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Hypoxic Stress-Enhanced Expression and Release of Adrenomedullin (AM) and Up-Regulated AM Receptors, while Glucose Starvation Reduced AM Expression and Release and Down-Regulated AM Receptors in Monkey Renal Cells

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Summary

The proliferative peptide adrenomedullin (AM) has a wide distribution in a variety of tissues and cells. The mechanism how the AM gene is regulated in cells is not yet known. The renal cortex, renal vascular smooth muscles, glomeruli and tubular epithelial cells are very sensitive to hypoxia. Renal hypoxia produces acute renal tubular necrosis and markedly induces AM expression in damaged cells. However, little information is available regarding the possible pathophysiological production and release of renal tubular AM. Regulation of membrane-bound AM receptors in renal cells has not yet been systematically studied. To elucidate the potential pathological role of human AM we examined the production and release of AM, as well as the characteristics of surface membrane AM receptors in cultured monkey renal tubular epithelial cells (RC) exposed to hypoxia, induced with endothelin-1, and subjected to glucose deprivation. Exposure of RC to hypoxia (1 % O_2, 5 % CO_2 in N_2), and to phorbol 12-myristate 13-acetate (PMA) increased production and secretion of AM and increased specific [^{125}I]AM binding on RC. Metabolic stress (1 % glucose in the cultivation medium) and preincubation of RC with rival peptide endothelin-1 significantly reduced immunoreactive-AM in a conditioned medium and whole cell surface membrane AM binding on RC. Altogether, our data suggest that the AM is involved in the adaptation of renal tubular cells to hypoxia. Increased expression of AM mRNA and regulation of AM receptors in metabolic stress may function as an important autocrine/paracrine regulator(s) of renal tubular epithelial cells.

Key words

Adrenomedullin expression and release • Ligand binding studies • Renal tubular epithelial cells • Radioimmunoassays • Hypoxia • Glucose repression

Introduction

As an example of the oxygen-regulated gene located in the 5´ flanking region a few considerable binding sites important for binding of regulatory proteins and nuclear factors (Ishimitsu *et al.* 1994), adrenomedullin (AM) is probably a more significant contributor to human pathology than had previously been expected. Recently, AM expression was shown to increase markedly in kidney with arterial stenosis (Sandner *et al.* 2004). Hypoxia was reported to stimulate AM production in renal mesangial cells (Nagata *et al.* 1999). In renal carcinoma, an overexpression of AM was shown to be directly involved in the regulation of angiogenesis (Wang *et al.* 2001). Experimental AM gene delivery attenuated renal damage and cardiac hypertrophy in Goldblatt hypertensive animals (Fujita 2005). AM expression cells was strongly induced by reduced oxygen tension. A loss of polarization of tubular epithelial cells with their detachment is known as an early response to acute renal ischemia and renal tubular necrosis (Fujigaki *et al.* 2003). The overexpression of AM in renal tubular cells may, under appropriate conditions, also protect renal tubuli in renal ischemia. However, the role underlying autocrine/paracrine AM mechanism(s) in renal cells remains undefined. In tubular cells, the production and secretion of AM in response to hypoxia may be adaptive or inflammatory. Either activation or disruption of AM signaling may significantly contribute to renal cell pathology. In earlier studies we reported significant Ca^{2+} signaling, the counteractive β-adrenergic and endothelin subtype-ET_A/subtype-ET_B receptor signaling in RC (Dřímal 1989, 1994, Dřímal and Boháčik 1994, Dřímal and Koprda 1996, Dřímal *et al.* 2000). In order to justify the use of these cells also for adrenomedullin study, we showed satisfactory expression of AM in RC in preliminary studies. We therefore used hypoxia and metabolic stress in cultured monkey distal (convoluted tubuli) renal tubular epithelial cells (RC) as a suitable *in vitro* model mimicking regulations surface membrane AM receptors on hypoxic and metabolically stressed cells.

