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# Epigenetic Modification of the Renin-Angiotensin System in the Fetal Programming of Hypertension

Irina Bogdarina, Simon Welham, Peter J. King, Shamus P. Burns, Adrian J.L. Clark

**Abstract**—Hypertension is a major risk factor for cardiovascular and cerebrovascular disease. Lifelong environmental factors (eg, salt intake, obesity, alcohol) and genetic factors clearly contribute to the development of hypertension, but it has also been established that stress in utero may program the later development of the disease. This phenomenon, known as fetal programming can be modeled in a range of experimental animal models. In maternal low protein diet rat models of programming, administration of angiotensin converting enzyme inhibitors or angiotensin receptor antagonists in early life can prevent development of hypertension, thus implicating the renin-angiotensin system in this process. Here we show that in this model, expression of the AT<sub>1b</sub> angiotensin receptor gene in the adrenal gland is upregulated by the first week of life resulting in increased receptor protein expression consistent with the increased adrenal angiotensin responsiveness observed by others. Furthermore, we show that the proximal promoter of the AT<sub>1b</sub> gene in the adrenal is significantly undermethylated, and that in vitro, AT<sub>1b</sub> gene expression is highly dependent on promoter methylation. These data suggest a link between fetal insults to epigenetic modification of genes and the resultant alteration of gene expression in adult life leading ultimately to the development of hypertension. It seems highly probable that similar influences may be involved in the development of human hypertension. (*Circ Res.* 2007;100:520-526.)

**Key Words:** hypertension ■ angiotensin receptors ■ fetal programming ■ DNA methylation

Although hypertension is recognized as 1 of the major contributing factors to cerebrovascular and cardiovascular disease,<sup>1</sup> its pathogenesis remains incompletely understood. Genetic and environmental factors clearly contribute to this,<sup>2</sup> but on current models fail to account entirely for the disease. There is now substantial epidemiological evidence that intrauterine stress may program the later development of the disease.<sup>3</sup> The phenomenon of fetal programming can be modeled effectively in several mammalian species.<sup>4</sup> Most investigators have used models in which mothers are subjected to relative undernutrition during pregnancy. Offspring from these pregnancies exhibit later development of hypertension, insulin resistance, glucose intolerance and frank diabetes, the extent of each of these depending on the species and experimental model.<sup>4</sup>

Administration of a low protein diet (8% protein in place of 18% protein in normal rat chow with the calorific content of protein made up in the form of carbohydrate) to pregnant rats either until term or weaning has been widely used in the investigation of the pathogenesis of hypertension. This model produces offspring of reduced birth weight in which elevated systolic and diastolic blood pressures, as measured by tail cuff methods or by indwelling carotid artery catheters, can be identified as early as 4 weeks of age.<sup>5</sup> Hypertension can be

prevented in this model by administration of angiotensin converting enzyme inhibitors or angiotensin receptor antagonists, but not by nifedipene, between 2 and 4 weeks of age.<sup>6,7</sup> This is highly suggestive of a role of the renin-angiotensin system (RAS) in disease pathogenesis.

The consequence of the fetal insult in this and other programming models is that the phenotype is altered in a stable, but subtle manner. Such a phenotypic alteration may be achieved either by a change in the number or distribution of differentiated cells, or by changes in gene expression by individual cells, and there is evidence to support both occurrences.<sup>8</sup> Following embryo implantation, the majority of the genome is demethylated.<sup>9</sup> The process of differentiation and development is accompanied by the selective methylation of genes that are not needed for function of the differentiated cell. As this process of DNA methylation takes place in utero and in early postnatal development, it is a good candidate for disturbance by environmental interference, and thus provides a potential mechanism for fetal programming. Whereas methylation patterns are generally considered to be established in early postnatal life and persist thereafter, there is evidence from human monozygotic twin studies that methylation patterns can change with ageing.<sup>10</sup>

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It has been shown that a maternal low protein diet is associated with reduced global methylation, and it may be that it is deficiency of specific amino acids, eg, glycine, required to generate methyl donors, that underlies such changes.<sup>11</sup> Supplementation of low protein diets with glycine or folate reverses the programming effect of those diets.<sup>12,13</sup> However such a mechanism may lack gene specificity, whereas other data argues that it is specific genes that are susceptible to this effect.<sup>14–17</sup>

Thus in this study we set out to test the hypothesis that alteration of DNA methylation of 1 or more RAS component genes might underlie the alteration of gene expression that culminated in the development of hypertension.

