOVA. P < 0.05 was considered significant.

**RESULTS**

**Effect of hypoxic hypoxia and functional anemia on AM mRNA in rat kidney regions.** The response of AM to hypoxia in rat kidney regions was determined. Induction of AM mRNA in the kidney cortex was relatively moderate (2.5-fold increase), and only significant after exposure to CO (Fig. 1). In the outer medulla, CO inhalation led to an increase of AM mRNA ~5.5 times above control. Normobaric hypoxia and CoCl\(_2\) also increased AM mRNA level significantly in the outer medulla (Fig. 1). In the inner medulla, AM mRNA was further increased by 0.1% CO compared with the outer medulla (Fig. 1). Hypoxic hypoxia and CoCl\(_2\) enhanced AM mRNA levels more strongly in the inner compared with the outer medulla (Fig. 1). In none of the experiments did the AM antisense riboprobe hybridize nonspecifically to yeast tRNA and the probe was completely digested in the absence of template mRNA (Fig. 1A, lane tRNA).

**Distribution of AM immunoreactivity in control and hypoxic rat kidneys.** To address cellular localization of AM, we applied a polyclonal rabbit anti-rat AM antibody to sections of perfusion-fixed control kidneys and kidneys obtained after CO admixture to the inspiratory air (0.1% for 6 h). The antibody was validated to be specific by ELISA. Thus binding of the AM antibody to rat AM peptide precoated wells was dose-dependently blocked by preincubating the antibody with increasing amounts of rat AM peptide (Fig. 2).

In the kidney cortex, significant labeling for AM was associated with distal convoluted tubules, connecting tubules and weakly with cortical collecting ducts (Fig. 2, A and C). In particular, the luminal aspect of the cells was strongly immunopositive. No immunopositive staining was noted in proximal
convoluted or straight tubules or in the cortical part of the thick ascending limb of the loop of Henle. Collecting ducts in the outer medulla showed modest but significant labeling, whereas IMCDs were strongly immunopositive for AM (Fig. 2B). The thin limbs of Henle’s loop were also positive for AM in the inner medulla. The epithelial cells lining the surface of the papilla showed distinct labeling for AM (Fig. 2B). In the absence of primary antibody, no immunolabeling was detected (Fig. 2E). In kidneys from rats treated with CO, there were no grossly discernible changes in the distribution of AM immunoreactive peptide, whereas labeling was more intense com-

Fig. 2. Localization of AM immunoreactive peptide and mRNA in rat kidney. A: in kidney cortex from control rats, AM immunolabeling was associated with distal nephron segments and cortical collecting ducts (CCD). B: in the inner medulla from a control rat, AM immunoreactivity was associated with the collecting ducts and thin limbs of Henle’s loop. The epithelium covering the papilla was positive for AM. C: in the absence of a counterstain, AM labeling was segment specific and predominantly observed in the apical part of the epithelium. D: in response to CO inhalation for 6 h, significant immunoreactivity for AM was associated with inner medullary (IM) collecting duct (IMCD), thin limbs of Henle’s loop, papillary epithelium, and interstitial cells. OMCD, outer MCD. Insets: AM-immunoreactivity in papillary epithelium and urothelium of the renal pelvic wall. E: in the absence of primary antibody no labeling was detected. E: ELISA showing specificity of AM antibody used for immunostaining. Wells were precoated with rat AM peptide. A dilution series of rat AM peptide was incubated with anti-rat AM antibody for 1.5 h. The mixture was then added to the wells and washed several times. Bound IgG was visualized by horseradish peroxidase-coupled secondary antibody. The y-axis shows the absorbance at 492 nm. Absorbance, and thereby AM antibody binding to AM-precoated wells, was concentration-dependently prevented by peptide preabsorption. F: PCR analysis of AM mRNA in microdissected rat collecting duct system. AM (223 bp), aquaporin-2 (AQP-2; 811 bp), and β-actin (194 bp) cDNAs were amplified by PCR for 32 cycles from template cDNA corresponding to 1 mm of collecting duct. In negative controls, a similar amount of template was amplified from RT reactions done in the absence of RT (−RT), or water was added to the mixture instead of cDNA (−cDNA). Amplification of 50 ng cDNA from rat inner medulla served as positive control. Molecular size marker: φX174/Hae III DNA fragments.