Methods

African Green Monkey Renal Tubular Epithelial Cell lines (RC) were kindly provided by Drs. S. and J. Pastorek (Virological Institute of the Slovak Academy of Sciences). Unless otherwise specified, cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM), 4.5 g/l of D-glucose, supplemented with 10 % fetal blood serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and non-essential amino acids (0.1 mmol). RC were maintained at 37 °C in a humidified atmosphere under normoxic (5 % CO_2 in air) and hypoxic (24 h, 1 % O_2 balanced with N_2 and 5 % CO_2) conditions. Some experiments were performed with cells synchronized by the addition of growth factors to cells made quiescent by incubation with low serum. RCs were trypsinized and plated (2 x 10^5 cells) into 6-well tissue culture clusters (Costar, Cambridge, MA). To produce actively growing cells subconfluent populations of RC were then stimulated to proliferate by feeding with 2 ml/well of a medium containing 10 % of serum and 0.1 μCi/ml [^{3}H]thymidine (21.0 Ci/mmol, Amersham Biosciences). To produce quiescent cells, subconfluent populations were changed to -ree medium with 0.1 % FBS. A set of cultures (as described above) was changed to serum-free medium, washed in HEPES-buffered physiological salt solution (Buffer I, PSS in mmol/l): NaCl 135, KCl 4.2, $CaCl_2$ 1.25, $MgCl_2$ 1.0, KH_2PO_4 0.44, NaH_2PO_4 0.34, $NaHCO_3$ 2.6, HEPES 20.0, glucose 5.5 containing protease inhibitors (5 μg/ml pepstatin-A, and 0.1 mmol/l phenylmethylsulfonylfluoride, 0.01 mmol/l phosphorami-don, pH 7.4) and preincubated with pharmacologically active substances, as indicated. Twenty-four hours later, the cultivation medium and RC were collected for radioimmunoassay, radioreceptor assays and activation analysis.

Radioimmunoassays of human adrenomedullin

AM present in the culture media was extracted by using Sep-Pak C_{18} cartridges (Waters, Milford, MA, U.S.A), eluted with Buffer II (60 % acetonitrile, HPLC grade, in 1 % trifluoracetic acid and the extract was reconstituted with RIA-Buffer (Buffer III,0.05 mol/l sodium phosphate,pH 7.4, 1 % bovine serum albumin, 0.5 % Triton X100, 0.08 mol/l NaCl, 0.025 mol/l EDTA, 0.05 % NaN_3). Commercial Competitive AM-RIA Kit (Penninsula Laboratories, Belmont, CA, U.S.A) and General Protocol with two overnight incubations were used.

Adrenomedullin mRNA assays

Reverse transcription-polymerase reaction (RT-PCR) testing of RC cells for AM mRNA. The total RNA was extracted from RC after disrupting the specimens using an Ultra-Turrax homogenizer (Janke and Kunkel,