## Materials and Methods

### Animals

All animal procedures were conducted in an approved facility in accordance with the Scientific Procedures (Animals) Act 1986, UK, and were approved by the Institutional Animal Use Ethics Committee. The model of fetal programming and the phenotypic characteristics of adult MLP rats have been described in detail.<sup>4</sup> Briefly rat dams (Wistar, Charles River, UK) were placed on either the normal 20% protein (control) rat chow or 8% protein (MLP, protein replaced with carbohydrate) at conception. Offspring were randomly culled to 8 in each litter at birth (4 male, 4 female) and then weaned on to identical (20% protein) rat chow at 3 weeks of age. Tissues (liver, lung, kidney, whole brain, adrenal, heart) were harvested from all members of a litter at 1, 4, or 12 weeks of age, and were immediately deep-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis. Up to 3 litters were studied in each set of analyses.

### RNA Isolation and Quantitation

Total RNA was isolated (QIAGEN, Crawley, UK), quantitated, and reverse-transcribed. Real-time QPCR was performed using a 2-step cycling protocol:  $95^{\circ}\text{C}\times 10$  minutes, then 40 cycles of  $95^{\circ}\text{C}\times 30\text{s}$  and  $59^{\circ}\text{C}\times 1$  minute. Q-PCR analysis was conducted in optical 96-well plates with optical caps, using the MX4000 (Stratagene). Primers and probes were designed to be intron-spanning wherever possible. All probes were 5' labeled with 6-FAM as a reporter dye and TAMRA as the 3' quencher. The 18S ribosomal RNA was used as an internal reference control. Amplification plots were analyzed using MX4000 software version 3.0 (Stratagene). RNA expression data were given as copy number of gene of interest/ $\mu\text{g}$  of RNA. Standards used were PCR fragments purified from polyacrylamide gel electrophoresis. All PCR reactions were performed in triplicate. Gene specific primer and probe sequences are shown in Table I of the online data supplement available at <http://circres.ahajournals.org>.

### Immunoblotting

Rat adrenals were homogenized in RIPA buffer. Laemmli buffer without mercaptoethanol was added to equal amounts of supernatants (50  $\mu\text{g}$  of protein). Proteins were incubated for 30 minutes at  $55^{\circ}\text{C}$  before being separated on SDS-PAGE and blotted onto PVDF membranes. Membranes were incubated with a monoclonal AT1 receptor antibody (TONI-1, 1:400, Abcam, Cambridge, UK) or rabbit anti-MAPK (1:1000, NEB, Hitchin, UK) as a loading control and immune complexes were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Chalfont St Giles, UK).

### Genomic DNA and Bisulphite Modification

Genomic DNA was isolated from whole adrenal or adrenal cortex using DNAsol reagent (Invitrogen, Paisley, UK) or QIAamp minikit (QIAGEN). DNA was then digested with restriction enzymes *EcoRV* and *Bgl II* (NEB), deproteinised with phenol/chloroform and ethanol precipitated. DNA was treated with sodium bisulphite according to modifications of the original protocol<sup>17</sup> and as described.<sup>18</sup> DNA was

ethanol precipitated, dissolved in 50  $\mu\text{L}$  water and used immediately for PCR or stored at  $-20^{\circ}\text{C}$ . PCR conditions were:  $94^{\circ}\text{C}\times 12$  minutes, then 40 cycles of  $94^{\circ}\text{C}\times 30\text{s}$ ,  $52^{\circ}\text{C}\times 1$  minute,  $72^{\circ}\text{C}\times 30\text{s}$  and finally 1 cycle of 7 minutes  $\times 72^{\circ}\text{C}$ . The reaction mixture contained  $1\times$  Ampli Gold PCR buffer, 0.2 mmol/L dNTPs, 2 mmol/L  $\text{MgCl}_2$ , 1  $\mu\text{mol/L}$  primers, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Warrington, UK) and 2 to 5  $\mu\text{L}$  DNA template. Primer sequences are shown in supplemental Table II of the online data supplement available at <http://circres.ahajournals.org>

