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Human proximal tubular cell responses to angiotensin II analyzed using DNA microarray

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Abstract

Angiotensin II has been shown to exert complex effects on proximal tubular cell function and growth. To assess some of the direct effects on proximal tubular cells, changes in gene expression of selected cellular pathways were determined after exposure to angiotensin II. We used DNA microarrays to analyze multiple gene expression responses to increasing angiotensin II concentrations. Human proximal tubular cells were grown in flasks, and the presence of angiotensin type 1 receptor was confirmed by Western blot analysis. At passages 4–6, these cells were exposed to angiotensin II and harvested 4 h later and mRNA of the cells was extracted; 2 μg of mRNA was fluorescently conjugated for cDNA microarray hybridization. A custom-made DNA microarray was designed by selecting 300 human genes from 10 different functional systems and amplifying clones using polymerase chain reaction. Cells were subjected to 10 and 100 nM angiotensin II with paired untreated cells as controls. RNA was isolated, reverse transcribed, labeled and hybridized to the arrays and the ratios calculated. Ratios of ≥ 2.0 and ≤ 0.5 were considered significant. Coordinated changes were observed in genes of the hepatocyte nuclear factor 3 family (NHF3; HNF3A, HNF3B and HNF3G), in the E2F genes (E2F1, E2F3) and the interferon regulatory factors IRF1 and IRF5. Induction of the expression of transcription factors points towards complex regulation of gene expression upon angiotensin II exposure. Three genes involved in the dampening of oxidative stress were enhanced. Taken together, brief exposure of human tubular epithelial cells to angiotensin II elicited a marked induction of nuclear factors, antioxidant genes and hormones and hormone receptor genes. The quick activation of transcription factors by angiotensin II indicates that angiotensin II can directly initiate a cascade of expressional events in proximal tubular cells.

Keywords: Angiotensin II; Gene expression; Microarray; Transcription factor; Renal cell, human; Proximal tubule

1. Introduction

Knowledge regarding the cellular and molecular actions of angiotensin II has expanded greatly in recent years. The simple model of angiotensin II as a vasoconstrictive and sodium-retaining hormone has been replaced by complex schemes involving many signaling pathways and interactions with other hormone systems. Several receptors have been recognized (Timmermans et al., 1993); cell type-dependent signaling pathways have been described and interactions with other vasoactive hormones such as prostanoids (Inagami et al., 1999; Navar et al., 1996) and endothelin-1 (Moreau et al., 1997) have been elucidated. Furthermore, angiotensin II regulates growth factors (Wolf and Wolf, 2001), adhesion molecules (Prasad et al., 2001), inflammatory factors (Kranzhofer et al., 1999) and cell-cycle factors (Braun-Dullaeus et al., 1999). Finally, angiotensin II activates endothelial nitric oxide synthase (eNOS) (Braam, 1999) and NADPH-oxidase, via p22phox (Fukui et al., 1997). By doing so, angiotensin II has important consequences for the redox-state of the cell (Dalton et al., 1999). Thus, angiotensin II influences multiple systems in various cell types through direct and indirect mechanisms and isolated cell type are necessary to determine direct actions of angiotensin II.

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For the purpose of analyzing proximal tubular cell responses to brief angiotensin II exposure, we designed and manufactured a cDNA microarray. Several observations led to this microarray-assisted analysis of proximal tubular cell responses to angiotensin II. Some studies have suggested that angiotensin II effects can elicit positive feedback on angiotensinogen mRNA and protein leading to further angiotensin II generation and other elements of the renin–angiotensin system (Navar et al., 1999) and other systems (Kaysen et al., 1999), which could lead to a marked induction of genes. The widespread responses triggered by angiotensin II suggest that coordinated effects on transcription factors may regulate the initial steps of changes in gene expression. It is thus conceivable that at a very early stage, the expression of multiple transcription factors is altered in response to angiotensin II, however, limited information is currently available regarding this possibility. Accordingly, the aim of the present study was to assess the responses of human renal proximal tubular cells to brief exposure to angiotensin II. Specific questions were (1) are genes of the renin–angiotensin system itself activated or depressed by angiotensin II, (2) can angiotensin II directly activate transcription factors and (3) are genes related to compensatory mechanisms activated by angiotensin II itself. The genes on the microarray were chosen so that they included key factors from a number of functional systems in the cell related to these questions in proximal tubular cells.