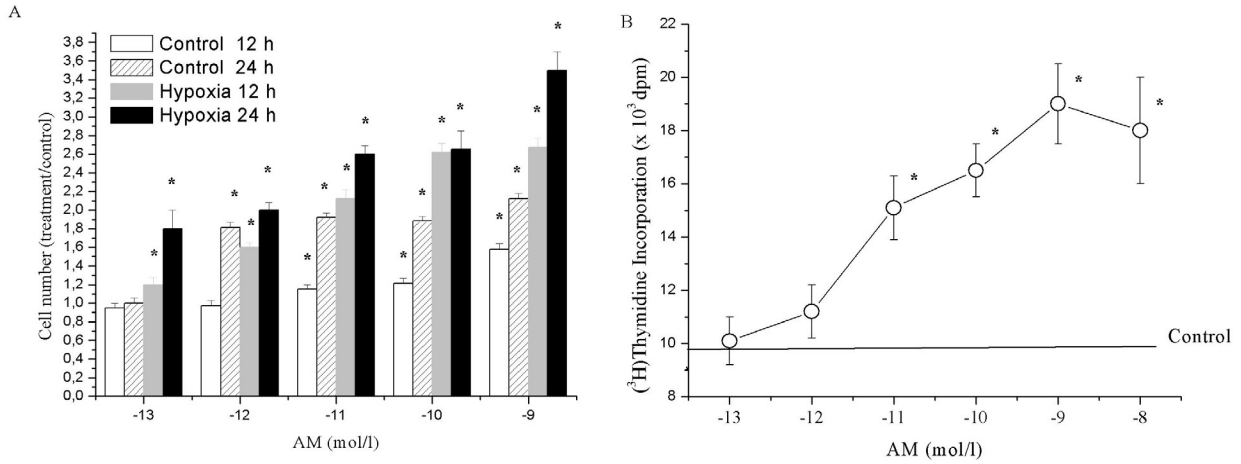

Fig. 1A Stimulation of proliferation of renal tubular cells (RC) by human adreno-medullin(1-52), (AM). The increase in cell number was measured in two intervals (12 and 24 h) under normoxic (empty and sparse-lined columns) and hypoxic (1 % O_2, 5 % CO_2 in N_2) conditions (hypoxia is represented by light-gray and black columns) in DMEM without fetal calf serum, supplemented with the indicated concentration (mol/l) of AM. Pepstatin-A (5 µg/ml), phenylmethylsulfonyl fluoride (10^{-4} mol/l) and aprotinin (0.4 µmol/l) added to the medium had no effect on the number of cells (n=40). Values are mean ± SEM. * Significant change when compared to control ($P<0.05$). **1B** Effects of human AM on (^{3}H)-thymidine incorporation in RC cultured in DMEM supplemented with 10 % fetal calf serum (n=12). AM significantly increased the amount of incorporated (^{3}H)-thymidine (24 h), * Significant change ($P<0.05$).

Staufen, Germany). The total RNA was reverse-transcribed with PCR primers designed to amplify the cDNA seqences coding for human AM (upstream primer, 5-AAGAAGTGGAATA-AGTGGGCT-3 downstream primer, 5-TCGGGCCTCCGAAACC-ATGA using the method described by Fujita *et al.* (2002). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with the presence of [α-^{32}P] deoxycytidine triphosphate using a Gene-Amp Kit (Roche, Branchburg, NJ, USA). Human AM was obtained from Bachem AG Bubendorf (Switzerland). The levels of AM mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which served as an internal control. The expression level of AM of the RC line under hypoxic conditions was indicated as the ratio of the hypoxic and normoxic sample.

Cell proliferation and viability

Stable RC subcultures were seeded in a 96-well plate at $5x10^3$ cells per cm^2 and cultured for 7 days. [^{3}H]thymidine uptake was used as an indicator of cell proliferation. Cell counts and thymidine incorporation were performed both in actively growing and quiescent cell populations. Cell viability was measured by [3-(4-5-dimethylthiazol-2yl)-5-βcarboxy-methoxyphenyl)-2-(4-sulphoxyphenyl-2H tetrazolium]-dye reduction assays. RC were inoculated into 24-well plates (Costar Cambridge) and cultured in a medium supplemented with 0.1 % serum for 48 h. Then cells were removed and fixed in 60 % aqueous methanol at - 20 °C overnight. Then they were washed with 97 % ethanol, air dried. Viability of cells was assessed by Trypan Blue Dye exclusion test.

Ligand binding studies

Grown, confluent cultures of RC were used in saturation studies. Whole-cell binding assays were performed (measurements in triplicate) at room temperature in HEPES-buffered physiological salt solution. RC were incubated for 60 min with ten increasing concentrations of human ([^{125}I]AM, (from 0.9 to 2.9 nmol/l) and nonspecific binding was determined in the presence of 10^{-6} mol/l of unlabeled human AM. At the end of the incubation period, cells were separated by rapid filtration on Wathman GF/C glass fiber filters, washed three times with 1.5 ml of cold assay buffer, then filters were collected in 10 ml scintillation cocktail (Bray) and counted on 2500 TR Liquid Scintillation Analyzer (Tricarb, Packard) at 65 % efficiency. Protein concentration was measured according to Bradford (1976).

Data analysis

Statistical analysis was performed by using Inplot, Ligand and Origin Packs. All data are expressed as the mean ± S.E.M., differences and correlations were considered significant at a value of $P<0.05$).

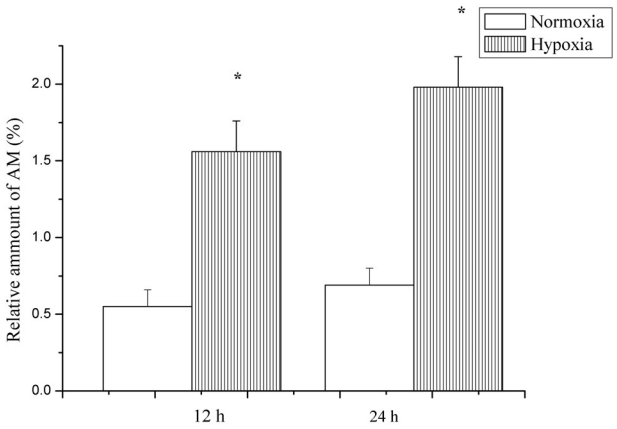

Fig. 2. Radioimmunoassays of immunoreactive AM in the conditioned media of RC cells cultured under normoxia (control) and hypoxia (1 % O_2, 5 % CO_2 in N_2), incubated (12 and 24 h) in DMEM. *Significant increase (P<0.01) in accumulated immunoreactive AM in cultured media of hypoxic RC. Values are mean ± S.E.M.