### Pyrosequencing

PCR and sequencing primers for pyrosequencing were designed using PSQ Assay design software (Biotage AB, Uppsala). One of the PCR primers was biotinylated, and the biotinylated strands were purified and sequenced using the PSQ<sup>TM</sup> 96MA 2.1 instrument (Biotage AB). The primer sequences are listed in supplemental Table II. Calibration curves were recorded using five mixtures of PCR products (0, 25, 50, 75 and 100% methylation) prepared from cloned fully methylated and unmethylated gene promoter region of the rat  $\text{AT}_{1b}$  receptor.

### TOPO-TA Cloning and Sequencing

Gel sliced PCR products were passed through SNAP columns (Invitrogen) and used immediately for TOPO-cloning according to the manufacturers instruction (Invitrogen). Transformed TOP10 *E. coli* cells were selected on LB-ampicillin (100  $\mu\text{g}/\mu\text{L}$ ) agar and subjected to bacterial colony PCR. 15 independent clones containing the appropriate sized insert for each amplified fragment were sequenced on an ABI 3700 automated DNA sequencer (Applied Biosystems) in accordance with the manufacturer's instructions.

### $\text{AT}_{1b}$ Promoter Analysis

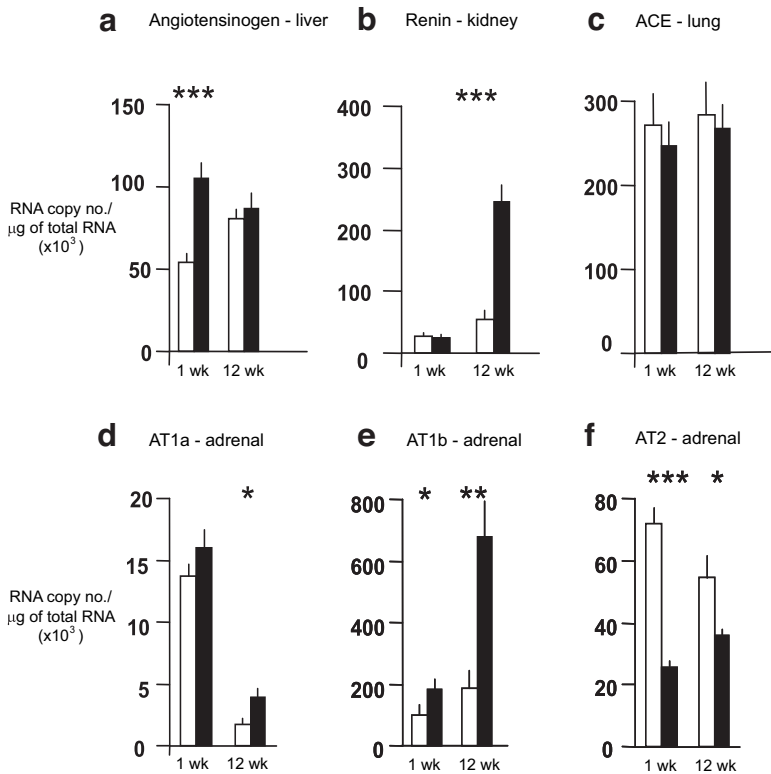
A 1.2kb fragment (positions 277 to 1611 from Genbank U01033) containing the rat  $\text{AT}_{1b}$  receptor promoter was cloned into pGL3 basic (Promega). The promoter was methylated in vitro with 10 U of *SssI* or *HhaI* (NEB) according to the manufacturers instructions. Controls included a mock methylated construct. 150 ng of each plasmid were then cotransfected with 20 ng of the pRL-CMV *Renilla* control vector (Promega) into mouse Y1 cells. After 24 hours cell lysates were prepared and luciferase activity was measured using the Dual-Luciferase reporter assay (Promega) and a Wallac Victor<sup>2</sup> 1420 Multilabel counter (Perkin Elmer, Finland). Reporter activity was calculated by normalizing the reporter luciferase value to that of the *Renilla* control vector. Site-directed mutagenesis of C residues at CpG sites 1 to 3 in the modified  $\text{AT}_{1b}$  promoter (positions 277 to 1454 from Genbank U01033) was performed using the QuickChange Site-directed mutagenesis protocol (Stratagene) according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing.

