

# High Ambient Glucose Augments Angiotensin II-Induced Proinflammatory Gene mRNA Expression in Human Mesangial Cells: Effects of Valsartan and Simvastatin

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## Key Words

Angiotensin II · Hyperglycemia · Glomerular mesangial cell · Proinflammatory action

## Abstract

**Background:** Hyperglycemia may potentiate the adverse renal effects of angiotensin II (AII). In the kidney, the major target of AII action is the glomerular mesangial cell, where its hemodynamic and proinflammatory action contributes to renal injury. AII action is mediated by several types of cell receptors. Among those, the AT<sub>1</sub> receptor has been best studied using specific AII receptor blockers (ARBs). These agents have emerged as major new modalities in the prevention and amelioration of renal disease where the ARB renoprotective anti-inflammatory properties could be more important than previously appreciated. Like the ARBs, statins may also modulate inflammatory responses that are renoprotective and complement their cholesterol-lowering effects. **Aim:** The aim of this project was to (i) identify a repertoire of proinflammatory mesangial cell AII-inducible mRNAs; (ii) determine if the AII-induced proinflammatory mRNA responses depend on ambient glucose, and (iii) test the anti-inflammatory effectiveness of an ARB, valsartan, either alone or in combination with a statin, simvastatin. **Results/Conclu-**

**sions:** Using high-density microarrays and real-time PCR we identified several AII-inducible proinflammatory mesangial genes that exhibited augmented mRNA responses in high-glucose milieu. Valsartan blocked the AII-induced mRNA expression of proinflammatory genes (i.e. MCP-1, LIF and COX-2) maintained in normal and high glucose. These observations add to the mounting evidence that ARBs have anti-inflammatory effects in the kidney, a beneficial effect that may be more important in protecting renal function in diabetic patients. While simvastatin inhibited expression of some mRNAs encoding chemokines/cytokines, it enhanced expression of mRNA encoding COX-2, a key mediator of inflammation. Thus, the non-cholesterol effects of statins on inflammatory responses appear complex.

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## Introduction

Angiotensin II (AII) is a multifunctional peptide found in organisms as evolutionarily distant as invertebrates and man [1, 2]. In invertebrates the renin-angiotensin system (RAS) regulates not only osmoregulation but also inflammatory responses [1–3]. Thus the immunoregulatory role of RAS is as ancient as its vasoconstrictive

tive action. Still, it is only recently that the proinflammatory action of AII has been more widely appreciated [4–8]. Many of the AII inflammatory responses are mediated by reactive oxygen species (ROS) [9] resulting in the activation of NF- $\kappa$ B, a transcriptional factor central to the inflammatory response [8, 10].

Angiotensin type 1 (AT<sub>1</sub>) and type 2 (AT<sub>2</sub>) are the two major classes of receptors that mediate the action of AII [11, 12]. The AT<sub>1</sub> receptor is expressed ubiquitously, but the tissue expression of AT<sub>2</sub> is more restricted [11–13]. In mesangial cells the number of AT<sub>1</sub> receptors expressed on the cell surface far exceeds that of AT<sub>2</sub> [14]. Although signaling through AT<sub>1</sub> is thought to be the major contributor to the glomerular inflammatory lesion [15], signaling through AT<sub>2</sub> may also play a role in renal damage [16, 17].

Although the etiology of micro- and macrovascular complications of diabetes is multifactorial, low grade inflammation is increasingly being recognized as one of the systemic hallmarks not only associated with increased cardiovascular complications but also with diabetic nephropathy [18]. In diabetic nephropathy the dysfunctional mesangial cell is one of the major effectors of glomerular injury. When exposed to the diabetic metabolic milieu, mesangial cells display growth abnormalities, increased expression and synthesis of prosclerotic growth factors and cytokines, accumulation of normal components of extracellular matrix, as well as increased synthesis of proinflammatory ligands [19–21].

Based on the abundant evidence spanning from in vitro studies in various renal cell types to large clinical trials, RAS and its major effector AII have been implicated in the pathophysiology of diabetic nephropathy. There is increasing evidence that the proinflammatory action of AII is directly involved where the mesangial cell plays a key role in the pathogenesis of diabetic nephropathy. Inhibition of RAS with angiotensin-converting enzyme inhibitors or AII receptor blockers (ARBs) plays a pivotal role in the treatment of this disorder, and these beneficial effects in the diabetic kidney are at least in part attributable to amelioration of ROS generation and proinflammatory signal transduction [22].

Thus, studies in a variety of experimental settings, including studies in patients with diabetes, have suggested synergistic actions of hyperglycemia and AII in diabetic renal pathophysiology [15, 23, 24]. On the molecular level, these synergistic actions involve stimulation of proinflammatory signal transduction pathways and genes. High ambient glucose by itself activates some of the same proinflammatory signaling mediators as those triggered

through the AT<sub>1</sub> receptor, such as ROS, NF- $\kappa$ B, JAK/STAT, and others [16, 17, 23, 25, 26]. These observations suggest a possibility that the glucose milieu could modulate AII-mediated expression of genes that drive progression of diabetic nephropathy, such as the proinflammatory cytokines, enzymes and transcription factors.

The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, or statins, are potent inhibitors of cholesterol biosynthesis that are used extensively to treat hypercholesterolemia [27]. Although traditionally the beneficial effects of statins were thought to result from the competitive inhibition of cholesterol synthesis [28], it has become increasingly apparent that statins exert additional cellular effects [29]. These studies indicate that statins exert cholesterol-independent effects by modulating signal transduction pathways and suggest that, like the ARBs, statins modulate inflammatory responses independent of HMG-CoA reductase inhibition [30]. Indeed, evidence is accumulating that statins confer renoprotection in a variety of glomerular diseases, including diabetic nephropathy [31–33], and in models of AII-induced injury, statins have been shown to be beneficial [34–37].

