

# Effects of amino acids and glucose on mesangial cell aminopeptidase a and angiotensin receptors

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## Effects of amino acids and glucose on mesangial cell aminopeptidase a and angiotensin receptors.

**Background.** High protein diets and diabetes increase renal renin angiotensin system (RAS) activity, which is associated with glomerular injury. Aminopeptidase A (APA) is a cell surface metalloprotease that degrades angiotensin II (AII) in the mesangium. Mesangial cells (MC) also possess receptors for AII; the type 1 (AT<sub>1</sub> receptor) promotes proliferation and fibrosis, while the type 2 (AT<sub>2</sub> receptor) opposes these effects. We evaluated whether amino acids and glucose alter expression of APA, AT<sub>1</sub> receptor and AT<sub>2</sub> receptor in a manner that further augments RAS activity.

**Methods.** Confluent rat MC were grown in serum-free media for 48 hours prior to exposing to experimental conditions: control (C), high amino acids (HA, mixed amino acid solution added to raise concentrations 5- to 6-fold over C), high glucose (HG 30, mM glucose). Semi-quantitative RT-PCR was used to assess mRNA for APA, AT<sub>1</sub> receptor, AT<sub>2</sub> receptor, and  $\beta$ -actin. Values are expressed relative to  $\beta$  actin.

**Results.** Both HA and HG reduced APA mRNA (HG  $1.13 \pm 0.19$ , HA  $1.12 \pm 0.16$  versus C  $1.27 \pm 0.16$   $P < 0.05$ ,  $N = 8$ ). HA increased AT<sub>1</sub> receptor mRNA (HA  $2.11 \pm 0.43$  versus C  $1.14 \pm 0.28$   $P < 0.05$ ,  $N = 8$ ). HG increased AT<sub>2</sub> receptor mRNA (HG  $1.31 \pm 0.43$  versus C  $0.82 \pm 0.33$   $P < 0.05$ ,  $N = 6$ ).

**Conclusions.** A reduction of APA, in response to high levels of amino acids or glucose, could contribute to increased AII as a result of decreased degradation in MC. The effect of amino acids to increase AT<sub>1</sub> receptor expression may further enhance adverse hemodynamic and pro-fibrotic actions of AII. Conversely, glucose increased AT<sub>2</sub> receptor expression, which could modulate responses mediated by the AT<sub>1</sub> receptor.

## INTRODUCTION

The development of diabetic nephropathy is associated with augmentation of the renal renin angiotensin system (RAS) and increased production of angiotensin II (AII). Increased intrarenal AII promotes glomerular hyperfiltration and hypertension, as well as extracellular matrix production via stimulation of transforming growth factor-beta (TGF- $\beta$ ) production, a key cytokine in the fibrosis pathway [1]. Two receptor subtypes, the angiotensin type 1 (AT<sub>1</sub>) receptor and the type 2 (AT<sub>2</sub>) recep-

tor, mediate most of the known effects of AII. In the kidney, AT<sub>1</sub> receptors promote vasoconstriction, cell growth, and expression of genes for matrix proteins via increased TGF- $\beta$  expression [1, 2]. Less is known about the AT<sub>2</sub> receptor, except that it generally opposes the effects of AT<sub>1</sub> receptor [3]. In addition to augmented production, the amount of AII may also be increased by reduced degradation. Aminopeptidase A (APA) is a metalloprotease that degrades AII by N-terminal cleavage, producing angiotensin III. A recent study reported that overexpression of APA in murine mesangial cells (MC) reduced the proliferative response to AII [4]. APA is postulated to be an important regulator of glomerular AII activity [5–7].

Patients with diabetes often consume relatively high levels of dietary protein in an effort to control weight and carbohydrate intake. However, high protein diets are known to stimulate the renal RAS and, in animal models of diabetes, accelerate renal injury [8]. Conversely, low protein diets are protective against progression of renal disease in both animals and humans with diabetes [8, 9]. Protein restriction in other experimental models of renal disease also reduces proteinuria, fibrosis, and TGF- $\beta$  [10]. Renal injury accelerated by excess protein may be mediated by increased RAS activity, at least in part.