2. Material and methods

2.1. Cell culture model

Human proximal renal cortical cells (hPRTCs) were isolated by Clonetics (San Diego, CA) from kidneys unsuitable for transplantation. Differential trypsinization resulted in cell fractions highly purified for proximal tubular cells. Cells were cultured in conventional T-flasks, in Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12) supplemented with fetal calf serum, and an antibiotic cocktail (Kaysen et al., 1999; Hammond et al., 1999, 2000). We have studied hPRTCs up to 10 passages and through that time course they maintain many proximal tubular features including proximal tubular enzyme markers (gamma-glutamyl transpeptidase, alkaline phosphatase, and leucine aminopeptidase), remain polarized with apical microvilli, and express the proximal tubular receptors cubulin and megalin (Hammond et al., 2000; Kaysen et al., 1999). They maintain active sodium-dependent transport of phosphate and glucose.

2.2. DNA microarray selection of genes and preparation of spotting solution

The gene array used for these experiments was designed from key factors in 10 different functional systems associated with the renin–angiotensin system: antioxidant, cell membrane, channels, growth factors, hormones and receptors, inflammatory factors, nuclear factors, signaling, transcription machinery and transporters (Table 1). For each of the 287 genes, ~1000 bp clones were obtained from Research Genetics (Huntsville, AL) and the bacteria with the vector grown overnight at 37 °C in a shaker. Two strategies for polymerase chain reaction (PCR) amplification were applied: directly from the culture and after isolation of the vector. For the first, 5 μl of culture suspension were added to a PCR mix consisting of 10 μl 10× reaction buffer (100 mM Tris–HCl, 500 mM KCl, Triton® X-100 1%; Promega, Madison, WI), 8 μl 25 mM $MgCl_2$, 2 μl 10 mM 5′-T7T3 and 3′-T7T3 primer, 1 μl 25 mM dNTP cocktail, 0.6 μl Taq (Promega) and 73.4 μl H_2O. For the latter, 2 μl of vector were added to the PCR mix, and the volume of H_2O increased to 76.4 μl. Following PCR, DNA was precipitated using standard methods and resuspended in dH_2O, and the amount of obtained DNA was verified using a 0.9% agarose gel and ethidium bromide. Vectors were isolated from the cultures using a kit (Qiagen, plasmid isolation kit) in case the PCR from the culture did not yield a single band on the gel. To obtain sufficient DNA for spotting, about eight runs were necessary. Spotting was performed from four 96-well plates, after determination of the adequate spotting volume. The use of spotting volumes from 25 μl and higher yielded consistent spots. For the spotting of the chips used for the experiments, a spotting volume of 50 ul was applied. Clones were re-suspended in 3× sodium chloride, sodium citrate (SSC) at a concentration of 0.20 μg/μl and 5 nl drops were spotted on to polylysine coated slides (Telechem, San Jose, CA) using the OmniGrid spotting robot (GeneMachines, San Carlos, CA) forming 200 μm diameter spots. All clones were spotted in duplicate. After spotting, the slides were dried and the 5′ terminal amino groups were covalently linked to the slides by UV-cross linking (65 mJ).

2.3. Isolation of mRNA and labeling of the probes

Cells were harvested in the TRIzol reagent and total RNA extracted according to the manufacturer's instructions. mRNA was extracted using the μMACS magnetic separator (Milveny Biotec). The protocol developed and successfully applied by the Stanford group was used (Ash/Max protocol, http://cmgm.stanford.edu/pbrown/). Briefly, 2 μg of mRNA was reverse transcribed using a polyT primer and Superscript™ reverse transcriptase and Cy3 or Cy5 fluorescent dUTP incorporated. Then, the sample was filtered and the volume reduced to exactly 20 μl. COT-1 DNA and yeast tRNA are added to optimize hybridization conditions. After addition of SSC, the samples were hybridized at 65 °C for 12–16 h, washed and dried. All samples were labeled with Cy5. A mixture of all the cDNA clones was labeled with Cy3, and served as a hybridization control.