Until now a comprehensive study of the mesangial cell repertoire of inflammatory genes activated by AII, and the modulatory effects of high glucose on these AII-induced mesangial cell responses has not been done. To address this issue, we used high-density microarrays and real-time PCR to identify several AII-inducible proinflammatory mesangial gene mRNAs that exhibit augmented responses in a high-glucose milieu. Furthermore, we evaluated whether these proinflammatory responses were influenced by the AT<sub>1</sub> receptor blocker, valsartan, or the HMG-CoA inhibitor, simvastatin, common treatments for diabetic nephropathy.

## Material and Methods

### Cells

Primary human glomerular mesangial cells (6–9 passages) were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, Utah, USA). Culture medium was supplemented with 16% (vol/vol) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 8  $\mu$ g/ml insulin, 1 mM sodium pyruvate and 10 mM HEPES buffer. Cells were incubated at 37°C in humidified 5% CO<sub>2</sub>–95% air.

### Study Design

AII enhances renal production of a host of proinflammatory polypeptides including TNF- $\alpha$ , MCP-1 and IL-6 [38, 39]. The increased synthesis of these cytokines/chemokines, at least in part,

**Table 1.** Design of the real-time PCR experiment

Glucose	Untreated				AII			
Normal (100 mg/ml)	UNT	VAL	SMV	VAL+SMV	UNT	VAL	SMV	VAL+SMV
High (450 mg/ml)	UNT	VAL	SMV	VAL+SMV	UNT	VAL	SMV	VAL+SMV

AII = Angiotensin II (100 nM); UNT = untreated; VAL = valsartan (100 nM); SMV = simvastatin (1  $\mu$ M).

reflects NF- $\kappa$ B-mediated transcription observed as early as 30–60 min following AII stimulation [39]. Thus to identify inducible proinflammatory genes in human mesangial cells we have chosen to assess transcript levels at 0 and 60 min following treatment with AII. A wide range of AII levels in renal interstitial fluid has been reported [40–43]. For example, in rats the intrarenal AII concentration was reported to be in the range of 1–4 nM [42], while in the dogs the levels have been reported to be as high as 740 nM [41]. The concentration of AII in the human mesangium is not known. We have chosen to use 100 nM AII [44, 45].

For microarray profiling, serum-deprived (24 h) cells maintained in normal glucose (100 mg/ml) and preincubated with or without Val (100 nM, 30 min) were treated with or without AII (100 nM, 60 min).

For real-time PCR, cells maintained in either normal (NG; 100 mg/dl) or high (HG; 450 mg/dl) glucose (72 h) were serum deprived for 24 h and then stimulated with AII (100 nM) for 60 min in normoglycemic or hyperglycemic conditions in the absence or presence of ARB valsartan (100 nM) or the HMG-CoA inhibitor simvastatin (1  $\mu$ M). The study conditions are summarized in table 1.

#### Reagents

AII purchased from AnaSpec (San Jose, Calif., USA) was dissolved in water  $10^{-4}$  M. Valsartan (a gift from Novartis) was dissolved in ethanol to a concentration of  $10^{-4}$  M and aliquots were stored frozen ( $-20^{\circ}$ C). Simvastatin, carboxylate-activated (Cat. No. 567020, Calbiochem, San Diego, Calif., USA), was dissolved in ethanol to a concentration of  $10^{-3}$  M and aliquots were stored frozen ( $-20^{\circ}$ C).

#### RNA Extraction

TRIzol was used to purify RNA according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, Calif., USA). Total extracted RNA was dissolved in 20  $\mu$ l sterile water and stored at  $-80^{\circ}$ C. The RNA concentration was measured with a spectrophotometer (Bio Mate3, Thermo Fisher Scientific) and the 260/280 ratio of RNA was  $>1.7$ .

#### Microarrays

Mesangial cell transcripts were profiled using the Affymetrix Human Genome U133 Plus 2.0 gene array (HG-U133 Plus). A single GeneChip<sup>®</sup> array contains probe sets representing more than 47,000 transcripts derived from approximately 33,000 well-substantiated human genes. The probes were made by one round of linear amplification by the Eberwine method used at the Center for Expression Arrays (CEA), University of Washington [46]. Labeled probes were hybridized using the Affymetrix protocol.

**Table 2.** PCR primer list

MCP-1 (CCL2)	Gene ID 6347	fwd	5'-TGTTGATGTGAAACATTATGCC-3'
		rev	5'-AATGATTCTTGCAAAGACCCTC-3'
LIF	Gene ID 3976	fwd	5'-ATTCAGTGATGCTGTGCAGG-3'
		rev	5'-ATCACCTCATCTCCCTGTGG-3'
FGF5	Gene ID 2250	fwd	5'-TCCTAAACCTTTGGTGGCTG-3'
		rev	5'-GTTCAAGAATGAGGGCAAGG-3'
COX-2 (PTGS2)	Gene ID 5743	fwd	5'-TCCCTGAGCATCTACGGTTT-3'
		rev	5'-TACTCTGTTGTGTTCCCCGA-3'
FOSL1	Gene ID 8061	fwd	5'-ATGTGGGATACTGTCCAGGC-3'
		rev	5'-CATCGCAAGAGTAGCAGCAG-3'
TIEG	Gene ID 7071	wd	5'-ATCCTGGGTGGCTACAGATG-3'
		rev	5'-GTGCAGAGTTCAAAGCCTCC-3'
IER3	Gene ID 8870	fwd	5'-CTCCTACTTTGCCGCAGTTC-3'
		rev	5'-CGTCCTCCTAGGTGATGGAG-3'
EGR-1	Gene ID 1958	fwd	5'-TGAACAACGAGAAGGTGCTG-3'
		rev	5'-GGTCATGCTCACTAGGCCAC-3'
$\beta$ -Actin	Gene ID 60	fwd	5'-AGAGCTACGAGCTGCCTGAC-3'
		rev	5'-AAGGTAGTTTCGTGGATGCC-3'

Four separate experiments were done using 14 Affymetrix chips. GeneTraffic software (<http://www.stratagene.com>) was used for microarray data management and statistical analysis (ANOVA).