Fig. 3. Effect of ischemia on AM mRNA expression in rat kidney. Graphs display the quantitative evaluation of AM mRNA abundance in rat kidneys in response to ischemia. Open bars represent control animals and filled columns ischemic animals. In the occlusion-reperfusion (ORP) experiments, the left kidney was occluded and the right kidney was left untouched. Sham animals were anesthetized and surgically prepared without occlusion and AM expression was measured in both kidneys. AM mRNA level was determined by real time quantitative PCR in kidney regions in response to severe anemia for 6 h (A; n = 6), in whole kidney after occlusion for 1 h, followed by reperfusion for 6 h (B; n = 6) and 24 h (C; n = 6). OM, outer medulla. The results are GAPDH-normalized mean values ± SE. *P ≤ 0.05.
pared with control rat kidneys. Thus the most prominent labeling was associated with the inner medullary collecting ducts, thin limbs, and interstitial cells (Fig. 2D). Significant labeling was observed in epithelium covering the surface of the papilla and the pelvic wall (Fig. 2D, inset). To examine whether the AM gene was transcribed locally, we employed RT-PCR analysis of microdissected collecting duct segments from control rats. Significant AM expression was detected in all three segments investigated (Fig. 2). The collecting duct marker AQP-2 was expressed in all tested segments confirming correct identification during dissection (Fig. 2). β-Actin was amplified in all samples with a molecular size compatible with cDNA template and not genomic DNA (Fig. 2). In the absence of cDNA or of RT, no products were amplified. Thus the AM gene was expressed and translated along rat collecting ducts under control conditions.

**Effect of global transient ischemia and permanent localized ischemia on AM expression and distribution in rat kidney.** Next, we studied changes in AM expression in response to various maneuvers of ischemic hypoxia. After two consecutive bleedings, the rats lived for 6 h with severe anemia (hematocrit was reduced from 49.2 ± 2.2 in control rats to 21.4 ± 2.6). Anemia resulted in an 85.6-fold induction of erythropoietin mRNA in kidney (not shown). AM mRNA abundance was significantly elevated in all major regions and the largest relative increase was observed in the inner medulla (Fig. 3). Immunolabeling of anemic kidneys showed a labeling pattern that was similar to that observed after CO inhalation depicted in Fig. 2D (not shown). Subsequently, AM mRNA level was studied in kidneys where the renal artery was occluded for 1 h, followed by reperfusion for 6 and 24 h (occlusion-reperfusion). Reperfusion for 6 h after the global ischemic insult was associated with a significant stimulation of total kidney AM mRNA abundance, which subsided 24 h after the insult (Fig. 3). Sham manipulation of the renal artery did not change kidney AM expression after 6 and 24 h (Fig. 3). Kidneys that were labeled for AM 24 h after release of occlusion exhibited marked tubular damage, particularly in the outer medulla where tubular segments were necrotic; epithelial cells were detached, nuclei were pyknotic, there was interstitial edema and tubular casts, and cells had lost signs of differentiation (Fig. 4, A and B). Detached cells were not labeled for AM 24 h after the injury, whereas other tubules that had maintained integrity were AM positive (Fig. 4A). In the absence of primary antibody, no labeling was observed (Fig. 4B). Thus there seems to be a differential sensitivity of nephron segments and collecting ducts to transient ischemia, which is reflected in differential expression of AM.