Table 1
Official symbols of the selected gene set per functional system

Adhesion	Antioxidant	Apoptosis	Cell cycle	Cell	Signaling	Transport
FAK	GSS	APAF1	CCNA1	membrane	CALM1	ATP1A1
ICAM1	GSTA1	ATM	CCNB1	ADD1	CALM3	ATP1A3
ICAM2	GSTA2	BAX	CCNC	ADD2	GNA11	ATP1B1
ICAM3	GSTA3	BCL2	CCND1	ADD3	GNA15	ATP1B2
SELE	GSTA4	CASP8	CCND2		GNAI1	ATP1B3
SELL	GSTM1	CASP9	CCND3	matrix	GNAI2	ATP1G1
SELP	GSTP1	CDKN1A	CCNF	COL1A1	GNAL	ATP2A3
VCAM1	GSTT1	CDKN1C	CCNH	COL1A2	GNAS1	ATP2B4
CAT	GSTT2	DAP3	CDC2	COL4A1	GNAT1	CA2
	HMOX1	DAPK3	CDK2	COL4A2	GNB1	NHE3
	HMOX2	FADD	CDK3	COL4A4	PDE2A	ROAT1
	MTHFR	IGFBP3	CDK4	COL4A5	PDE4A	SLC2A2
	NCF1	IRLB	CDK5	DCN	PDE4B	SLC2A4
	NCF2	MDM2	CDK5R2		PDE4C	SLC2A5
	SOD1	PRKDC	CDK6		PDE5A	SLC4A4
	SOD2	RB1	CDK7		PIP5K1A	SLC7A1
	SOD3	TNFRSF6	CDK9		PLCG2	SLC7A2
		TP53	CDKN2A		PRKCA	SLC9A1
					PRKCB1	
					PRKCG	
					PRKCI	
					PRKCM	
					PRKCSH	

Channels	Growth factors		Hormones	Hormones	Inflammatory factors	Nuclear factors
ACCN3	FGF1	ADA	EPO	CSF1	CREB1	JUNB
AQP1	FGF2	ADK	EPOR	IFNG	CREB2	JUND
AQP2	FGFR1	ADM	INS	IFNGR1	CREBBP	MYC
AQP3	HGF	ADORA1	INSR	IFNGR2	E2F1	NFKB1
AQP4	IGF1	ADORA2B	KLK1	IL10RA	E2F3	NFKB2
AQP5	IGF1R	ADORA3	LTA4H	IL10RB	ELK1	PRKM1
AQP7	IGF2	ADRA1A	LYZ	IL1A	ETS1	PRKM10
AQP8	IGF2R	ADRA1B	MLR	IL1B	ETS2	PRKM3
AQP9	PDGFA	ADRA2C	NOS1	IL1R1	FOS	PRKM4
CACNA1B	PDGFB	ADRB2	NOS2A	IL1R2	FOSB	PRKM7
CACNA1C	PDGFRA	AGT	NPR1	IL2RB	GTF3A	PRKM8
CACNA1D	PDGFRB	AGTR1A	NPR2	IL6	HNF3A	PRKM9
CACNA1S	TGFB1	AGTR2	NPR3	IL6R	HNF3B	RARB
KCNJ8	TGFB2	ALOX12	P2RY1	SCYA2	HNF3G	RARG
P2RX1	TGFB3	ALOX5	P2RY11	SCYA5	HSF1	RELA
P2RX4	TGFBR2	AVPR1A	P2RY2		HSF2	RELB
P2RX5	TGFBR3	BDKRB1	PGR		IKBKG	SP1
SCNN1B	VEGF	BDKRB2	PTGER1		IRF1	SP2
SUR	VEGFB	CHRM3	PTGER3		IRF2	SP3
SUR2	VEGFC	CHRNA1	PTGFR		IRF3	SP4
VDAC1		CHRNB1	PTGIR		IRF4	SRF
VDAC2		CYP27B1	PTGIS		IRF5	STAT2
VDAC3		CYP4A11	PTGS1		IRF6	STAT3
		DCP1	PTGS2		IRF7	STAT6
		ECE1	REN		JAK1	TBP
		EDN1	TBXA2R		JAK2	TNF
		EDN2	TBXAS1		JAK3	
		EDN3	THRA		JUN	
		EDNRA	THRB			
		EDNRB	VDR			