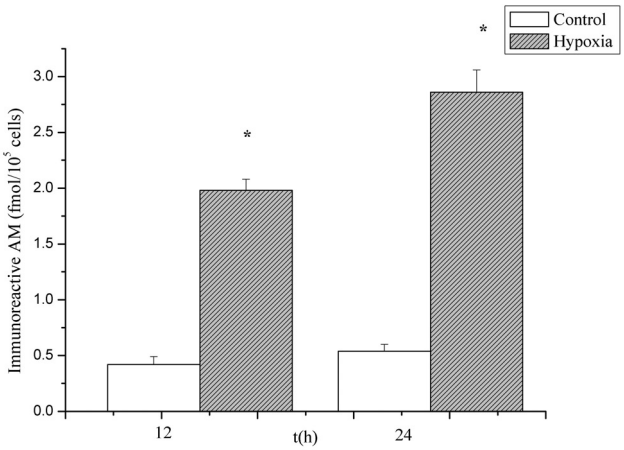

Fig. 3. Change of AM mRNA in normoxic and hypoxic renal tubular cells. AM mRNA was analyzed by AM reverse transcription-PCR; (RT-PCR) and was elevated by threefold. *Statistical significance (P<0.05) (n=5). Values are mean ± SEM.

Chemicals

Human $AM_{(1-52)}$, (Calbiochem), $AM_{(22-52)}$, (Bachem), Amprep Minicolumns (Amersham Pharmacia Biotech), angiotensin-II (Calbiochem), BAY 11-7082 (Calbiochem), human endothelin-1 (Sigma), lipopolysaccharide (Sigma), L-NAME (Calbiochem), 13-O-tetrahydro-decanoyl-phorbol-13-acetate (Serva), Sep-Pack C_{18} Cartridges (Tumor Necrosis Factor-α, Bachem), vinblastine (Calbiochem).

Radiochemicals

Human [^{125}I]AM (Amersham), human [^{125}I]AM-Radioimmunoassay Kit (Peninsula Lab.Inc., Belmont, Ca. U.S.A.), [^{3}H]thymidine (Amersham).

Results

Two different, but complementary, experimental designs were used to assess the effect of human AM on proliferation (Fig. lA). In the first group of experiments (Control), AM was added to normoxic subconfluent cultures of RC and its effect on saturation was measured by determination of cell numbers per dish. AM in the concentration 10^{-11} mol/l significantly increased cell numbers after 12 and 24 h incubation periods. After 24 h of incubation with the concentration 10^{-9} mol/l of AM, cell numbers almost doubled. When RC were made hypoxic by incubation for 12 h and 24 h in DMEM with 1 % of oxygen, the concentration 10^{-13} mol/l of AM significantly increased cell numbers, and the concentration 10^{-11} mol/l of AM doubled the cell numbers. The concentration of 10^{-11} mol/l of AM significantly increased [^{3}H]thymidine incorporation after 24-h incubation (Fig. 1B).

AM production in RC and immunoreactive AM in media of renal cells

As shown in Figure 2 immunoreactive AM accumulated in culture media of control RC up to 24 h. Under hypoxic conditions (1 % of O_2) for 12 and 24 h (Fig. 3), the respective AM levels in the medium of RC were 3.8-5.7 fold higher than with normoxia (P<0.01). RNA of satisfactory quality was obtained from five experiments with monkey RC. Renal tubular cell lines in the present study expressed low basal levels of AM. AM mRNA expression was found markedly increased (3-fold increase in Fig. 3) in hypoxic RC. Preincubation with actinomycin-D (1.0 μg/ml) significantly lowered AM mRNA expression (–42±19 %, P<0.05) in hypoxic RC line in the present study.

Whole cell saturation binding experiments with [^{125}I]AM in RC

Specific binding of [^{125}I]AM was measured as a function of ligand concentration in polarized RC. Aliquots of RC were incubated with ten gradually increasing concentrations of [^{125}I]AM for 60 min. The course and characteristics of saturation curves in our control experiments confirmed the presence of a single class of binding sites with high affinity (K_D = 0.82±0.15 nmol/l and B_{max} = 2.37±0.16 fmol/mg of protein, Fig. 3).