Diabetic animals that are allowed to be hyperglycemic also demonstrate an overall increase in the renal RAS [11]. Animal and clinical studies have proven the benefit of ACE inhibitors for decreasing progression of diabetic nephropathy, independent of blood pressure [12, 13]. Recently, Singh et al. reported that endogenous production of AII was increased in cultured rat MC exposed to high glucose, suggesting that MC themselves have the requisite RAS components [14]. The purpose of this study was to evaluate whether elevated levels of amino acids and glucose alter expression of the AT<sub>1</sub> receptor, the AT<sub>2</sub> receptor, and APA in a manner that could further augment AII activity in MC.

**Key words:** diabetes, high protein, metalloprotease.

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**Table 1.** Primer sequences used in PCR

Gene name	Genbank number	Primers	Position	Size (bp)
APA	S73583	F 5'-CATGAAGTCCGTCCTCCGATGAGC-3' R 5'-GTCACGATATTTCCAAACCACTGG-3'	36-57 399-422	387
$\beta$ -Actin	J00691	F 5'-AACCTTAAGGCCAACCGTGAAGAAG-3' R 5'-TCATGAGGTAGTCTGCTAGGT-3'	1659-1682 2342-2363	241
AT <sub>1</sub> receptor	M74054	F 5'-TTGGAACAGCTTGGTGGTGTAT-3' R 5'-CCAGAAAAGAAGAAGAAAAGCAC-3'	269-290 874-879	629
AT <sub>2</sub> receptor	D16840	F 5'-TTGCTGCCACCAGCAGAAAAC-3' R 5'-TGTGGGCTCCAAACCATTC-3'	159-178 1263-1283	1125

## METHODS

### Cell culture and experimental conditions

Rat MC were prepared from glomeruli isolated by differential sieving of the renal cortex excised from Sprague Dawley rats (a gift from William Couser, MD, and Richard Johnson, MD). Cells were verified to be MC by positive immunostaining with  $\alpha$ -smooth muscle actin, desmin, Thy-1.1, and negative staining with anti-RECA-1. Studies were conducted in MC at passages 14 to 19. For all experiments, cells were seeded at 10,000 cell/cm<sup>2</sup> in 100 mm plates (Nunc, Cambridge, MA). Cells were grown in Dulbecco's Minimal Essential Medium (DMEM, Life Technologies, Gaithersburg, MD), supplemented with penicillin-streptomycin (100 units each/ml) and 10% fetal calf serum (Summit, Ft. Collins, CO). MC were passaged at confluence using 0.025% trypsin in phosphate-buffered saline. Confluent cells were made quiescent in serum-free DMEM for 48 hours, followed by incubation for 48 hours in DMEM with the following: 1) control (C); 2) high levels of glucose [30 mol/L glucose (HG)]; and 3) high levels of amino acids [Travasol 10% amino acid solution, Baxter, Deerfield, IL; supplemented with l-arginine in order to raise concentrations 5- to 6-fold higher than DMEM (HA)]. Cell viability was assessed under each experimental condition at the initiation of the study using the MTT assay [15].

### Reverse transcription and polymerase chain reaction

PCR was used to assess steady state levels of AT<sub>1</sub> receptor, AT<sub>2</sub> receptor, APA, and  $\beta$ -actin mRNA. Total RNA was extracted and purified using the Tri reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription (RT) was carried out using 2.5  $\mu$ g of total RNA annealed with random hexamers in 25  $\mu$ L (First-strand cDNA Synthesis Kit™, Novogene, Madison, WI). PCR was performed using cDNA templates directly from the RT reactions, 0.2  $\mu$ L for  $\beta$ -actin and APA, and 1  $\mu$ L for AT<sub>1</sub> and AT<sub>2</sub> receptors. Primers (Ransom Hill Biosciences, Romona, CA) were used at 100 pmol/L (Table 1). Primer pairs for AT<sub>1</sub> receptor and AT<sub>2</sub> receptor were synthesized as reported [16, 17] and primers for APA and  $\beta$ -actin were designed using primer design software (P/C GENE; Intelligenetics, Palo Alto, CA). Magnesium concentrations were 2 mol/L, except for AT<sub>2</sub>

