A haplotype of angiotensinogen gene that is associated with essential hypertension increases its promoter activity in adipocytes

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Abstract

Obesity is associated with hypertension and other cardiovascular diseases especially in the African-American population. Human angiotensinogen (AGT) gene has seven single nucleotide polymorphisms (SNPs) in 1.2 kb region of its promoter. Recent studies have shown that variant –217A is associated with hypertension in African-American and Chinese population. Nucleotide sequence of the hAGT gene has shown that variant –217A almost always occurs with variants –532T, –793A and –1074T (forming haplotype AAT) and variant –217G almost always occurs with variants –532C, –793G and –1074G (forming haplotype GGG). Since hAGT gene is expressed in the adipose tissue and its expression in this tissue may play a role in hypertension, we have analyzed the role of haplotypes AAT and GGG on the expression of this gene in adipocytes. We show here that a reporter construct with haplotype AAT of the hAGT gene has increased promoter activity on transient transfection in pre-adipocytes and differentiated adipocytes as compared to the reporter construct containing GCGG haplotype. Increased expression of the AGT gene containing haplotype AAT in the liver and adipocytes may be a contributing factor for hypertension.

Keywords: Hypertension; African-Americans; Angiotensinogen gene; Single nucleotide polymorphism; Glucocorticoids; Adipocytes

1. Introduction

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure (Burt et al., 1995; Fukamizu et al., 1990; Kannel, 2000; Mosterd et al., 1999). The renin–angiotensin system (RAS) plays an important role in the regulation of blood pressure. Angiotensin-II is one of the most potent vasopressor agents (Corvol et al., 1995; Corvol and Jeunemaitre, 1997) and is synthesized from its precursor molecule angiotensinogen (AGT) by the proteolytic action of renin and angiotensin-converting enzyme. Increased plasma AGT levels are associated with essential hypertension especially in African-American population (Bloem et al., 1997; Walker et al., 1979). The role of AGT in hypertension has been further substantiated by recent experiments where over-expression of the AGT gene increases blood pressure in transgenic mice (Kim et al., 1995; Kimura et al., 1992). In experimental as well as clinical studies, chronic administration of renin–angiotensin inhibitors has proven effective in lowering blood pressure in hypertension (Corvol et al., 1997).

Obesity is also associated with hypertension (Kopelman, 2000) and there is a positive correlation between plasma AGT levels (Bloem et al., 1997) and body mass index in different human populations (Cooper et al., 1998). Adipose tissue contains all the components of the renin–angiotensin system (Engeli et al., 1999) and is an important source of AGT (Engeli et al., 1999). An increased synthesis of AGT in adipose may explain high circulating levels of this protein in obesity. In support of this hypothesis, increased transcription of the AGT gene has been observed in the adipose tissue of obese human subjects (Van et al., 2000). Frederich et al. have shown that AGT mRNA level was threefold higher in the epididymal fat pad of the genetically obese mice (ob/ob and db/db) as compared to lean controls (Frederich et al., 1992). In addition, over-expression of the AGT gene in the adipose tissue has been shown to increase plasma AGT level and produce hypertension in transgenic mice (Massiera et al., 2001).