### Statistical Analysis

Expression levels were compared using student's 2-tailed t test and methylation density in control and MLP adrenals was compared using  $\chi^2$ .

## Results

Programmed and control animals from 10 control (5 male, 5 female) and 12 MLP (7 male, 5 female) offspring from 3 litters in each group were obtained as described and were harvested for liver, lung, kidney, brain, heart, and adrenal either at 1 or 12 weeks after birth. Real-time RT-PCR assays for angiotensinogen, angiotensin converting enzyme (ACE), renin, and the  $\text{AT}_{1a}$ ,  $\text{AT}_{1b}$ , and  $\text{AT}_2$  angiotensin receptors were performed. Absolute values for RNA copy number of angiotensinogen in the liver, renin in the kidney, ACE in the lung and the 3 angiotensin receptors in the adrenal are shown in Figure 1. Detailed results of all analyses are shown in



**Figure 1.** Altered expression of RAS genes in MLP offspring. Real-time RT-PCR was used to quantify angiotensinogen, renin, ACE, and the AT<sub>1a</sub>, AT<sub>1b</sub>, and AT<sub>2</sub> angiotensin receptor mRNAs in various control and MLP offspring rat tissues. Notable shifts in expression at either 1 or 12 weeks of age, or both, were demonstrated (a) for angiotensinogen in liver, (b) renin in kidney, and (d, e, and f) the 3 angiotensin receptors in the adrenal. Note the different range of the y axis scale. No changes in ACE expression were apparent (c). Data are expressed as RNA copy number per μg of total RNA. White columns, control animals; black columns, MLP animals. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001.

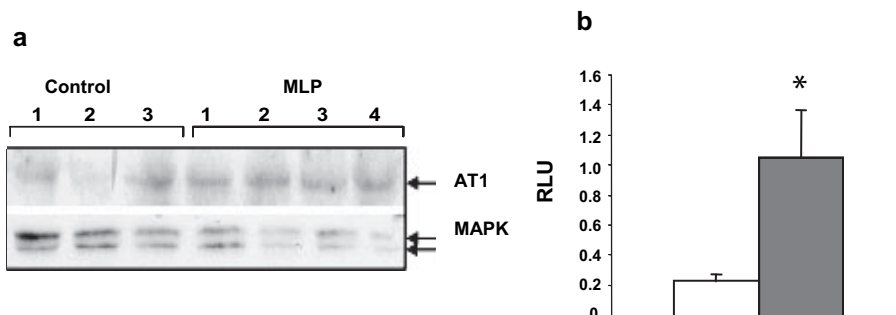
supplemental Table III. The only genes which showed a significant increase in expression in maternal low protein offspring (MLP) at both 1 and 12 weeks were the AT<sub>1a</sub> angiotensin receptor in the female, but not the male kidney, and the AT<sub>1b</sub> angiotensin receptor in the adrenal gland. RNA copy number for this gene is considerably greater than that for the other angiotensin receptors in this tissue. In contrast, the AT<sub>2</sub> receptor showed significant reduction of expression in the adrenal at both 1 and 12 weeks in MLP offspring.

Confirmation that these changes resulted in changes in receptor protein was provided by immunoblotting of adrenal lysates using an antibody that recognizes both AT<sub>1a</sub> and AT<sub>1b</sub> receptors which reveals significant differences on densitometry (Figure 2). The histological appearance of the adrenal was also investigated and shows no significant change in structure or zonal distribution (Figure 3).