2.4. Angiotensin II assay (Braam et al., 1993)

For angiotensin II measurements, cell homogenates in 90% ethanol were evaporated to dryness under nitrogen and redissolved in buffer. The samples were applied to phenyl-bonded solid phase extraction columns. Angiotensin II was eluted with methanol/ultrapure water (9:1 vol/vol). Standards (Peninsula, Belmont, CA) and unknowns were incubated for 48 h at 4 °C with ^{125}I-labeled angiotensin II (Amersham, Arlington Height, IL) and rabbit

anti angiotensin II antiserum (Arnel, New York, NY). Cross reactions of the antibody against angiotensin I and C-terminal angiotensin II fragments were <1% and <0.01%, respectively, except for angiotensin III, which has a cross reactivity of 90%. Bound and free angiotensin II were separated by second antibody precipitation with Sac–Cel (I.D.S., Washington, UK). The pellets were counted in a cobra Autogamma counter (Packard Instruments, Warrenville, IL) and concentrations were calculated using logit-log transformation. The sensitivity of the assay was 0.5 fmol/assay tube. Nonspecific binding of radiolabeled angiotensin II in the radioimmunoassay was <2%. The intra-assay variance was 9% and the interassay variance was 15%.

2.5. Experimental design

Primary human proximal tubular cells were cultured to passage 6. The medium use was DMEM/F12 (Sigma, St. Louis, MO) supplemented with 2% fetal calf serum (Life Technologies, Grand Island, NY), and an antibiotic cocktail (ciprofloxacin and fungizone, Life Technologies, Grand Island, NY). When the passage 6 cells were near confluent, the media was changed to serum free for 24 h prior to the experiment. The angiotensin II was diluted in 1 ml of serum free media and added to the 35 ml of media which had already been in the T-225 flask overnight. Four hours was chosen as the initial time for study as Ingelfinger et al. observed changes in angiotensin-dependent gene expression at this time, and we wanted to catch the early activation events in a time course similar to suspension culture studies (Kaysen et al., 1999). At time zero, the flasks were divided with one group exposed to vehicle and the other group with angiotensin II. Angiotensin II was diluted to yield final concentrations in the culture flasks of 10 and 100 nM ($n=4$ cultures for each concentration). The angiotensin II was added to the culture medium and the fluid gently mixed. After 4 h, 1 ml of culture medium was sampled and added to 9 ml of 90% ethanol for determination of angiotensin II levels. Cells were harvested from control and angiotensin II flasks, respectively, and mRNA extracted as mentioned above. An experiment was included if it yielded enough mRNA to perform one labeling and hybridization.

2.6. Microarray quantitation and statistics

The microarray slides were scanned using a ScanArray 3000 (General Scanning, Watertown, MA). Laser intensity and photomultiplier tube (PMT) sensitivity were held constant for the Cy5 and Cy3 channels at 60% and 90%, respectively, and triple scans were performed for each slide. All slides were scanned at a density of 10 μm. Stored images were analyzed using Imagene analysis software (Biodiscovery, Los Angeles, CA). For both the Cy3 and Cy5 images, the average intensity and background was used for the calculations.

Each gene was spotted in duplicate and the signal minus background was averaged from the two spots. The mean signal minus background for each gene was calculated per group, that is, for control slides and slides from the 10 and 100 nM angiotensin II experiments, and a correction factor calculated for the control, so that the median $\log_2$ of the ratio for angiotensin II and control samples was zero. A $\log_2$ value of the angiotensin II over control ratio >1 or <−1 was considered as relevant.

3. Results

3.1. Presence of angiotensin type 1 receptors and angiotensin II concentrations in culture medium

We have previously demonstrated the presence of angiotensin type 1 receptors in this cell line by commercial cDNA array (Kaysen et al., 1999), semi-quantitative reverse transcription PCR (RT-PCR) (Kaysen et al., 1999) and Western blot analysis (Hammond et al., 2000). The presence of functional angiotensin type 1 receptor protein in the current cell cultures was confirmed by Western blot analysis and flow cytometry antibody binding (data not shown). Angiotensin II concentrations in the culture medium had declined to very low concentrations after 4 h. Angiotensin II levels in the supernatant of the cells exposed to 100 nM angiotensin II was 23 ± 4 pM after 4 h.