Hypertension is a polygenic disease and it has been suggested that genetic factors play an important role in this disease. AGT gene locus is associated with human essential
hypothesis in different ethnic groups (Jeunemaitre et al., 1992). Human AGT gene has seven single nucleotide polymorphisms (SNPs) in 1.2 kb region of its promoter (Jeunemaitre et al., 1997; Nakajima et al., 2002). These SNPs are: A/G at −6, C/A at −20, A/G at −217, C/T at −532, C/T at −776, A/G at −793, and T/G at −1074. We have recently shown that −217A allele of the AGT gene is associated with hypertension especially in African-American population (Jain et al., 2002). Reporter constructs containing human AGT gene promoter with nucleoside A at −217 have increased promoter activity on transient transfection in human liver cells. An oligonucleotide containing human AGT gene promoter with nucleoside A at −217 binds more strongly to CCAAT/enhancer binding protein (C/EBP) family of transcription factors (Jain et al., 2002) and glucocorticoid receptor (GR) (Jain et al., 2005). The role of −217A allele in hypertension has also been suggested by Wu et al who have shown that this allele is associated with hypertension in Chinese population (Wu et al., 2004). Nucleotide sequence of the 1.3hAGT gene has shown that variant −217A almost always occurs with variants −532T, −793A and −1074T (forming haplotype AAT) and variant −217G almost always occurs with variants −532C, −793G and −1074G (forming haplotype GCGG). Since AGT gene is expressed in adipocytes and obesity is associated with an increased risk of hypertension, our main objective in this paper was to study the effect of haplotypes AAT and GCGG on the expression of the hAGT gene in adipocytes. We show here that reporter constructs containing haplotype ATAT have increased promoter activity on transient transfection in pre-adipocytes and differentiated adipocytes. In addition we show that reporter construct containing six copies of an oligonucleotide with nucleoside A at −217 (present in haplotype ATAT) has increased glucocorticoid-induced promoter activity on transient transfections in adipocytes as compared to the same reporter construct with nucleoside G at −217 (present in haplotype GCGG).

2. Experimental procedures

2.1. Plasmid construction

Reporter construct pHAGT1.3luc with either AAT haplotype or GCGG haplotype was constructed by PCR amplification of human AGT gene from two different subjects containing ATAT or GCGG haplotypes in their genomic DNA using TATGCTAGTCAGTGCTCATCTTACGGCAAC as the forward primer and CAAGTGACTAAGTGAGTCTGAGGTGGGCCCCGGCTTA as the reverse primer (Fukamizu et al., 1990; Gaillard et al., 1989). The amplified fragment contained the nucleotide sequence −1206 to +37 and was subcloned in the pGL3 basic vector that lacks eukaryotic promoter and enhancer sequences (Promega, Madison, WI). Nucleotide sequences of reporter constructs were confirmed by sequence analysis. Reporter constructs (217A)_6-luc and (217G)_6-luc were constructed by random hexamerization of oligonucleotides CAGTG and CCGGTCACTCAGTCATCATCAGTG and CCTGCACCAGCTGACT GTGTCGACAGTG (position of A/G polymorphic site is underlined) and blunt ended ligation of hexamers in the SmaI site of pGL3 promoter vector. Expression vector RSV-β-gal was obtained from Promega. Restriction enzymes were purchased from New England Biolab (Beverly, MA). Plasmid DNAs for transient transfection were prepared by Qiagen midi or maxi plasmid kits (Chatsworth, CA) using conditions described by the manufacturer. PolyFect transfection reagent was also purchased from Qiagen.

2.2. Cell culture and transient transfection

For induction of adipogenic differentiation, 3T3-L1 cells were grown to confluence (designated as day 0) and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, 10 µg/ml insulin, 1 µmol/l dexamethasone, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) for 2 days. At the end of day 2, dexamethasone and IBMX were removed. Cells began to accumulate cytoplasmic triacylglycerol by day 3. Preadipocyte control cells were maintained in DMEM containing 10% calf serum. Control cells did not contain lipid droplets at any time. For transient transfections, reporter DNA (1.0 µg), and RSV β-gal DNA (0.05 µg) were mixed with pBluescript DNA to a final weight of 1.5 µg of DNA. Transient transfections were performed using the manufacturer protocol. For co-transfection experiments, expression vectors containing RSV-GR (0.2 µg) were added to the reporter constructs. After 24 h of transfection, cells were treated for an additional 24 h with dexamethasone (100 nmol/l). Cells were harvested 48 h post-transfection and whole cell extracts were prepared by resuspension in 100 µl of lysis buffer (Promega). An aliquot of the cell extract was used to measure luciferase activity by Turners Design Luminometer TD 20/20 using a luciferase assay system (Promega) as described by the manufacturer. Luciferase activity was normalized with the β-gal activity as described previously (Narayanan et al., 1998).