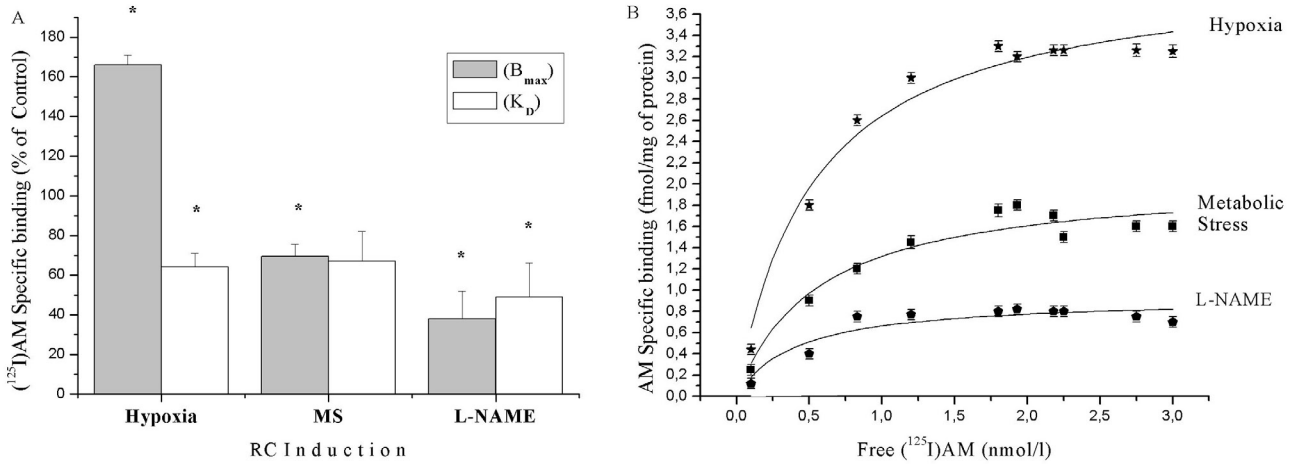

Fig. 4A. Characteristics of specific human [^{125}I]AM$_{(1-52)}$ binding: total number of specific binding sites (B_{max}) and the corresponding dissociation constant (K_D). Both binding parameters were identified on RC exposed to: 1) human mitogen and presumably concurrent paracrine hormone, peptide endothelin-1 (ET-1; 0.1 µmol/l), 2) cytotoxic cytokine tumor necrosis factor-α (TNF-α 50 pg/ml), and 3) phorbol-12-myristate-13-acetate (PMA; 1.0 µmol/l). Values are mean ± S.E.M. * Significant difference when compared with control ($P<0.01$). **4B.** Representative whole cell saturation binding isotherms of human [^{125}I]AM$_{(1-52)}$ on control cultured RC and RC exposed to endothelin-1 (ET-1, 0.1µmol/l), or phorbol-12-myristate-13-acetate (PMA, 1.0 µmol/l). Range of free ligand employed was in low nmolar concentration (0.15-3.0 nmol/l). Incubation was performed in the absence or in the presence of 10 µmol/l of human AM$_{(1-52)}$. Values are mean ± S.E.M.