3.2. PCR of clones, spotting volume and demonstration of an active surface of the chip

Ninety-one percent of the clones subjected to PCR yielded sufficient DNA and had a single band, albeit that of 40 clones the vector had to be isolated. The remainder of the clones did not yield PCR product, showed smear on the gel or had more than one band. These clones were excluded from the analysis. To show activity of the spots on the chip, 1 ul of each clone was added to constitute a mixture of all the clones on the chip. Then, the mixture was labeled and hybridized to the chip. It should be noted that the concentration of the clones in the mixture was not yet adjusted to be 200 μg/ul. This experiment demonstrated that the surface of the chip was active (Fig. 1). Comparison of a 10 times and 100 times dilution of the mixture demonstrated that the signal was proportional to the applied amount of probe (Fig. 2). Comparison of the signal intensity in the Cy3 and Cy5 channels using the same probe and the same PMT sensitivity and laser intensity yielded a consistent difference in signal intensity between the channels (data in Fig. 2 are corrected for this channel difference).

3.3. Gene expression under control conditions

The 25 genes that showed the highest signals under control conditions are shown in Table 2. Note that the

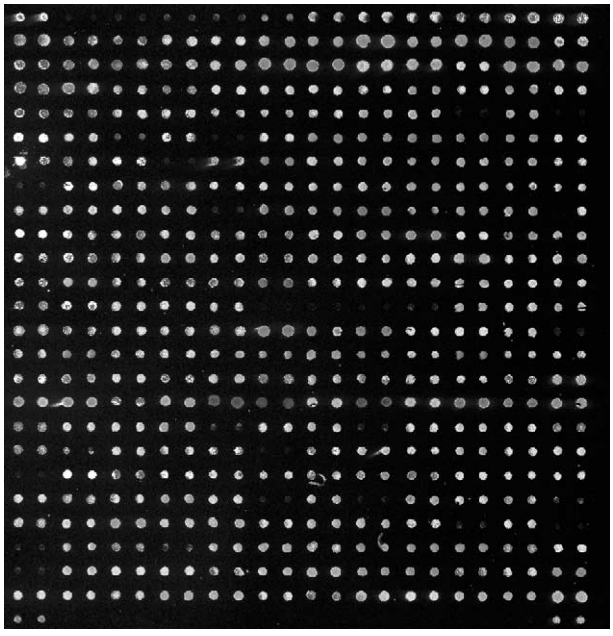

Fig. 1. Microarray hybridized with a Cy3-labeled mixture of all the clones printed on the microarray. Those spots that are barely visible represent clones that would not amplify.

samples were taken from 8 separate untreated cultures. There was considerable variation among the different cultures. No particular groups of genes were prominently present in the genes with the highest signals.

3.4. Gene expression following 4 h exposure to different concentrations of angiotensin II

Changes in expression of the clones on the chip were evaluated for 10 and 100 nM angiotensin II after 4 h of

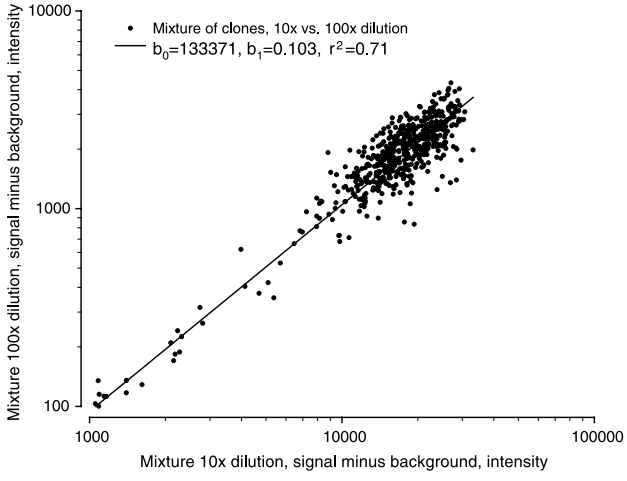

Fig. 2. Comparison of the obtained signals after hybridization of the microarray with a 10-times diluation of the mixture of the clones that was used to test the surface activity of the microarray. A 10-fold lower concentration of each of the labeled clones was associated with a 10-fold lower signals, as can be appreciated from the regression statistics.