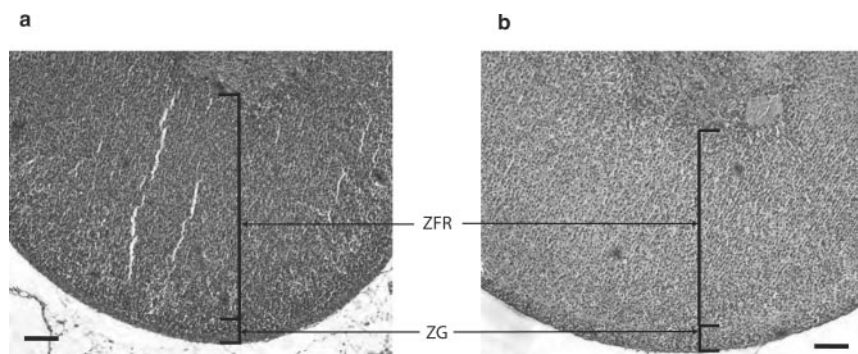
As the principal site of AT<sub>1b</sub> expression in the rat is the adrenal zona glomerulosa (ZG)<sup>20,21</sup> we prepared adrenal

capsules (which contain the majority of the ZG cell layer) from 4 week old programmed and control animals and used pooled tissue from one male and one female for methylation analysis to focus on the 3 CpG sites in the proximal AT<sub>1b</sub> promoter. Bisulphite converted DNA was subcloned and 30 individual clones from each group were sequenced This showed significantly less methylation at the three potentially methylatable sites in the proximal AT<sub>1b</sub> promoter ( $\chi^2=7.37$ ; *P*<0.01) (Figure 4a and b). The alteration of AT<sub>1b</sub> gene expression was confirmed in these same samples using real-time RT-PCR (Figure 4c).

In view of the possibility that pooling might obscure the variation between samples these studies were repeated in 4 week old animals from three independent litters in which each animal's whole adrenals were treated independently. Bisulphite conversion, PCR and subcloning was performed as before and revealed methylation at 21.8% of CpG sites in control animals against 7.4% of sites in programmed animals.



**Figure 2.** a, Immunoblot of AT<sub>1a</sub> and b receptor in whole adrenal from control and MLP rats at 12 weeks age. Total MAPK is used as a loading control. b, Densitometric quantitation of (a), corrected for protein loading and mean values+SD for 3 control (white bar) and 4 MLP adrenals (shaded bar) are shown. \**P*<0.05



**Figure 3.** Adrenal histology from control (a) and MLP rat (b) at 10X magnification. ZG=zona glomerulosa; ZFR=zona fasciculata and reticularis. Scale bar represents 100  $\mu\text{m}$ .

These results are shown in Figure 4d and are significantly different when assessed as a group (all 3 sites considered,  $\chi^2=7.73$ ,  $P<0.05$ ) or in the case of sites 1 and 3 when considered individually (site 1,  $\chi^2=11.88$ ,  $P<0.01$ ; site 3,  $\chi^2=7.65$ ,  $P<0.05$ ). Pyrosequencing analysis of this same region confirmed a similar significant reduction in methylation of each of the three sites (data not shown).

Analysis of 17 CpG sites in the  $AT_{1a}$  promoter and first exon using Pyrosequencing showed only occasional methylation of site 13 (position 3278 in rat  $AT_{1a}$  sequence; accession number S66402) and approximately 20% methylation of site 14 (position 3264), both located in exon 1. There was no difference in methylation frequency at this site between 5 MLP and 4 control offspring. There was no methylation of any other CpG site in this promoter in the adrenal.

The influence of methylation of the  $AT_{1b}$  promoter on gene expression was demonstrated by transfection of 1.2 Kbp of the  $AT_{1b}$  promoter coupled to a luciferase reporter gene into mouse adrenocortical Y1 cells. Treatment of this promoter with the site-specific *HhaI* methylase or the nonspecific CpG methylase *SssI* before transfection results in methylation of either 1 or all 6 CpG sites respectively in this fragment, and in the latter case is associated with markedly reduced promoter activity. When the 3 proximal sites analyzed for methylation were mutated so that they could no longer be modified by *SssI*, significant loss of promoter activity was observed. Methylation of the 3 remaining upstream CpG sites with *SssI* had no further influence. The contribution of each of the three proximal sites, and notably site 3 is revealed by analysis of the effect of their individual mutation without methylation (Figure 5).