Next, the effect of permanent localized ischemia, namely infarct, on renal AM was assessed. A segmental artery was occluded and kidneys were perfusion fixed after 6 and 24 h and then labeled for AM. Tissue AM mRNA level was not determined in this model because of the heterogeneity in PO₂ across the tissue. Occlusion of a segmental artery resulted in a wedge-shaped necrotic area with the base at the capsular surface. Microscopically, there was a sharp border between

Fig. 4. Immunohistochemical labeling of ischemic rat kidney for AM. A: rat kidney after 1-h occlusion and 24 h of reperfusion labeled for AM. B: section from same kidney as in A reacted in the absence of primary antibody. C: rat kidney stained for AM 24 h after total occlusion of a segment artery. Micrograph displays the border zone between infarct and viable tissue. AM labeling was observed particularly in the infarct border zone and adjacent to kidney capsule. Bar = 200 μm. D: high-power view of the infarct border zone in a rat kidney 24 h after infarct. AM immunoreactivity was seen in few tubular cells below the capsule, in capsular cells and was associated with all cells in the adjacent parenchyme. E: section of rat kidney medulla 24 h after infarct stained for AM. Subsets of parallel tubules and collecting ducts were positive for AM in a restricted part of medulla and no obvious necrotic cells were observed. Bars in A, B, D, and E = 50 μm.
normal and infarcted tissue characterized by hyperemia and a progressive loss of tissue architecture in the infarct area, most prominent after 24 h (Fig. 4C). There was intense immunoreactivity for AM associated with the vast majority of tubular and vascular cells in the border region and labeling intensity gradually waned with distance from the infarct (Fig. 4, C and D). At sites distant from the infarct, AM localization and staining intensity was not different from that observed in control kidney (Fig. 4C). Of note, a thin rim of strongly AM-positive cells was found in and just underneath the renal capsule over the infarcted area (Fig. 4D). Often, the outermost part of a tubule was AM positive, whereas cells deeper within the parenchyme in the same tubule were necrotic (Fig. 4D). This pattern of distribution probably reflects a limited oxygen supply from capillary capillaries sufficient to sustain survival of adjacent cells. In the medulla, large necrotic areas were not observed, but cone-shaped assemblies of strongly AM-immunopositive tubules and collecting ducts were typically encountered (Fig. 4E).

Effect of hypoxia on AM expression in primary cultures of rat IMCD cells. In five separate cultures of IMCD cells, AM expression was detected after four days of primary culture (Fig. 5). The differentiation marker AQP-2 was expressed in the cells during culture (Fig. 5). Real-time PCR-analysis showed that AM and β-actin mRNA abundances did not change with time in the control situation, but hypoxia (1% ambient oxygen) significantly increased AM mRNA levels after 2, 4, and 6 h (Fig. 5) (n = 3 experiments; 5 rats per experiment and 3 wells per condition in each experiment).

Cellular localization of AM in human kidney control tissue and in tissue from patients with arterial stenosis and renovascular hypertension. To explore clinical correlates of experimental renal hypoxia, we employed immunohistochemical labeling of human kidney tissue for AM. In the control kidney, immunoreactivity for AM was associated with distal convoluted tubules, the parietal layer of Bowman’s capsule, and collecting ducts (Fig. 6A). With the use of nonimmune serum, no labeling was noted (Fig. 6B). Immunoreactivity was not associated with glomerular capillaries (Fig. 6, A), arteries and arterioles (Fig. 6C) or proximal convoluted tubules (Fig. 6A). In the medulla, collecting ducts were strongly positive for AM, and thin limbs of Henle’s loop were also labeled (Fig. 6D). The ischemic kidneys (Fig. 6, E–H) displayed a wide spectrum of pathological changes: glomerular atrophy (Fig. 5, E and F), glomerular sclerosis (Fig. 6, E and F), thickened Bowman’s capsule (Fig. 5F), tubular atrophy (Fig. 6, E–H) and vascular medial and intimal thickening and some arteriolar hyalinosis (Fig. 6, F and G). The major part of tubules and vessels were positive for AM (Fig. 6, E, F, and H). Glomeruli displayed a variable degree of intraglomerular labeling for AM (Fig. 6F). AM labeling was associated with smooth muscle in the media of arterioles and arteries (Fig. 6, E–G). Vascular smooth muscle cells were negative for AM in control kidneys (Fig. 6C). In the medulla, the inner medullary collecting ducts were positive for AM (Fig. 5H).