#### Reverse Transcription

First strand cDNA was synthesized by priming 1  $\mu$ g total RNA with 10  $\mu$ M random hexamers (Promega Co, Madison, Wisc., USA) then by heating at  $65^{\circ}$ C for 10 min, and snap-cooling on ice. Reverse transcription ( $37^{\circ}$ C for 1 h) was performed in the presence of 10 mM each of dATP, dCTP, dTTP and dGTP (Invitrogen Corp.), 4  $\mu$ l 5 $\times$  first strand buffer (Invitrogen Corp.), 0.1 M DTT (Invitrogen Corp.), 200 units of Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Corp.), and 20 units of RNase inhibitor (Invitrogen Corp.). Following the reaction the sample was heated at  $94^{\circ}$ C for 5 min, cooled on ice and, after adding 180  $\mu$ l water, samples were stored  $-80^{\circ}$ C.

#### Real-Time PCR

PCR primers were designed using the Primer3 software (<http://frodo.wi.mit.edu/>). The reaction mixture contained 5  $\mu$ l 2 $\times$  SYBR Green PCR Master Mix (ABI), 2.5  $\mu$ l DNA template and 0.3  $\mu$ M primers (10  $\mu$ l final volume) in 384-Well Optical Reaction

**Table 3.** Summary of statistical analysis

Comparison	PTGS2 (COX-2)	CCL2 (MCP-1)	EGR-1	FOSL1	LIF	TIEG (KLF10)	FGF5	IER3
HG vs. LG control	<0.001	<0.001	<0.001	<0.001	<0.001	NS	<0.01	NS
NG AII vs. NG control	<0.001	0.001	<0.001	NS	<0.001	<0.001	NS	<0.001
HG AII vs. HG control	<0.001	0.001	<0.001	0.1	<0.001	<0.001	NS	<0.001
HG AII vs. NG AII	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.02	0.01
NG AII Val vs. NG AII	<0.001	<0.001	<0.001	NS	<0.001	0.001	NS	0.01
HG AII Val vs. HG AII	<0.001	<0.001	<0.001	NS	<0.001	0.001	NS	0.02
NG AII Val Sim vs. NG AII Val	<0.001	<0.001	NS	NS	NS	<0.001	<0.001	NS
HG AII Val Sim vs. HG AII Val	<0.001	<0.001	NS	<0.001	<0.001	<0.001	<0.001	NS
$\Delta$ HG AII vs. cont - $\Delta$ LG AII vs. cont	NS	NS	NS	NS	<0.05	NS	NS	NS
NG vs. NG Sim	<0.05	<0.001	NS	NS	<0.001	<0.001	<0.001	<0.05

NG = Normal glucose; HG = high glucose; AII = angiotensin II; Val = valsartan; Sim = simvastatin; NS = statistically not significant; cont = control.

Plate (ABI). Amplification (three steps, 40 cycles), data acquisition and analysis were done using the 7900HT Real-Time PCR system and SDS Enterprise Database (ABI). Standard curves were generated for each primer set by serial dilution of human genomic DNA. Primer sequences are shown in table 2. mRNA levels were calculated relative to untreated cells grown in normal glucose and all results were corrected to the  $\beta$ -actin transcript.

#### Statistics

Real-time PCR mRNA results were compared by four-way ANOVA for each gene [47]. The model included main effects for normal versus high glucose, presence or absence of AII, presence or absence of valsartan and presence or absence of simvastatin along with all two-way interactions. mRNA levels were log transformed due to skewness and robust standard error estimates were used to protect against departures from the constant variance assumption. The level of significance was taken to be 0.05, and p values were not adjusted for multiple comparisons due to the exploratory nature of the analysis. The statistical analysis is summarized in table 3.

## Results

Expression microarray profiling of the RNA identified 27 genes that were induced by AII and 7 that were suppressed (100 nM, 60 min treatment; ANOVA  $p < 0.05$ ; fig. 1). In all cases the AII-induced effects were blocked by pretreatment of cells with the AT<sub>1</sub> receptor blocker valsartan (100 nM). In agreement with reported dominant expression of AT<sub>1</sub> [12], our results suggest that in primary human mesangial cell culture AII-responsive gene expression is largely mediated by the AT<sub>1</sub> receptor. Several of the AII-responsive genes identified by micro-

arrays encode proteins, such as cytokines, enzymes and transcription factors that could be involved in inflammatory responses. Among the candidate genes, mRNAs encoding proteins potentially involved in inflammatory responses, including MCP-1 [8, 48], LIF [49], PTGS2 (COX-2) [9], FOSL1 [50] and NFIL3 [51] were previously shown to be induced in response to AII treatment. The list of the genes and link to iHOP (Information Hyperlinked over Proteins) is <http://www.ihop-net.org>.