## Discussion

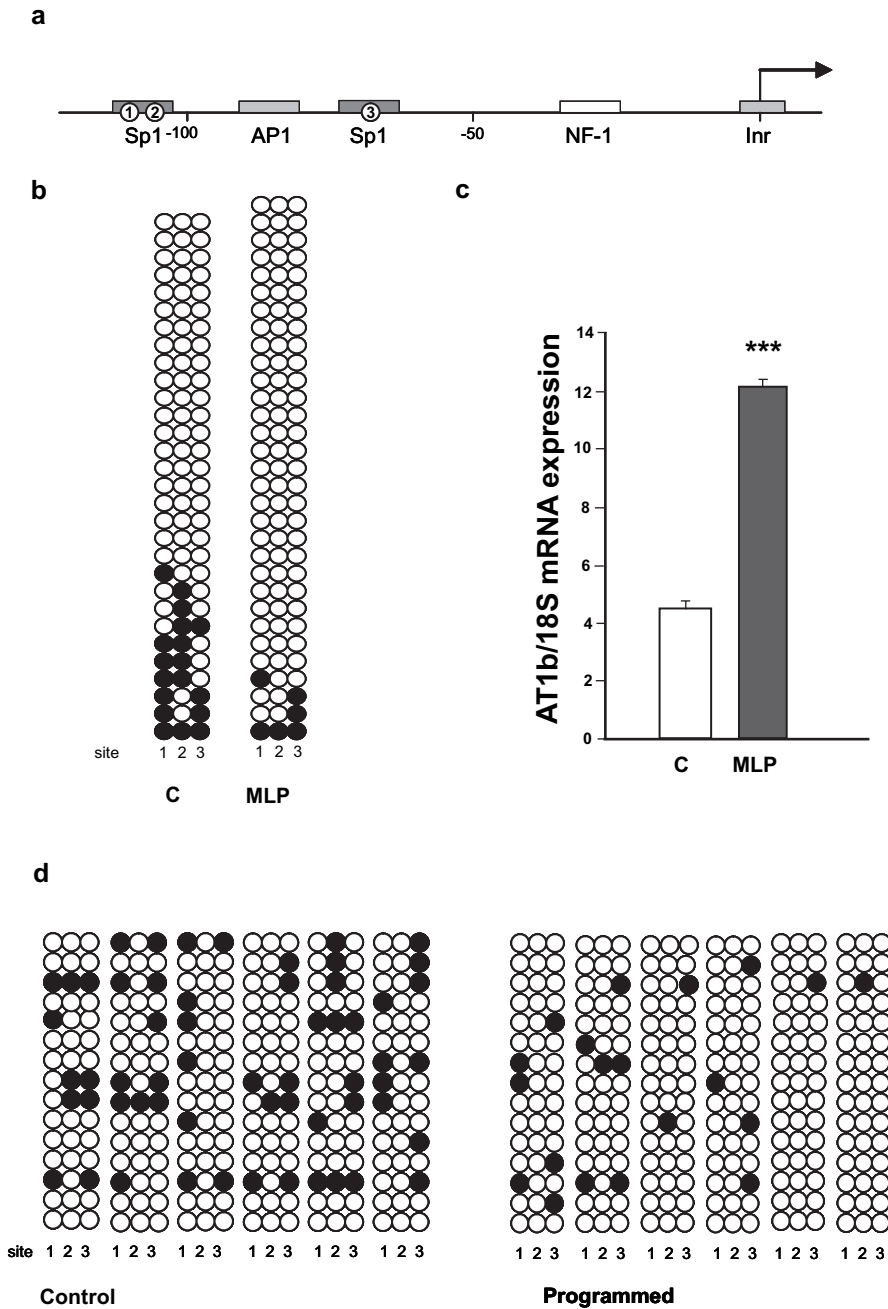
We show here that offspring of mothers fed a low protein diet develop increased expression of the  $AT_{1b}$  receptor mRNA and protein in the adrenal. The antibody used in immunoblotting will detect the  $AT_{1a}$  receptor equally well, but as the relative expression of  $AT_{1b}$  at the mRNA level in these adrenal glands is much greater we believe that the majority of receptor protein identified on immunoblotting is the  $AT_{1b}$  form. Similar findings of increased  $AT_1$  receptor expression have been reported in sheep following maternal protein deprivation.<sup>22</sup> Moreover it has also been shown that the MLP rat adrenal is more responsive to angiotensin II (Ang II).<sup>23</sup> Importantly we show that these changes are apparent very early in the life of programmed offspring and persist until at least 12 weeks of age. Certain genes