Table 2
Genes with the highest signals (corrected for background and expressed as 1/1000th of the total intensity of the signals on the microarray) under control conditions

Group	Gene	Average	S.E.M.
Antioxidant	GSTT2	2.92	1.05
	SOD1	3.37	0.79
	GSTP1	3.87	1.3
	GSTA2	5.12	3
Apoptosis	CASP8	2.46	0.81
	FADD	8.06	2.1
	IGFBP3	8.19	2.63
Cell cycle channels	CDK4	7.62	2.51
	VDAC2	2.68	1.38
	P2RX5	3.21	0.84
	ACCN3	10.43	7.76
	CACNA1B	12.87	3.49
Growth factors hormones	PDGFB	2.48	0.4
	ADM	2.5	0.92
	EPOR	2.53	0.94
	NPR3	2.55	0.26
	AGTR1A	2.85	0.6
	DCP1	3.07	0.62
	NOS1	3.83	1.4
	CHRNA1	4.64	0.59
	EPO	6.8	2.91
	ADORA2B	7.55	5.51
Inflammatory factors	IL1A	2.61	0.63
	IL10RB	3.05	1.27
	IL6	3.07	0.83
	IFNGR2	4.15	0.96
	SCYA2	5.45	1.94
Matrix	COL4A1	7.5	4.34
	COL4A2	7.65	2.29
Nuclear factor	RARB	2.65	1.12
	HSF1	2.68	0.73
	RELA	3.25	0.89
	ETS1	3.54	0.6
	PRKM7	4.6	1.03
	PRKM3	8.3	3.15
Signalling	PRKCA	2.57	0.87
	PRKCG	2.63	0.62
	PRKCB1	3.57	1.29
	GNAS1	6.27	1.99
Transporters	CA2	2.49	0.11
	ATP1B3	4.54	1.26

Table 3
Genes in proximal tubular cells that displayed a more than 2-fold change upon 4 h exposure to 10 nM angiotensin II

Group	Gene	$S-B_{Ang}$	S.E.M.	$S-B_{Con}$	S.E.M.	Ratio	S.E.M.
Apoptosis	IGFBP3	30,596	9170	15,608	3277	2.53	0.88
Hormones	ADM	1526	248	1224	370	2.13	0.84
Matrix	COL1A1	1015	270	984	307	2.18	1.18
Nuclear	IRF5	1228	223	1133	295	2.33	1.37
factor	HSF2	2638	1843	1556	660	2.52	1.26
Signalling	PIP5K1A	1609	550	1231	371	2.24	1.1
	CALM1	2415	788	1705	456	2.39	1.14
	PDE4C	1238	298	1135	316	3.02	2.07
	PDE2A	1152	196	1043	277	3.56	2.42
Transporters	ATP2A3	1961	670	1614	460	4.38	3.28

$S-B$: Signal minus background; Ang: angiotensin; Con: control.

Table 4
Genes that displayed a more than 2-fold change upon 4 h exposure to 100 nM angiotensin II