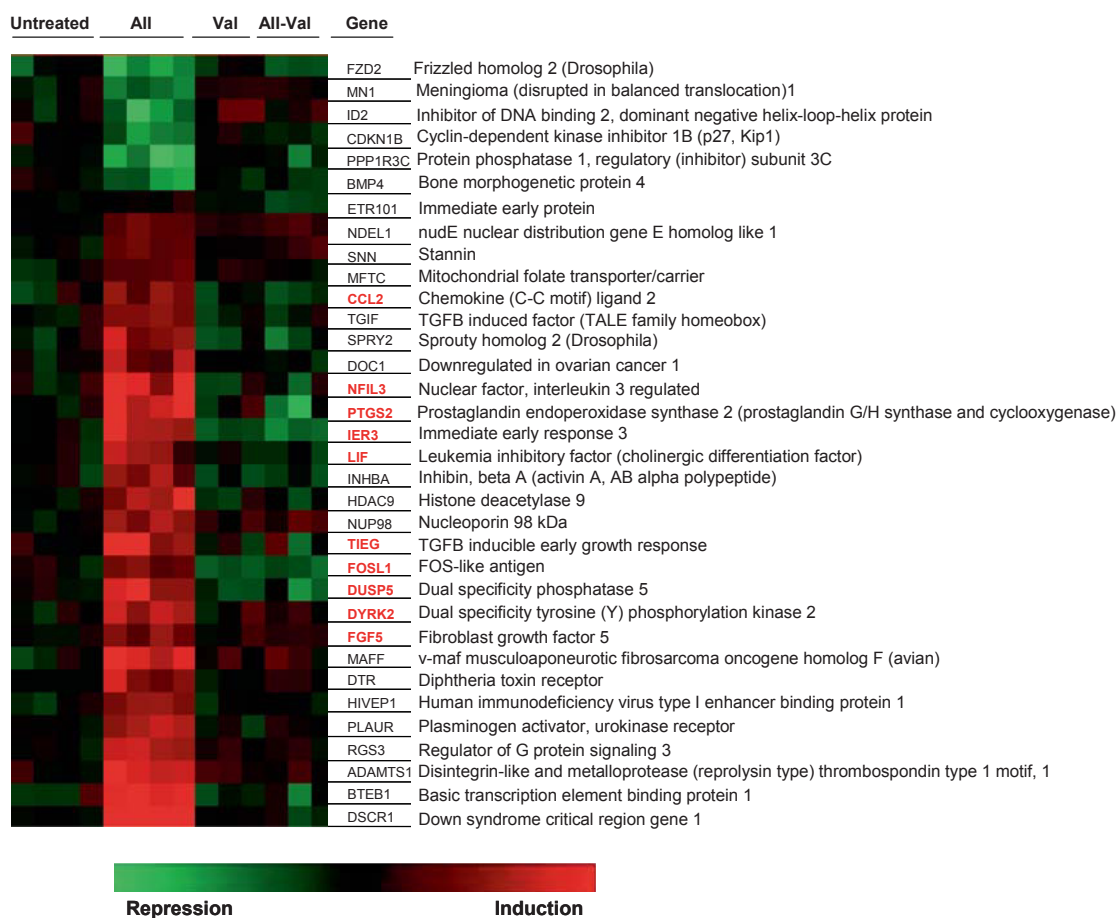
Next we used real-time PCR (i) to confirm microarray data, (ii) to test if ambient glucose modulates these mRNA responses, and (iii) to compare the effectiveness of AT<sub>1</sub> blockade with or without pretreatment with a statin, simvastatin. The outline of the treatment groups is shown in table 1. The AII-induced inflammatory gene mRNAs can be grouped into three classes, cytokines/chemokines, enzymes, and transcription factors. Each functional class of genes will be presented as a group.

A summary of the statistical analyses is shown in table 3 and to the right of figures 2–4.

#### Cytokines/Chemokines

Leukemia inhibitory factor (LIF) belongs to the IL-6 family of cytokines that share the common co-receptor gp130 and activate similar cellular responses [49]. Several studies reported AII-induced LIF and IL-6 gene expression in different cell types [49, 52–54]. Importantly, it has been shown that increased systemic [18] and renal [55] expression of IL-6 is associated with diabetic nephropathy. Thus, the involvement of the IL-6 family of cytokines in mediating adverse cardiovascular [49] and





**Fig. 1.** Heat map showing AII-induced transcripts in untreated and valsartan-pretreated (Val) primary human mesangial cells. Serum-deprived (24 h) cells maintained in normal glucose (100 mg/ml) and preincubated with or without Val (100 nM, 30 min) were treated with or without AII (100 nM, 60 min). Total RNA extracted was labeled for hybridization of high-density oligonu-

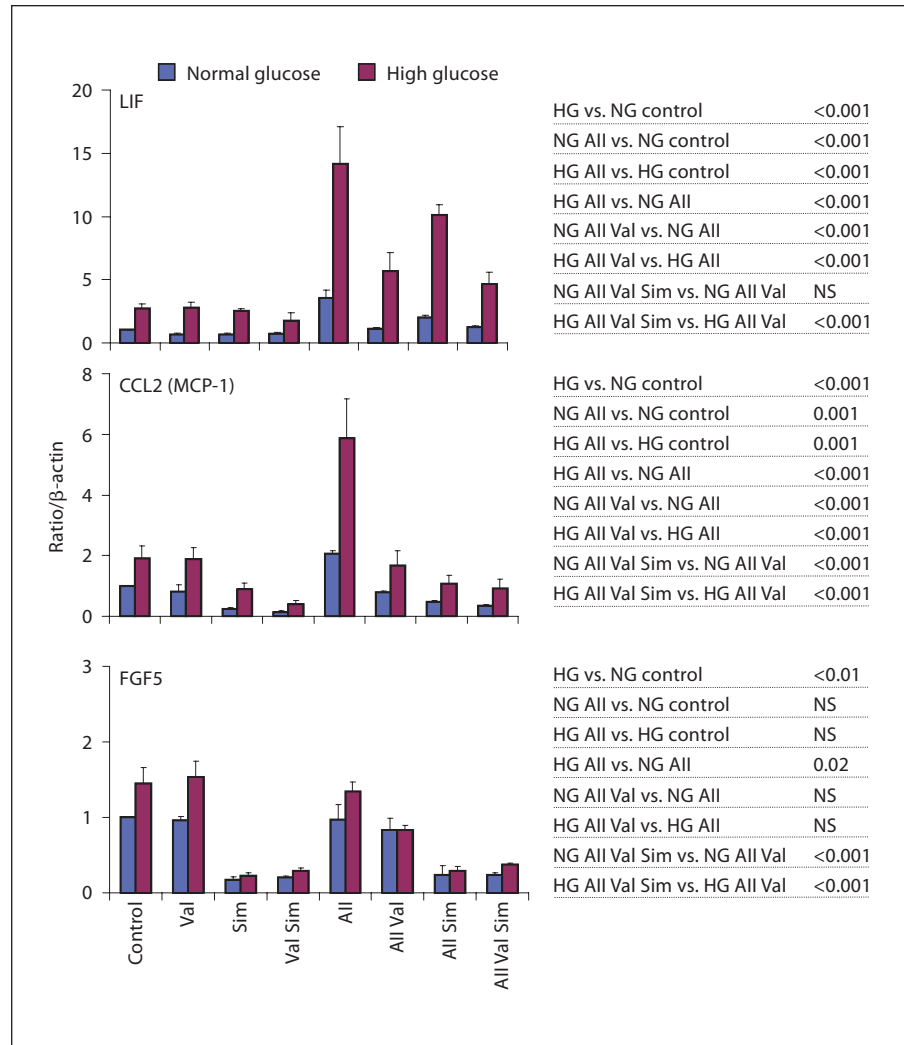
cleotide microarrays (Material and Methods). Data were acquired and analyzed using the Gene Traffic program. The list of genes and link to iHOP (Information Hyperlinked over Proteins) can be found at <http://www.ihop-net.org>. The AII-inducible genes encoding putative mediators of inflammatory responses are labeled in red in the online version.