(angiotensinogen, renin and  $AT_{1a}$  receptor in kidney and  $AT_{1a}$  and  $AT_2$  receptor in the adrenal) show significant changes in expression at 12 weeks of age which may relate to the early consequences of developing hypertension. Other genes such as angiotensinogen and the  $AT_{1a}$  receptor in the liver show increased expression at 1 week of age which has normalized by 12 weeks. We speculate that this may reflect recovery from the period of relative undernutrition. These studies were not set up to investigate sex differences in gene expression, but it is interesting to note that female rats show significantly increased  $AT_{1a}$  expression in the MLP kidney at both 1 and 12 weeks of age and this observation may be worthy of further investigation.

Interestingly, expression of the  $AT_{1b}$  receptor is similar or reduced in the spontaneously hypertensive rat when compared with control Wistar Kyoto rats, probably as an adaptive response.<sup>24</sup> Because this receptor mediates the action of the peptide Ang II to stimulate adrenal aldosterone production, it is likely to contribute to the subsequent development of hypertension. Furthermore, it has been shown that blockade of Ang II production with ACE inhibitors, or interaction with the  $AT_1$  receptor using a receptor antagonist will prevent development of hypertension in MLP offspring in contrast to nifedipene.<sup>6,7</sup> These findings confirm an important, if not unique, role for this receptor in this model. Investigation of the role of the  $AT_{1b}$  receptor in mice in which 1 or the other, or both  $AT_1$  receptors have been deleted suggests the  $AT_{1b}$  receptor contributes to blood pressure maintenance and in the absence of  $AT_{1a}$  receptors mediates a pressor response to angiotensin.<sup>25–27</sup>

Two possible explanations for increased receptor expression are conceivable. Expression of the  $AT_{1b}$  receptors in the rat adrenal is almost entirely restricted to the ZG, and fetal hyperproliferation of this cell type might be consistent with the increased quantity of  $AT_{1b}$  mRNA expressed in the adrenal. Differentiation of the rat ZG cells is first apparent just before birth<sup>28,29</sup> and although this might be influenced by the continuation of the low protein diet during suckling, the hypertensive phenotype can equally be observed in animals that receive the low protein diet in pregnancy alone. However histological examination of the adrenal (Figure 3) shows no apparent ZG hyperplasia and MLP animals cannot be distinguished from controls.

A second explanation for increased  $AT_{1b}$  receptor gene expression is that the fetal insult has induced a persistent modification of  $AT_{1b}$  gene expression. Altered DNA methylation has long been proposed as a candidate mechanism for fetal programming, although there are few examples in which