Effect of renal artery stenosis on human renal AM mRNA. Subsequently, we analyzed AM transcript abundance in cortex and outer medulla from four control kidneys and compared the level with four stenotic kidneys. First, a test of the human AM probe specificity showed that the assay was linear at least in the range of 5–20 µg of target total RNA from human kidney and that only a single hybridization product resulted with the expected molecular size (Fig. 7, top). Nonspecific hybridization with 20 µg of yeast tRNA was not observed and in the absence of template complete degradation of the probe was seen (Fig. 7, top). In subsequent assays we used 20 µg total RNA from patient samples for the AM assay and 5 µg for determination of β-actin. β-Actin mRNA abundance was significantly higher in the cortical stenotic tissue compared with controls (1,348 ± 112 and 714 ± 107 cpm; Fig. 7). We have no explanation for this effect, but a nonspecific decrease of RNA quality in the stenotic tissue can be excluded to contribute to the results. There were no differences between β-actin abundance in control and stenotic medullary tissue. By direct comparison of nonnormalized AM mRNA abundance, we did not detect any statistically significant difference in AM mRNA level between control kidneys and kidneys with arterial stenosis (Fig. 7, bottom). If corrected for β-actin expression, AM mRNA level was significantly decreased in both cortex (control 20 ± 3 vs. RVH 3 ± 1) and medulla (control 21 ± 4 vs. renovascular hypertension 6 ± 2 arbitrary units) of the stenotic kidneys. Thus it is safe to conclude that AM mRNA level was not increased by arterial stenosis in end-stage human kidney cortex and medulla.

Fig. 5. Effect of hypoxia on AM expression in primary cultures of IMCD. A: PCR amplification of rat AM and AQP-2 using template cDNA from cultured rat IMCD cells. Total RNA (50 ng) obtained at day 4 of culture from five separate preparations of IMCD cells was amplified. Total RNA from rat aorta was amplified as a positive control for AM. Molecular size marker: φX174/Hae III DNA fragments. B: AM expression in primary cultures of IMCD cells that were incubated at 21% oxygen (control) and 1% oxygen (hypoxia) for 2, 4, and 6 h. For TaqMan quantification, three independent preparations of IMCD cells were analyzed, each of them composed of medullas of five animals. In every preparation, three different dishes per condition and per time point were assayed. Data are given as relative expression (rE) of AM compared with β-actin in arbitrary units. *P ≤ 0.05.
DISCUSSION

We undertook the present study to examine whether there were regional differences in the regulation of AM by hypoxia in the rat and human kidney. At ambient $P_O^2$, AM expression was detected in the cortex and medulla and AM immunoreactivity was localized in distal nephron segments and collecting duct system, both in the rat and human kidney. In vivo, the applied models of inspiratory, anemic, and ischemic hypoxia led to differential increases of AM mRNA expression in rat kidney in keeping with expected oxygen gradients. In vitro, hypoxia enhanced AM expression in cultured rat IMCD cells. Although AM immunoreactive staining was marked both in tubules and vessels, AM mRNA abundance was not significantly elevated in human kidneys with arterial stenosis in either cortex or medulla.

Constitutive expression of AM mRNA and peptide in the kidney has been shown in several species, including the rat (31, 32) and dog (15), and AM has been detected in distal nephron segments and collecting ducts. Previous data from the human kidney have shown AM mRNA and peptide in tissue homogenates (12, 18, 19) and AM localization in collecting ducts (2). The present data showed a constitutive and equal expression of AM in normal human kidney cortex and medulla. Irrespective of the known differences in $P_O^2$, AM immunoreactivity was detected in cortical distal tubules and collecting ducts in all zones of human kidney, similar to rat kidney, which supports a conserved localization of AM in the kidney across mammalian species.