receptor (2.5 mol/L). PCR (25  $\mu$ L) was performed using hot-start, then denaturation for 30 seconds at 94°C, and elongation for 1:30 minutes at 73°C (Robocycler, Stratagene, LaJolla, CA). Primer annealing cycles were 1:30 minutes at 55°C for AT<sub>1</sub> receptor, AT<sub>2</sub> receptor, and  $\beta$ -actin, and 60°C for APA. Reactions were stopped in the exponential amplification phase. The average number of amplification cycles for each primer pair was: 32-36 for AT<sub>1</sub> receptor, 34-40 for AT<sub>2</sub> receptor, 18-22 for  $\beta$ -actin, and 28-32 for APA. PCR products were run on agarose gels (1.5%), soaked for 20 minutes in 0.05% ethidium bromide stain, and destained for 10 minutes. Digitalized image results were quantified by the Kodak Digital Science™ 1D Image Analysis Software (Eastman Kodak, Rochester, NY). Standard curves were generated using dilutions of cDNA from whole rat kidney for comparison to each experimental duplicate. Quantified data was compared to the linear amplification range of a standard curve on each gel. The "housekeeping gene,"  $\beta$ -actin was used to equalize for sample-to-sample variation in the efficiency of the reverse transcriptase reaction.

### Statistical analysis

Results were expressed as mean  $\pm$  SEM. Repeated measures analysis of variance was used to evaluate differences between the three treatment groups. Differences were considered significant if the *P* value was less than 0.05. All tests were two-tailed.