renal [18, 55] outcomes appears well documented. HG by itself increased LIF mRNA levels  $2.71 \pm 0.35$ -fold over NG ( $p < 0.001$ ; fig. 2). AII-increased LIF mRNA levels  $3.56 \pm 0.63$ -fold in NG ( $p < 0.001$ ) and  $14.15 \pm 2.93$ -fold in HG ( $p < 0.001$ ), and the AII-induced mRNA level was greater in HG (HG AII vs. NG AII,  $p < 0.001$ ; fig. 2). The AII-induced increase in high ambient glucose was greater than the sum of AII and HG alone ( $\Delta$ HG AII vs. control –  $\Delta$ LG AII vs. control,  $p < 0.05$ ; table 3), suggesting synergism. Valsartan inhibited the AII-induced LIF

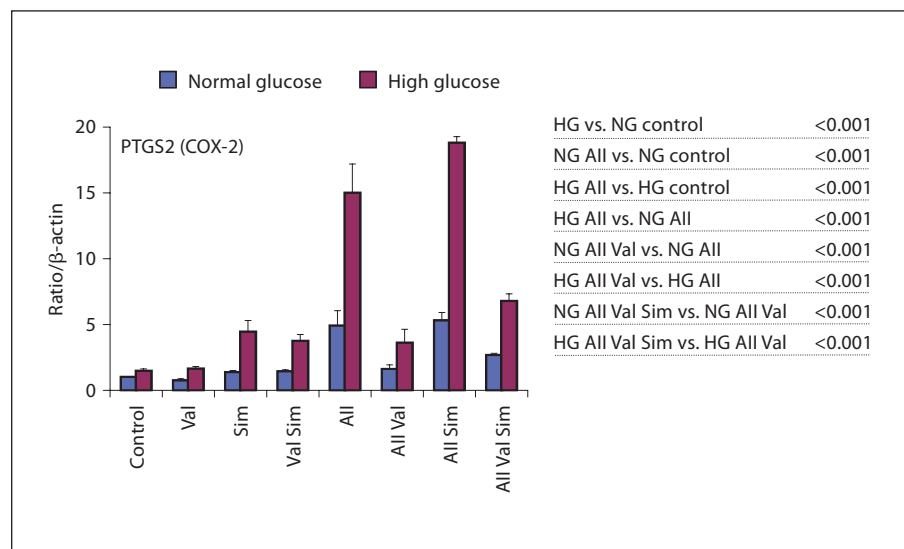
mRNA response in both NG ( $p < 0.001$ ) and HG ( $p < 0.001$ ) to  $1.12 \pm 0.05$  and  $5.66 \pm 1.46$  levels, respectively. Valsartan was a slightly more effective inhibitor of AII-induced LIF mRNA response compared to when used in combination with simvastatin in HG ( $p < 0.001$ ) but not NG ( $p = 0.071$ , NS).