Group	Gene	$S-B_{Ang}$	S.E.M.	$S-B_{Con}$	S.E.M.	Ratio	S.E.M.
Down							
Hormones	ADORA1	727	312	1842	366	0.46	0.26
Signalling	GNAL	582	566	912	523	0.47	0.17
Up							
Antioxidant	GSTA2	9813	5675	5757	2409	2.14	0.75
	SOD3	801	338	991	493	2.97	1.80
	GSTM1	595	90	419	100	3.39	2.16
	NCF1	4695	2915	1425	133	4.10	3.01
Apoptosis	CASP9	1293	1197	445	259	2.02	0.65
	DAP3	1188	605	972	479	2.26	1.01
	CDKN1C	4417	4038	1281	723	5.00	3.38
Cell cycle	CCNB1	594	413	1184	702	2.02	1.12
	CCNF	1531	493	1121	290	2.68	1.85
Channels	SCNN1B	1821	1177	1222	210	2.05	1.5
	CACNA1S	1557	1413	848	515	2.69	1.61
	AQP9	1953	512	1104	434	3.23	1.42
Growth factors	PDGFRA	3043	1113	1505	489	3.43	1.89
	FGF2	2661	1965	1541	909	3.95	2.34
Hormones	PTGFR	1677	589	1287	323	2.12	1.33
	EPO	11,264	3918	7971	2829	2.19	0.80
	THRB	1984	1361	767	312	2.33	0.42
	NOS1	9162	1971	3682	470	2.47	0.36
	EDNRA	2830	1456	1515	316	2.48	1.53
	P2RY1	5455	3023	1950	692	2.80	0.32
	KLK1	347	278	301	189	2.84	1.32
	P2RY2	2196	956	758	281	3.22	1.26
	PTGIS	1281	1127	805	542	3.97	1.44
	INS	878	111	382	137	4.19	1.31
Inflammatory factors	SCYA2	3897	1929	1810	450	2.01	0.49
	IL1B	1240	703	1512	779	3.74	2.92
Nuclear factor	HNF3A	215	127	178	98	5.29	1.99
	HNF3B	3081	1743	1724	464	2.01	0.84
	HNF3G	2065	1921	1233	719	2.96	0.45
	E2F1	258	171	208	120	2.29	1.13
	E2F3	376	182	267	145	6.29	3.72
	IRF1	2933	827	1459	340	4.33	2.90
	IRF5	4999	3825	1689	587	2.24	0.86
	ETS2	1894	1210	1007	474	2.06	0.30
	STAT4	1035	873	952	594	3.42	2.07
	ELK1	1695	277	1535	619	4.74	3.19
	JUN	1623	1213	829	401	4.95	3.75
Signalling	PRKCZ	112	44	306	169	5.64	4.54
	PRKM7	3404	1534	2882	1705	3.36	1.27
Transporters	NHE3	2646	1460	1219	278	2.49	0.98
	ATP1B2	381	319	743	438	2.88	1.45
	SLC7A1	345	209	194	84	3.06	2.04

$S-B$: Signal minus background; Ang: angiotensin; Con: control.

exposure. Table 3 lists those genes of which the signal was increased more than 2 times upon 4 h of exposure 10 nM angiotensin II. The most pronounced increase in signal was observed for genes coding for the phosphodiesterases II and IV and of a subunit of the Na^+/K^+-ATPase. No genes displayed a significant downregulation. Upon exposure to 100 nM angiotensin II 2 genes displayed decreased and 42 genes displayed increased signal intensities on the microarray (Table 4). Genes involved in the oxidant/anti-oxidant balance, as well as hormones and nuclear factors were well represented.

We specifically looked at the signals of the genes of the renin–angiotensin system. None of the genes showed a more than 2-fold change in response to angiotensin II. The signal of the angiotensin type 1 receptor increased to 1.9 times of control upon 100 nM angiotensin II.

4. Discussion

In the present study, a custom-made array was used to study gene expression of human proximal tubular cells in

culture following brief, 4 h, exposure to angiotensin II. In accord with studies showing that angiotensin II can stimulate expression of tubular angiotensin type 1 receptor mRNA and its protein (Ingelfinger et al., 1999; Cheng et al., 1995; Kobori et al., 2001), we observed that angiotensin II increased the signal for the angiotensin type 1 receptor gene 1.9-fold. Angiotensin II also stimulated the expression of several nuclear factors, suggesting that angiotensin II can trigger a cascade of expressional events leading to a complex expression pattern. Finally, increased expression of antioxidant genes suggests that angiotensin II has the capability of changing the redox status of the cell.