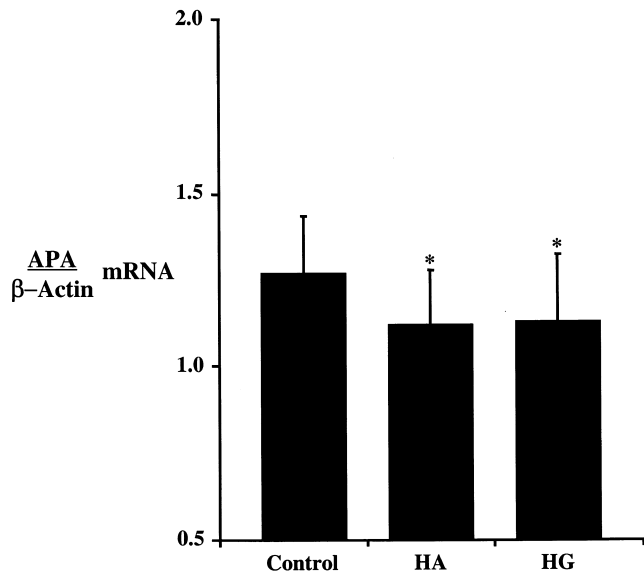
## RESULTS

### Effects of high levels of amino acids and glucose on aminopeptidase A mRNA

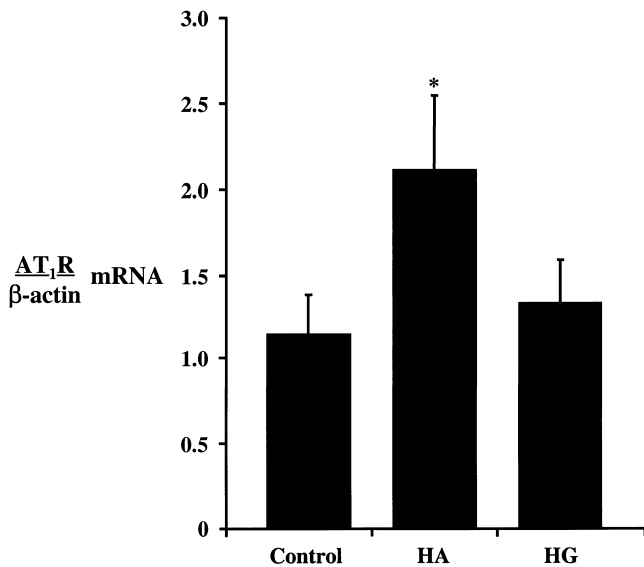
APA mRNA was assessed after 48 hours of experimental conditions (Fig. 1). In the group treated by HA, APA mRNA was reduced;  $1.12 \pm 0.16$  versus  $1.27 \pm 0.16$  in C, *P* = 0.03. HG also reduced APA mRNA to  $1.13 \pm 0.19$ , *P* = 0.02 compared to C (Fig. 1).

### Effects of high levels of amino acids and glucose on angiotensin type 1 receptor mRNA

HA treatment increased expression of the AT<sub>1</sub> receptor,  $2.11 \pm 0.43$  versus  $1.14 \pm 0.28$  in C, *P* = 0.03 (Fig. 2). In contrast, HG did not significantly change AT<sub>1</sub> receptor mRNA levels,  $1.33 \pm 0.25$ , *P* = 0.18 versus C (Fig. 2).



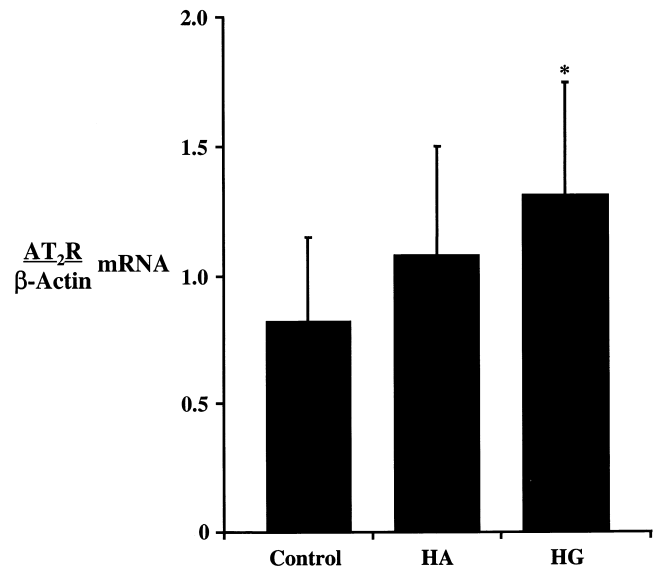
**Fig. 1.** Effects of high levels of amino acids (HA) and glucose (HG) on aminopeptidase A (APA) mRNA expression. Confluent MC were serum deprived for 48 hours then subjected to HA or HG conditions for 48 hours. Total RNA was isolated and mRNA was assessed by RT-PCR. The mRNA values are expressed in arbitrary units relative to  $\beta$ -actin for each sample. Bars indicate mean  $\pm$  SEM, \* $P < 0.05$ ,  $N = 8$ .



**Fig. 2.** Effects of high levels of amino acids (HA) and glucose (HG) on angiotensin type 1 receptor (AT<sub>1</sub>R) mRNA expression. Confluent MC were serum deprived for 48 hours then subjected to HA or HG conditions for 48 hours. Total RNA was isolated and mRNA was assessed by RT-PCR. The mRNA values are expressed in arbitrary units relative to  $\beta$ -actin for each sample. Bars indicate mean  $\pm$  SEM, \* $P < 0.05$ ,  $N = 8$ .