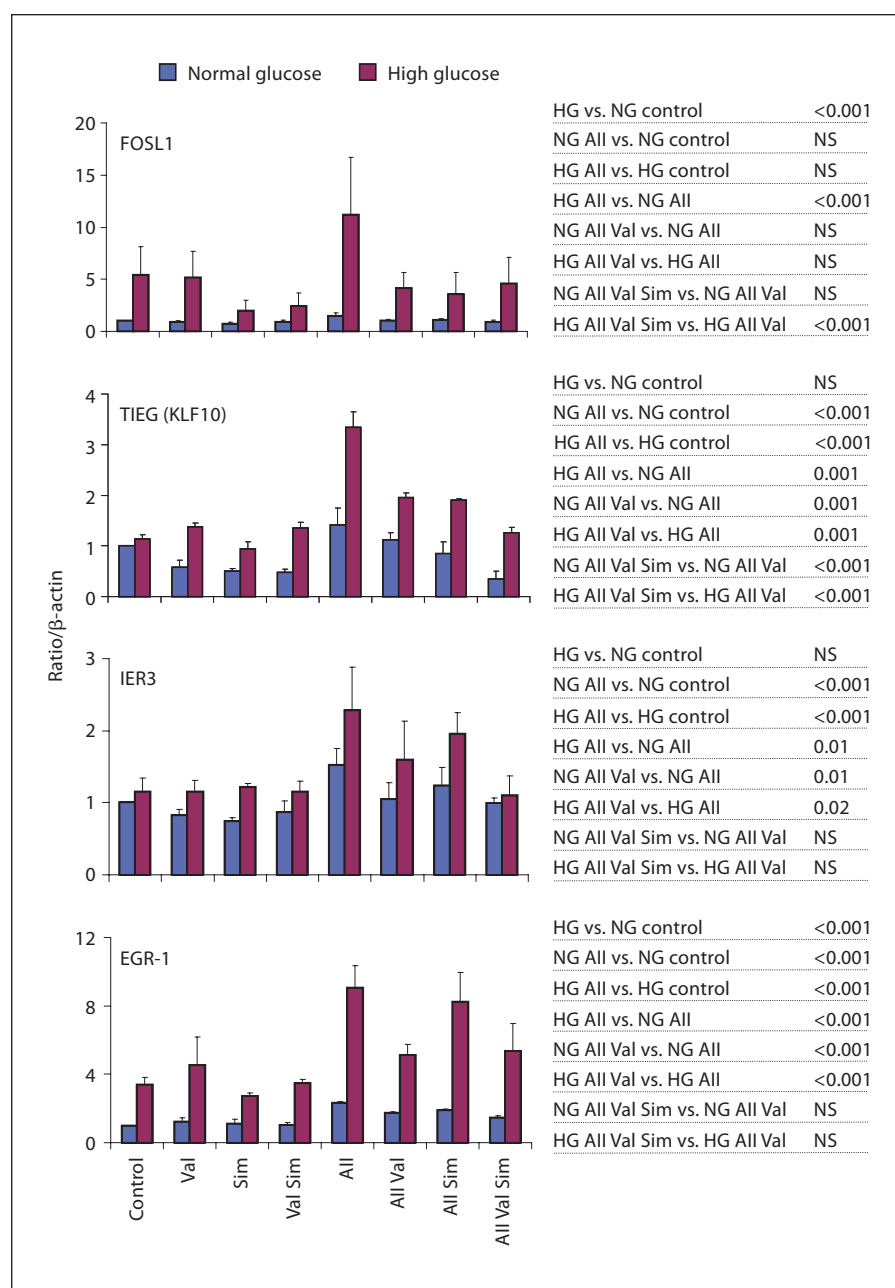
The chemokine MCP-1 (CCL2) is considered key mediator of AII-induced inflammation in the kidney and other organs [8, 15, 25, 48, 52, 53]. Production of MCP-1 by mesangial cells has been postulated to be part of an

**Fig. 2.** AII effects on cytokine mRNAs expressed in primary human mesangial cells maintained in normal and high glucose: effects of valsartan and/or simvastatin. Cells were grown to 75–85% confluence in NG (100 mg/dl) or HG (450 mg/ml), following serum deprivation (24 h) cells were incubated with or without 1  $\mu$ M simvastatin (24 h). After pretreatment with or without valsartan (100 nM, 30 min) cells were stimulated with or without AII (100 nM, 60 min; table 1). Total RNA was extracted, reverse transcribed and transcript levels were assessed by real-time PCR done in triplicates using specific primers. Results are reported as relative expression normalized to  $\beta$ -actin mRNA. Results shown as mean  $\pm$  SE from the data of three separate cell experiments. Statistical analysis is shown on the right and in table 3. NS = Statistically not significant.



**Fig. 3.** AII effects on mRNAs encoding enzymes expressed in primary human mesangial cells maintained in normal and high glucose: effects of valsartan and/or simvastatin. Results are reported as relative expression normalized to  $\beta$ -actin mRNA. Results shown as mean  $\pm$  SE from the data of three separate cell experiments. Statistical analysis is shown on the right and in table 3. NS = Statistically not significant.





**Fig. 4.** AII effects on mRNAs encoding transcription factors expressed in primary human mesangial cells maintained in normal and high glucose: effects of valsartan and/or simvastatin. Results are reported as relative expression normalized to  $\beta$ -actin mRNA. Results shown as mean  $\pm$  SE from the data of three separate cell experiments. Statistical analysis is shown on the right and in table 3. NS = Statistically not significant.

early inflammatory process that causes renal injury in diabetes. Transcription of MCP-1 in mesangial and other cell types is primarily regulated by NF- $\kappa$ B [15]. HG by itself increased MCP-1 mRNA levels by  $1.91 \pm 0.72$ -fold over NG ( $p < 0.001$ ; fig. 2). AII increased MCP-1 mRNA levels,  $2.07 \pm 0.08$ -fold in NG ( $p = 0.001$ ) and  $5.86 \pm 1.30$ -fold in HG ( $p = 0.001$ ; fig. 2). The AII-induced levels were greater in HG ( $p < 0.001$ ). Valsartan totally blocked the AII-induced MCP-1 mRNA response in both NG

( $p < 0.001$ ) and HG ( $p < 0.001$ ) to  $0.79 \pm 0.02$  and  $1.67 \pm 0.48$  levels, respectively. Pretreatment with simvastatin decreased the expression of MCP-1 mRNA ( $p < 0.001$ ; table 3) and prevented the AII-induced response. Addition of simvastatin to valsartan lowered AII-induced responses in both NG ( $p < 0.001$ ) and HG ( $p < 0.001$ ).

Fibroblast growth factors (FGFs), are a family of factors involved in many processes including wound healing

and embryonic development. In the glomerulus they appear to regulate mesangial cell proliferation [56, 57]. Real-time PCR did not confirm the AII-induced FGF5 mRNA levels ( $p = 0.357$  in NG,  $p = 0.317$  in HG).

The lack of real-time PCR confirmation of microarray data is not uncommon [58], underscoring the need to validate microarray data. Nonetheless, the real-time PCR revealed that simvastatin reduced FGF5 levels by more than 80%, an effect that was equally potent in NG and HG conditions ( $p < 0.001$ ; table 3).