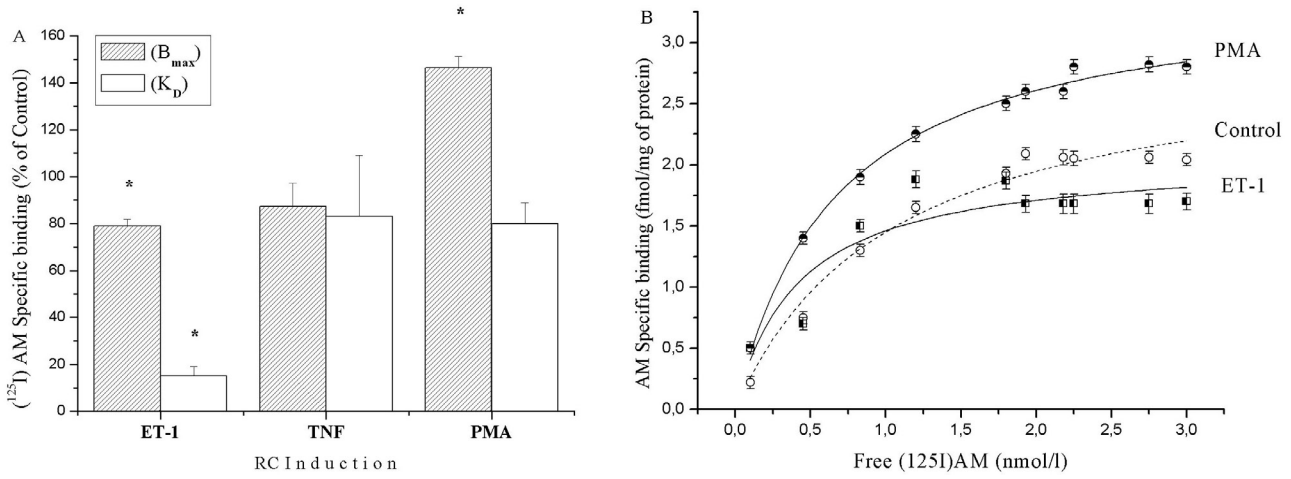

Fig. 5A. Representative characteristics of [^{125}I]AM specific binding (B_{max} and K_D), (both expressed in percentages of control) on RC subjected to: 1) hypoxia (24 h), 2) metabolic stress (MS; 1 % of glucose in the cultivation medium), and 3) competitive inhibitor of NO-synthase L-NAME. Note significant increase in the total number of [^{125}I]AM specific binding sites identified on RC subjected to hypoxia at reduced K_D in hypoxia, and significantly reduced specific binding sites of [^{125}I]AM identified on RC subjected to metabolic stress and to L-NAME. **5B.** Representative whole cell saturation binding isotherms of human [^{125}I]AM$_{(1-52)}$ on cultured RC subjected to: 1) hypoxia (24 h, 1 % O_2, 5 % CO_2 in N_2), 2) metabolic stress (24 h) induced with 1 % of glucose in the cultivation medium), or incubated (24 h) with the inhibitor of NO-synthase N^G–nitro-L-arginine-methylester hydrochloride (L-NAME) 0.4 µmol/l). The range of free radioligand employed was in low nmolar concentration (0.15-3.0 nmol/l). Values are mean ± SEM. Other explanation and symbols as in Fig. 4.

The Hill plot of the specific binding data (not listed) and Hill coefficient (n_H = 1.0) indicated the existence of a homogenous group of sites on the surface of RC. As shown in Figure 4 the total specific whole cell [^{125}I] AM binding increased significantly in cells preincubated with phorbol ester. Hypoxia significantly increased surface membrane specific AM binding in RC (Fig. 5). Metabolic stress induced by glucose deprivation (24 h) and L-NAME preincubation significantly reduced AM specific binding. The maximal amount of total cell-associated specific binding was significantly reduced after preincubation with ET-1 and remained unchanged after preincubation of RC with human recombinant tumor necrosis factor-α (50 pg/ml). Intracellular accumulation of AM was assessed after 120 min incubation by determining cell-associated [^{125}I]AM following an acid

wash, as previously described by Gansler *et al.* (1986) for other ligands and other cell lines. The experiments with acid wash to remove the surface-bound [^{125}I]AM showed that after 120 min of incubation approximately 42±16 % of activity was already retained in RC.