Unpaired t tests were performed to compare relative luciferase activities of reporter constructs containing haplotypes ATAT and GCGG of the AGT gene promoter in transfection experiments. All transfections were conducted in triplicates in four independent experiments as described (Jain et al., 2002). The fold increase was calculated from each set of transfection and then the mean value was derived from four different experiments. Statistically significant values (p<0.05) are shown by asterisks.

3. Results

3.1. Reporter construct 1.3hAGT-luc (ATAT) has increased promoter activity on transient transfection in NIH3T3-L1 cells as compared to the reporter construct 1.3hAGT-luc (GCGG)

The 1.2 kb promoter of the hAGT gene has seven SNPs as shown in Fig. 1. We have determined nucleotide sequence of

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1 It is important to mention that variant −6G always occurs with the haplotype GCGG but variant −6A occurs either with the haplotype ATAT or GCGG.
1.2 kb PCR-amplified promoter of hAGT gene from 86 African-American subjects and found that variant \(-217\) almost always occurs with variants \(-532\), \(-793\), and \(-1074\) (shown by asterisks in Fig. 1) (forming haplotype ATAT) and variant \(-217\) almost always occurs with variants \(-532\), \(-793\), and \(-1074\) (forming haplotype GCGG). Since hAGT gene is expressed in the adipose tissue and transgenic mice containing 1.3 kb of the hAGT gene promoter express this gene in adipocytes (Rahmouni et al., 2004; Yang et al., 1994), we wanted to examine whether reporter constructs containing haplotypes ATAT and GCGG alter the transcription of this gene in adipocytes. Since previous studies have shown that AGT gene expression starts after 3 days of differentiation and reaches to the maximum level after 6 days of differentiation (Saye et al., 1989), we performed transient transfections of reporter constructs 1.3hAGT-luc (AAT) and 1.3hAGT-luc (GGG) in NIH3T3 L-1 cells after 3 and 6 days of differentiation. Results of this experiment showed that promoter activity of ATAT haplotype was not statistically different in undifferentiated adipocytes (0 days of differentiation) (Fig. 2). However, promoter activity of ATAT haplotype was increased about 1.3-fold after 3 days of differentiation and about 1.7-fold after 6 days of differentiation as compared to the haplotype GCGG (Fig. 2).

3.2. Dexamethasone treatment increases the promoter activity of reporter construct (217A)\(_6\)-luc in NIH3T3-L-1 cells as compared to the reporter construct (217G)\(_6\)-luc

Since our previous studies have shown that A/G polymorphism at \(-217\) affects glucocorticoid-induced promoter activity in human liver cells (Jain et al., 2005), we were interested in examining whether this polymorphism also affects the glucocorticoid-induced promoter activity in adipocytes. To answer this question, we co-transfected reporter constructs (217A)\(_6\)-luc and (217G)\(_6\)-luc and expression vector RSV-GR were transiently co-transfected in NIH3T3 L-1 cells. After 24 h of transfection, cells were treated with 100 nM dexamethasone for another 24 h and promoter activity was analyzed as described. Dexamethasone-induced promoter activity was calculated by assuming the basal promoter activity as one.

![Graph showing relative luciferase activity](image1)

Fig. 1. Position of single-nucleotide polymorphisms in the promoter of hAGT gene. Variants that almost always occur together are shown by asterisks.

![Graph showing relative luciferase activity](image2)

Fig. 2. Reporter construct pHAGT1.3luc containing haplotype GCGG or ATAT was transiently transfected in NIH3T3 L-1 cells and cell extracts were prepared after 48 h of transfection promoter activity of ATAT haplotype was calculated by assuming the promoter activity of the haplotype GCGG as one. Statistically significant results are marked by asterisks.