### Enzymes

Cyclooxygenase-2 (COX-2) encoded by the *PTGS2* (prostaglandin-endoperoxide synthase 2) gene catalyzes the conversion of arachidonic acids to prostaglandins. In agreement with published reports in monocytes in vitro [59], and in the diabetic kidney in vivo [60, 61], HG increased COX-2 mRNA levels ( $1.46 \pm 0.17$ -fold over NG,  $p < 0.001$ ; fig. 3). Prostaglandins are synthesized in large amounts in the glomerulus in response to a host of ligands, including AII. Like AII, COX-2-derived metabolites have hemodynamic and mitogenic effects. AII potently induced COX-2 mRNA levels,  $4.90 \pm 1.15$ -fold in NG ( $p < 0.001$ ) and  $14.99 \pm 2.20$ -fold in HG ( $p < 0.001$ ; fig. 3). The AII-induced COX-2 mRNA levels were higher in HG compared to NG ( $p < 0.001$ ). Valsartan inhibited the AII-induced COX-2 mRNA response in both NG ( $p < 0.001$ ) and HG ( $p < 0.001$ ) to  $1.61 \pm 0.33$  and  $3.60 \pm 1.03$  levels, respectively. In contrast to the cytokine/chemokine effects, addition of simvastatin to valsartan increased AII-mediated COX-2 mRNA levels in both NG ( $p < 0.001$ ) and HG ( $p < 0.001$ ). In fact, simvastatin alone increased COX-2 mRNA levels, both in NG ( $1.36 \pm 0.09$ -fold,  $p < 0.05$ ) and in HG ( $4.43 \pm 0.88$ -fold,  $p < 0.001$ ).

### Transcription Factors

Fos-related antigen 1, FOSL1 or FRA1, is a member of the FOS family of transcription factors [62, 63] that are responsive to mitogens including AII [50] and insulin [62]. Increased expression of the FOS family of proteins has been reported in response to high glucose [64] and in diabetic nephropathy [65]. In agreement with these studies, ambient HG increased FOSL1 mRNA levels  $5.38 \pm 2.72$ -fold over NG ( $p < 0.001$ ; fig. 4). In AII-treated cells, FOSL1 mRNA levels averaged  $1.46 \pm 0.33$  in NG and  $11.16 \pm 5.56$  in HG (fig. 4), levels that were not statistically different from the untreated control. Nonetheless, statistical analysis of AII effects in HG versus NG showed that in the AII-treated cells the levels of FOSL1 mRNA

were higher in HG ( $p < 0.001$ ). This difference was not present in valsartan-pretreated cells. Thus, there is an interaction between AII and HG to increase FOSL1 mRNA levels.

TGF- $\beta$  activates expression of genes that encode factors promoting renal cell hypertrophy and stimulates extracellular matrix accumulation, lesions typical of diabetic nephropathy [66–68]. TGF- $\beta$ -inducible early growth response protein 1, TIEG (KLF10), is a Sp-1-like transcription factor. TIEG-1 has not previously been reported to respond to AII treatment. Unlike some of the other genes, HG by itself did not increase TIEG mRNA levels (HG vs. NG;  $p = \text{NS}$ ; fig. 4). AII increased TIEG mRNA levels  $1.41 \pm 0.33$ -fold in NG ( $p < 0.001$ ) and the AII-induced level was greater in HG,  $3.35 \pm 0.30$  (HG AII vs. NG AII  $p < 0.001$ ; fig. 4). Valsartan inhibited the AII-induced TIEG mRNA response in both NG ( $p = 0.001$ ) and HG ( $p = 0.001$ ),  $1.12 \pm 0.15$  and  $1.95 \pm 0.09$ , respectively. With the combination of valsartan and simvastatin the levels of AII-induced TIEG mRNA in NG and HG were lower than when each of the agents was used alone ( $p < 0.001$  in NG and HG).

The immediate early response 3 [IER3 (IEX1)] protein belongs to the NF- $\kappa$ B family of transcription factors. Expression of this gene is enhanced with cell proliferation and it may regulate apoptosis [69]. HG by itself did not increase IER3 mRNA levels (HG vs. NG;  $p = \text{NS}$ ; fig. 4). AII increased IER3 mRNA levels to  $1.52 \pm 0.52$  in NG ( $p < 0.001$ ) and in HG to  $2.28 \pm 0.61$  ( $p < 0.001$ ; fig. 4). In the presence of valsartan the AII-induced IER3 mRNA responses in both NG ( $p = 0.01$ ) and HG ( $p = 0.02$ ) were decreased. Addition of simvastatin did not appear to have an effect.

The EGR-1, early growth response-1, gene is a transcription factor of the immediate early response class [51]. Expression of EGR-1 is induced by a host of growth factors including insulin [70], AII [71] and high glucose [72]. Increased expression of EGR-1 has been linked to cardiovascular and renal disease [73, 74]. High ambient glucose increased EGR-1 mRNA levels  $3.38 \pm 0.44$ -fold compared to cells maintained in NG ( $p < 0.001$ ). AII increased EGR-1 mRNA levels,  $2.31 \pm 0.06$ -fold in NG ( $p < 0.001$ ) and  $9.06 \pm 1.28$ -fold in HG ( $p < 0.001$ ; fig. 4). The AII-induced levels were higher in HG (HG vs. NG,  $p < 0.001$ ). Valsartan inhibited the AII-induced EGR-1 mRNA response in both NG ( $p < 0.001$ ) and HG ( $p < 0.001$ ),  $1.72 \pm 0.10$  and  $5.11 \pm 0.64$ , respectively. Addition of simvastatin to valsartan had no effect on AII-induced responses.



## Discussion

Our study not only adds to the mounting evidence that AII activates expression of diverse classes of proinflammatory mediators [75, 76], but also provides new data that in human mesangial cells AII induction of proinflammatory gene mRNA expression is augmented in high ambient glucose. The AII effects are inhibited by valsartan and can be modulated by simvastatin.

We found that high ambient glucose (HG) altered constitutive (COX-2, LIF, MCP-1, FGF5, FOSL1, and EGR-1) and AII-inducible (COX-2, LIF, MCP-1, FGF5, FOSL1, EGR-1, IER3 and TIEG) levels of mRNAs encoding proinflammatory mediators.