Discussion

We investigated the expression and secretion of the regulatory peptide adrenomedullin and the regulation of surface membrane AM receptors in cultured renal tubular epithelial cells. Recent reports in the literature showed a high degree of homology between monkey AM and human AM, which renders the monkey an attractive model for pharmacological AM targeting (Zudaire *et al.* 2004). As mentioned above, monkey AM is almost identical with its human ortholog. This along with other findings allowed us to use it reliably in radioreceptor studies in man $AM_{(1-52)}$ and also the antibodies raised against human AM to detect AM and AM receptors *in vitro* in monkey RC lines. In order to minimize the risk of side effects related to the variability of indefinite phases of the cell cycle, we used cell synchronization in our study, i.e. an approach with timed addition of growth factors that enabled us to manipulate the cell cycle. Hypoxia and preincubation of RC with PMA increased and metabolic stress, peptides angiotensin II and endothelin-1 and NO-synthase inhibition significantly reduced the specific AM binding to RC. Vinblastine and actinomycin-D induced apoptosis and inhibited the AM binding. Little is known about AM gene expression and secretion of AM in renal cells. Evidence has accumulated showing that AM is more or less abundantly expressed in human tumor cells (Takahashi *et al.* 2002). In human renal cell carcinoma, a positive correlation was observed between AM mRNA expression and microvessel count (Wagner 2001). In normal human vascular smooth muscle preparation (glomerular mesangial cells) gene expression for AM was low and forskolin was without effect (Nagata *et al.* 1999), in astrocytes human AM mRNA expression showed only a small increase with interferon-γ (Takahashi *et al.* 2000) and in hypoxic canine kidney cells (Madin-Darby cell line) the expression of AM increased twofold (Nagata *et al.* 1999). In the present study, we observed that the monkey RC line was much more responsive to human AM under hypoxic conditions. Hypoxia of RC significantly increased immunoreactive AM in the conditioned media from 3.8-5.7 fold. Furthermore, hypoxia and preincubation with phorbol ester were the most potent inducers of gene expression. The present study clearly showed that the hypoxia in combination with exogenous human AM (in the concentration as low as 10^{-13} mol/l) significantly increased proliferation and induced AM gene expression. Consistent with previous reports on human tumor cell lines (Lai *et al.* 1998), we also demonstrated that AM mRNA induction was paralleled by delayed secretion of AM peptide from the cells to the conditioned media. The reduced ability of hypoxia to increase AM mRNA expression in our experiments with actinomycin could possibly mean that AM expression is also partially regulated at the transcriptional level.

Our results concerning the effects of hypoxia on AM mRNA expression in RC were also highly congruent with the gene expression of other human tumorigenic cells exposed to low oxygen, as reported by Garaoya *et al.* (2000). However, the change in the AM gene expression was less intensive than in hypoxic human hepatocytes (from 6.2 to 35, with a 15-fold geometric mean reported by Miller *et al.* (1996), but higher than that reported for canine kidney cells (Nagata *et al.* 1999). The average expression of AM mRNA in hypoxic RC of the present study increased 2.8±0.1 times. A complementary approach to the total number of specific [^{125}I]AM binding sites and to the affinity of AM for its receptors in our study (two representative characteristics of the whole cell [^{125}I]AM binding on RC) disclosed a significantly increased total number of AM binding sites (B_{max} of AM) identified on hypoxic and phorbol ester-exposed cells (155±5 and 143±4 %, respectively), due to the increased affinity of AM for AM receptors on RC (significant reduction of numeric values of K_D to 64.3±7 % and 80.4±9 % of its control value, $P<0.05$). Furthermore, this study showed that all three procedures: 1) exposure of RC to the maladaptive peptide endothelin-1 (rival peptide of AM, inducing generation of reactive oxygen species in cells, activation of nuclear factors and mitogenesis), 2) blockade of NO-synthase in RC with L-NAME, and also 3) exposure of RC to metabolic stress (deprivation of glucose in medium) significantly reduced the total number of surface membrane AM receptors on RC. Future studies should show whether the accompanying reduction in numeric values of K_D in our experiments with metabolically stressed cells were only reflections of certain redistribution of AM binding or whether they reflected a true change in the quality of surface membrane AM binding sites identified on RC. Our findings on the sensitivity of RC to hypoxia and AM are

additionally interesting as RC produce and secrete AM. When triggered to differentiation by PMA, in contrast to endothelial cells, RC are probably themselves an excellent target for AM. Our data showed that under normoxic conditions native RC were more resistant to AM than RC exposed to hypoxia. Hypoxic RC responded to addition of AM after some delay (24 h in the present study) by proliferation and expression of high levels of AM mRNA. AM was also found to be most probably metabolized in proximal tubules (Sonna *et al.* 2003), making the interpretation of results obtained in RC even more complicated. Only one of other studies on AM metabolism suggested that RC, which are particularly rich in neutral endopeptidase, are not supposed to metabolize AM (Lewis *et al.* 1997).

Our results may have several important implications: 1) human AM is a true survival factor for hypoxic renal cells, important in regulation of most renal cells and suppression of cytotoxic functions, 2) AM over-expression in hypoxic renal tubular cells may increase levels of secretion of other biologically active proteins involved in signal transduction, and 3) increase in expression of maladaptive proteins may increase oncogenic signal transduction in renal cells.

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Reprint requests

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