#### Effects of high levels of amino acids and glucose on angiotensin type 2 receptor mRNA

HG induced AT<sub>2</sub> receptor mRNA  $1.23 \pm 0.32$  versus  $0.82 \pm 0.33$  in C,  $P = 0.01$  (Fig. 3). However, HA did not significantly change AT<sub>2</sub> receptor mRNA levels,  $1.08 \pm 0.42$ ,  $P = 0.10$  versus C (Fig. 3).



**Fig. 3.** Effects of high levels of amino acids (HA) and glucose (HG) on angiotensin type 2 receptor (AT<sub>2</sub>R) mRNA expression. Confluent MC were serum deprived for 48 hours then subjected to HA or HG conditions for 48 hours. Total RNA was isolated and mRNA was assessed by RT-PCR. The mRNA values are expressed in arbitrary units relative to  $\beta$ -actin for each sample. Bars indicate mean  $\pm$  SEM, \* $P < 0.05$ ,  $N = 6$ .

#### DISCUSSION

In the present study, both HA and HG reduced expression of APA mRNA. Under the condition of HA, AT<sub>1</sub> receptor mRNA nearly doubled. To our knowledge, this is the first report of the effects of elevated amino acids on MC. These data suggest that high levels of amino acids, such as those that occur with a high protein diet, may further augment mesangial AII activity by both reduced degradation and increased expression of receptors that mediate its adverse hemodynamic and profibrotic effects. By contrast, HG increased AT<sub>2</sub> receptor mRNA, which may modulate the known effects of hyperglycemia to augment renal RAS activity.

MC are capable of generating RAS components, except for ACE [18, 19]. However, ACE is not required for AII generation [14]. Even though MC lack ACE, alternative pathways allow them to generate AII [20, 21]. The reduction of APA in response to HA and HG could further increase the amount of mesangial AII under conditions associated with high protein diets and diabetes. However, the reduction of APA mRNA may seem at variance with an in vivo study that demonstrated induction of APA expression in whole kidneys isolated from diabetic rats [22]. An explanation for these differences could be that, in diabetes, there is redistribution of expression of RAS components. For example, Anderson et al reported that ACE was increased in glomeruli and vasculature, despite a decrease in total renal ACE activity in diabetic rats [11]. Likewise, it is possible that APA may be reduced in the mesangium, despite being increased in other areas.

In our study, HA treatment of MC increased AT<sub>1</sub> mRNA expression nearly twofold. Assuming elevated mRNA levels are translated into an increase in functional receptors, high levels of amino acids could further enhance AII-mediated injury via a receptor mechanism. The relative increase in AT<sub>1</sub> receptors versus AT<sub>2</sub> receptors would be expected to produce adverse hemodynamic consequences and pro-fibrotic effects.

Results of our study showed that HG induced AT<sub>2</sub> receptor mRNA expression in MC. In comparison to activities mediated by the AT<sub>1</sub> receptor, only a few functions of the AT<sub>2</sub> receptor have been reported. However, activation of the AT<sub>2</sub> receptor generally produces effects that oppose the proliferative and fibrotic actions of the AT<sub>1</sub> receptor [3]. Interestingly, in vivo AT<sub>2</sub> receptor expression corresponds to active sites of cell growth, where AT<sub>2</sub> receptor activities may aid in control of proliferation and matrix protein formation [23–26]. Although hyperglycemia is well known to increase renal RAS activity, our data suggest that induction of the AT<sub>2</sub> receptor may modulate effects mediated by the AT<sub>1</sub> receptor. The relative contribution of AT<sub>2</sub> receptor induction to the pathogenesis of diabetic renal disease remains to be explored in experimental models.

In summary, both high levels of amino acids and glucose reduced expression of APA, which could contribute to increased AII by reduced degradation in MC. Expression of AT<sub>1</sub> receptors was nearly doubled when MC were exposed to increased levels of amino acids. This effect could enhance adverse hemodynamic and pro-fibrotic actions of AII. To the contrary, hyperglycemia induced the AT<sub>2</sub> receptor, an effect that may modulate responses mediated by the AT<sub>1</sub> receptor.

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