A low tissue $P_O^2$ is a well established stimulus for AM expression in whole kidneys and cultured renal cells (10, 22).
Our data suggest that in response to hypoxic or ischemic hypoxia the rather uniform distribution of AM in the rat kidney (14) was changed in keeping with oxygen gradients; e.g., marked positive staining in the inner medulla in response to hypoxic and anemic hypoxia and a marked increase of AM in the infarct border zone. Most of the cells in the border zone were positive for AM, suggesting an intrinsic capability of most renal cells to respond to hypoxia by induction of AM. In the model of transient ischemia, tubular damage and AM immunoreactivity was found predominantly in the outer medulla. This could reflect a more rapid and longer-lasting decrease of local \( P_{O2} \) during transient ischemia in this zone. In support of this assumption, there is a relatively high density of thick ascending limbs, which are metabolically very active in this region. Although the inner medulla is normally at the limit of hypoxia, it apparently better tolerates transient ischemic hypoxia (6, 36). Both in the infarct model and occlusion-reperfusion model, the inner medulla did not display overt necrosis but did stain strongly for AM. This observation probably reflects both a lower metabolic rate and a more developed collateral blood supply in the inner medulla compared with outer medulla.

A hypoxia-induced gradient of distribution within the kidney has been reported also for the oxygen-dependent hypoxia-inducible factor-1 (HIF-1) (30). HIF-1\( \alpha \) and -\( \alpha \) mRNA expression was limited to the inner medulla under normoxic conditions (36), and was significantly increased by hypoxia and anemia in inner medullary collecting duct, thin limbs, and interstitial cells (30). The present data on AM from cultured IMCD cells, and previous data on HIF (36), collectively show that the cells are directly sensitive to ambient oxygen independent of nervous activity, circulating hormones or osmolarity. On the basis of the significant co-localization and co-regulation of HIF-1 and AM by hypoxia in the kidney medulla, it is feasible that AM transcription is controlled by HIF-1 also at this site.

Several renal cell types, such as mesangial cells, Madin-Darby canine kidney cells, and renovascular smooth muscle cells display hypoxia-mediated AM induction in vitro (22). We did not observe AM in smooth muscle or in mesangial cells in rat kidneys, but in the human kidneys with arterial stenosis both mesangial cells, vascular smooth muscle cells and endothelial cells were positive for AM. In addition, the majority of tubules, in particular, the medullary collecting ducts, were positive for AM in these kidneys in contrast to a more restricted localization in control tissue. AM is elevated in plasma both in rats and humans with malignant hypertension and vascular damage; AM has also been detected in kidney medullary collecting ducts in this condition (27). We did not find any difference in AM mRNA level between human control kidney and kidneys with potential hypoxia due to arterial stenosis. The control tissue was selected from patients that received no chronic medication before nephrectomy and although the underlying disease, age, and gender differed, AM expression showed little variation between individual kidneys. It is not possible to determine the degree, if any, of hypoxia in the stenotic kidneys. In the present cases, the arterial stenosis was probably a chronic, long-lasting condition severe enough to provoke intractable hypertension and macroscopic atrophy of the kidney. The tissue was not necrotic and blood flow must have been sufficiently autoregulated to sustain medullary \( P_{O2} \) above the level necessary to enhance AM mRNA levels. On the basis of the present data, we cannot exclude that AM expression is also stimulated by hypoxia in the human kidney but the present and previous animal studies indeed suggest that severe ischemic hypoxia is necessary to induce AM in kidney and other organs (26, 28, 36). As to the physiological role of AM in the hypoxic kidney, we previously reported that AM lowers renal vascular resistance (13) and other reports have
shown that exogenous AM increases medullary blood flow and induces diuresis and natriuresis (5, 15, 20). In accordance, a higher renal vascular resistance and larger susceptibility to ischemic renal damage has been noted in genetically altered mice with constitutively lower plasma AM (25). AM gene delivery has beneficial effects on vessels, heart, and kidney in various hypertensive animal models with renal damage (4, 37). Together, these data show that hypoxia and ischemic hypoxia stimulate AM in the rat kidney according to expected gradients of tissue hypoxia. AM may contribute to protective paracrine mechanisms that sustain or increase medullary blood flow and lower epithelial oxygen demand.

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