In addition to intracellular glucose-specific pathways, HG levels may alter cell functions by increasing ambient osmolarity, an effect that is not unique to glucose. In some studies, osmotically active substances, such as mannitol, induced the same genes as HG, effects that may or may not represent shared hyperosmolarity-triggered pathways [77–82]. Regardless whether or not glucose and mannitol pathways are common, the hyperosmolarity is likely to play a role in gene induction in a variety of cell types because the postprandial osmolarity in diabetic patients is abnormally elevated [81–84].

Previous studies and our *in vitro* observations about levels of those mRNAs that encode factors involved in inflammatory responses suggest a possible model of how hyperglycemia could be aggravating the AII role in the development of diabetic nephropathy. The NF- $\kappa$ B/Rel family of transcription factors are among the most important intracellular mediators of inflammatory responses [16, 85, 86]. Hyperglycemia increases ROS production which in turn activates NF- $\kappa$ B [10, 59, 87]. Like high ambient glucose, AII also increases ROS production and activates NF- $\kappa$ B [10, 59, 87]. Both HG and AII have been shown to increase ROS production via NADPH oxidase [9, 77, 88–90]. There is evidence that HG- and AII-mediated activation of NADPH oxidase is mediated through the activation of protein kinase C (PKC) where the PKC- $\beta$  isoform may play a particularly important role [90]. Several of the AII- and HG-induced genes that we identified (fig. 2–4) including MCP-1 [8, 15, 25, 38], LIF/IL-6 [38], COX-2 [91], EGR-1 [92] and FOSL1 (Fra-1) [93] are activated by NF- $\kappa$ B. Thus, the greater AII-induced responses seen under high ambient glucose conditions may reflect augmented NF- $\kappa$ B responses. There are several potential NF- $\kappa$ B-dependent mechanisms that could be explain the synergistic (e.g. LIF) and additive (e.g. MCP-1) effects of AII and HG effects. (i) Both AII and HG ac-

tivate NF- $\kappa$ B and together the level of NF- $\kappa$ B activity is greater. Indeed, in addition to ROS, HG and AII synergistically activate signaling molecules acting upstream of NF- $\kappa$ B, such as PKC $\beta$  [94] and p38 MAP kinase [95]. Since PKC $\beta$  activates NADPH oxidase [90] this enzyme could be mediating HG-AII synergistic activation of ROS production. (ii) AII and HG activate other transcription factors that cooperate with NF- $\kappa$ B (e.g. EGR-1, KFL4 and FOSL-1). (iii) AII and HG increase synthesis of chemokines/cytokines that augment activation of NF- $\kappa$ B and other transcription factors via autocrine mechanism.

The ARB valsartan inhibited all AII-inducible genes studied here, suggesting that this agent has broad anti-inflammatory properties that appear as effective in high as in normal ambient glucose. The anti-inflammatory effects of ARBs likely reflect an indirect effect through the blockade of AT<sub>1</sub>R-mediated production of ROS intermediaries [88] and block of NF- $\kappa$ B induction which could result, in part, from the increased ROS levels [8, 87]. Because high ambient glucose renders the mesangial cells more sensitive to the AII proinflammatory action (fig. 2–4), the renoprotective RAS inhibition is particularly important in sparing the kidney from diabetes.

There is strong evidence that statins have organ-protective properties independent of their cholesterol-lowering effects [30, 36, 37, 96]. In experimental models of glomerulonephritis, lipid-induced nephritis and AII-induced injury, simvastatin suppressed mesangial cell proliferation, matrix expansion and inflammatory markers [37, 97–99], a process associated with decreased expression of cyclin-dependent kinase 2 (Cdk2), a key regulator of cell proliferation [100].

In our cultured human mesangial cell system, addition of simvastatin to valsartan further lowered some (e.g. MCP-1, LIF and TIEG) but not all (e.g. COX-2) AII-induced mRNAs. In addition, simvastatin by itself inhibited both HG-induced (FGF5, and MCP-1) and AII-inducible (FOSL1) gene expression. Taken together, these results suggest that some of the statin's renoprotective mechanisms are synergistic with RAS inhibitors, whereas others are mediated by pathways different from those of ARBs. One specific mechanism whereby statins ameliorate renal inflammation could be mediated by inhibition of small GTPases, such as RhoA [99, 101, 102]. However, we also found that simvastatin increased COX-2 mRNA expression, an effect that was greater in the HG environment (fig. 3). Statins have also been reported to increase COX-2 expression in macrophages, an effect mediated by ERK1/2 pathways [103]. Because hyperglycemia activates ERK1/2 [10], activation of this pathway thus

may explain the greater effect of simvastatin in HG. Taken together, these observations suggest that statins modulate inflammatory responses but, depending on the context, statins may have either anti- or proinflammatory-like effects.

It seems plausible that acting through both the same and different pathways the combined effects of ARBs and statins could be more renoprotective than using only one of the agents. Indeed, observations in some models of renal injury, such as Heymann nephritis, the combination of lisinopril with simvastatin was significantly more nephroprotective than monotherapies, including the inflammatory markers [104, 105]. Nonetheless, it should be noted that available evidence in experimental diabetic nephropathy about the potential superiority of combination of statins with RAS inhibitors is less clear. Although in streptozotocin-diabetic rats, this drug combination conferred superiority over monotherapies on renal function (prevention of albuminuria and rise in plasma BUN

and creatinine), no advantage of combination therapy was seen with respect to attenuating renal structural injury and renal expression of TGF- $\beta$  and VEGF in experimental diabetes [106].

In summary, our observations add to the growing evidence that ARBs and statins modulate renal cell inflammatory responses [4–6, 76]. However, in vivo studies measuring activities and protein levels encoded by the mRNAs identified in this and other studies are needed to fully explore the interaction between ARBs and statins in the diabetic kidney.

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