The first question focused on the expression of components of RAS in the view of a proposed positive amplification mechanism between angiotensin II, angiotensinogen and renin. It is now clear that the kidney has a complete RAS and that even the proximal tubule is capable of producing large amounts of angiotensin II (Navar et al., 1999; Braam et al., 1993). These findings have challenged the concept that angiotensin II enforces a negative feedback on its production and processing enzymes. It was demonstrated that chronic infusion of angiotensin II increased intrarenal angiotensin II levels, and that concomitant blockade of the angiotensin type 1 receptor largely prevented this increase (Kobori et al., 2001). The present study now demonstrates that brief exposure to angiotensin II may well increase the angiotensin type 1 receptor gene. This is in line with the observation by Ingelfinger et al. (1999) of increased expression of angiotensin type 1 receptor mRNA. Renin and angiotensinogen gene expression was not affected by such brief exposure and analyzed using microarray technique. This is in contrast with the observations by others, that exposure of a proximal tubule cell line to angiotensin II for 1 h increased angiotensinogen mRNA (Ingelfinger et al., 1999) and that chronic infusion of angiotensin II increased renal angiotensinogen mRNA and protein (Cheng et al., 1995). The lack of changes in angiotensinogen gene expression in the present study could be due to the limitation in sensitivity of the method. Indeed the changes observed by others are less than 2 times of control (Ingelfinger et al., 1999; Cheng et al., 1995). Time-series and pharmacological blockade of the angiotensin type 1 receptor, combined with expression analysis could yield further insight in this potentially important positive feedback mechanism. However, from the present findings, the possibility of a positive amplification mechanism in proximal tubular cells cannot be excluded.

The second question focused on early activation of transcription factors. Changes in nuclear factors after brief exposure to angiotensin II are interesting because these changes could evoke a cascade of expressional events. Brief exposure of the renal cells to 100 nM angiotensin II elicited a more than 2-fold induction in activity in 11 genes of the transcription factors. Coordinated changes were observed in genes of the hepatocyte nuclear factor 3 family (NHF3; HNF3A, HNF3B and HNF3G), in the E2F genes (E2F1, E2F3) and the interferon regulatory factors IRF1 and IRF5. The HNF3 family is involved in activation of numerous genes involved in glucose metabolism (Kaestner, 2000). More interestingly, HNF3B has been identified as a stimulator of transcription of the angiotensinogen-gene (Cui et al., 1998). Although in the present experiment we failed to indicate upregulation of angiotensinogen gene after 4 h of exposure to angiotensin II, one of its stimulators was already upregulated. The E2F gene family are important regulators of the cell cycle. Interestingly, a recent report suggested that angiotensin II-mediated growth stimulation of vascular smooth muscle cells is modulated by E2F1 (Fujita et al., 1999). Interferon regulatory genes are involved in cell cycle control; however, IRF1 has been implicated in expression regulation of the angiotensin AT2 receptor (Horiuchi et al., 1995).

Angiotensin II has been shown to activate many cell processes, leading to vasoconstriction, altered transport rate, proliferation, inflammatory responses and an increased level of metabolism. In the present study we also searched for dampening, defensive responses of the cell. Recent studies have demonstrated that angiotensin II also leads to oxidative stress (Fukui et al., 1997; Zhang et al., 1999). Angiotensin II has been shown to increase p22phox subunit of NADPH-oxidase (Fukui et al., 1997) and to lead to superoxide production in isolated cells, and blockade of the actions of angiotensin II leads to a decrease in oxidative stress (Fukui et al., 1997). In the present experiment, brief exposure of human renal epithelial cells to angiotensin II led to an increase in expression of NCF1. The product of this gene is known as p47phox, which is an essential component of NADPH-oxidase and is present in the kidney (Jones et al., 1995). Angiotensin II infusion was associated with increased expression of the extracellular superoxide dismutase, type 3, and two members of the glutathion-*S*-transferase family, genes all coding for enzymes acting as anti-oxidants. The expression pattern observed in the present study suggests that angiotensin II can indeed activate pro-oxidant genes, however, this may be balanced by the induction of anti-oxidant enzymes.

Taken together, brief exposure of human tubular epithelial cells to angiotensin II elicited a marked induction of nuclear factors, antioxidant genes and hormones and hormone receptor genes. The quick activation of transcription factors by angiotensin II indicate that the hormone is capable of starting a cascade of expressional events, which can explain the widespread actions of angiotensin II.

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