![Graph showing relative luciferase activity](image3)

Fig. 3. Reporter constructs (217A)\(_6\)-luc and (217G)\(_6\)-luc and expression vector RSV-GR were transiently co-transfected in NIH3T3 L-1 cells. After 24 h of transfection, cells were treated with 100 nM dexamethasone for another 24 h and promoter activity was analyzed as described. Dexamethasone-induced promoter activity was calculated by assuming the basal promoter activity as one.
analyzed after dexamethasone treatment. Results of this experiment (Fig. 3) showed that promoter activity of reporter construct (217G)_6-luc was increased 1.8-fold whereas the promoter activity of reporter construct (217A)_6-luc was increased 4.2-fold after dexamethasone treatment. We next examined the effect of glucocorticoid treatment on the promoter activity of reporter constructs (217A)_6-luc and (217G)_6-luc in 6 days differentiated adipocytes. Results of this experiment (Fig. 4) showed that dexamethasone treatment increased the promoter activity of GCGG haplotype by 6-fold and of ATAT haplotype by 26-fold in differentiated adipocytes.

4. Discussion

The main observation of this study is that haplotype ATAT (which contains −217A in the promoter of the hAGT gene) has increased promoter activity on transient transfection in pre-adipocytes and differentiated adipocytes as compared to haplotype GCGG (which contains −217G in the promoter of the hAGT gene). Our results also show that A/G polymorphism at −217 affects the glucocorticoid-induced promoter activity of the reporter constructs in adipocytes. Previous studies have suggested that obesity is associated with increased plasma AGT levels (Bloem et al., 1995, 1997) and hypertension in different human populations (Cooper et al., 1998). Adipose tissue is a major source of AGT and transgenic mice that over-express the AGT gene specifically in the adipose tissue have increased circulating levels of AGT and develop hypertension (Massiera et al., 2001). Previous studies have also shown that fasting results in a significant reduction and re-feeding results in an increased adipose tissue-specific AGT gene expression in Sprague-Dawley rats (Frederich et al., 1992). Surprisingly, increased expression of the AGT gene in adipose tissue was also associated with increased blood pressure in these rats (Frederich et al., 1992). Moreover, the effect of feeding was specific on adipose-specific AGT gene expression since this effect was not observed in the liver. These studies therefore suggested a possible link between obesity, expression of AGT gene in the adipose tissue, and increased blood pressure in rats.

Glucocorticoids play an important role in differentiation of adipocytes. Glucocorticoid treatment rapidly increases the synthesis of C/EBP-β and -δ in adipocytes which plays a crucial role in the expression of adipogenic genes (MacDougald et al., 1994). Although very little is known about transcriptional regulation of the AGT gene in adipocytes, previous studies have shown that glucocorticoids are the single most important regulator of AGT gene expression in mouse adipocytes (Aubert et al., 1997). A recent study has confirmed that glucocorticoids play an important role in adipose tissue-specific expression of the AGT gene since increased synthesis of cortisol by over-expression of 11β-hydroxysteroid dehydrogenase (11β HSD-1) gene in adipose tissue of transgenic mice resulted in visceral obesity, increased AGT gene expression in adipose, and increased blood pressure (Masuzaki et al., 2001). Omental adipocytes display higher levels of GR as compared to subcutaneous adipocytes and increased expression of AGT gene by glucocorticoids may be responsible for its increased expression in visceral adipose tissue (Rebuffe-Scrive et al., 1990). Previous studies have also shown that GR physically interacts with STAT (Biola et al., 2000; Stocklin et al., 1996; Zhang et al., 1997), C/EBP (Gotoh et al., 1997) and CREB (Imai et al., 1993), and increases the expression of a number of genes that contain binding sites for these transcription factors. In addition, glucocorticoids rapidly increase the expression of C/EBPβ and C/EBPδ that in turn increase the expression of genes containing C/EBP binding sites such as PEPCK (MacDougald et al., 1994). Since previous studies have shown the presence of CRE (Narayanan et al., 2000), STAT-3 (Sherman and Brasier, 2001) and C/EBP (Narayanan et al., 1998) binding sites in the promoter of hAGT gene, increased binding of GR to −217A variant of the hAGT gene may explain increased expression of the hAGT gene in human subjects containing ATAT haplotype. It will be important to examine whether hAGT gene expression is increased in visceral adipose tissue of human subjects containing ATAT haplotype as compared to the GCGG haplotype.

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References