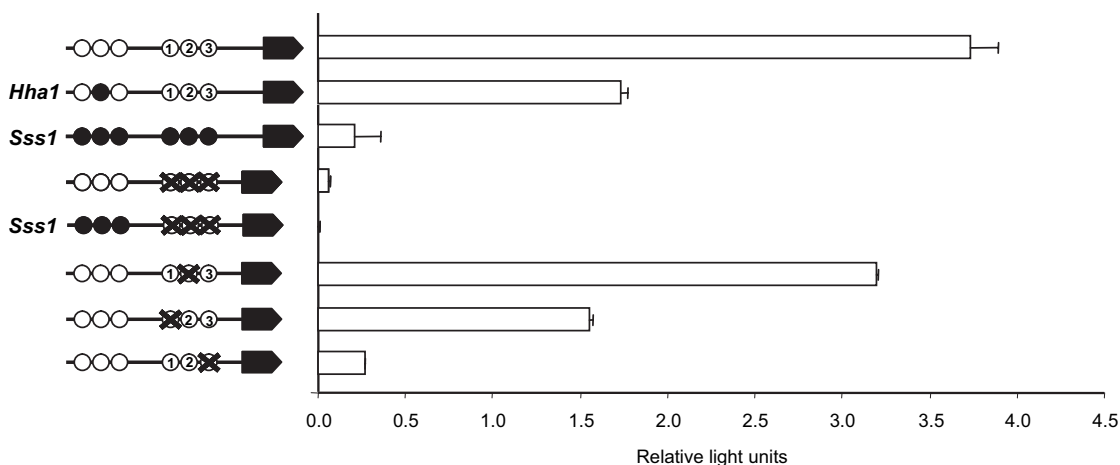
**Figure 4.** Methylation analysis of the AT<sub>1b</sub> promoter. **a**, Structure of the AT<sub>1b</sub> proximal promoter region showing location of putative transcription start site, consensus sequences for potential transcription factors and CpG methylation sites (open circles) analyzed in this study numbered 1 to 3. Inr=Initiator element. **b**, Methylation of adrenal capsule AT<sub>1b</sub> gene in control and MLP animals. ○=unmethylated; ●=methylated. One male and 1 female from each group had adrenal capsules harvested and used in this analysis. **c**, Expression of AT<sub>1b</sub> mRNA in these same adrenal capsule samples expressed relative to 18S RNA. Control rats, white bar; MLP rats, shaded bar. \*\*\**P*<0.0001. **d**, Methylation of AT<sub>1b</sub> gene proximal promoter in individual adrenals from six different animals in each group at 4 weeks of age. Each column represents an individual animal's adrenal in which methylation status is determined at each of the 3 sites of interest. Fifteen clones were examined from each adrenal.

this has been directly demonstrated by bisulphate sequencing<sup>14,16</sup> or inferred from methylation sensitive PCR analysis,<sup>15,17</sup> and at least one case in which increased gene expression appears to be independent of DNA methylation.<sup>19</sup> We have shown here that the AT<sub>1b</sub> gene promoter, which quite clearly is highly susceptible to regulation by DNA methylation, is indeed less methylated in programmed animals. The present work demonstrates a direct link between maternal environmental factors that cause hypertension and epigenetic modification of a gene promoter.

It is interesting to note that the proportion of AT<sub>1b</sub> methylation in the adrenal capsule, representing a relatively pure ZG cell preparation (Figure 4b), is not highly different from that in the whole adrenal (Figure 4d). The most likely explanation for this is that all adrenocortical

cells develop from a stem cell in which the AT<sub>1b</sub> methylation pattern has been set.<sup>30</sup> Thus the methylation pattern in the ZG cell would be reflected in cells in the fasciculata and reticularis cell layers, even though these cells do not express the receptor.

The molecular mechanisms that underlie such an alteration in methylation pattern as a result of fetal programming remain to be determined. One proposal is that undernutrition leads to deficiency of methyl donors and, conceivably, certain genes might be more susceptible to this influence than others.<sup>8</sup> A substantial body of evidence suggests that programmed phenomena can result from excessive glucocorticoid action either when directly administered to pregnant dams or as a result of maternal stress.<sup>31</sup> Whether such an occurrence can influence the normal process of DNA meth-



**Figure 5.** Influence of methylation on the AT<sub>1b</sub> promoter. Luciferase activity of AT<sub>1b</sub>-luc transiently transfected into mouse Y1 cells in the presence or absence of methylation of either 1 or 6 CpG sites in the proximal promoter using *Hha1* methylase (*Hha1*) or *Sss1* methylase (*Sss1*) respectively. Mutagenesis of the 3 proximal sites<sup>1-3</sup> to render them nonmethylatable markedly reduces promoter activity, and methylation of the remaining promoter makes no further contribution to reduced activity. Mutagenesis of individual CpG sites in the proximal promoter reveals the considerable impact of site 3. Mean luciferase activity is expressed relative to cotransfected renilla luciferase activity ( $\pm$ SEM).  $n=6$ .  $\circ$ =unmethylated site,  $\bullet$ =methylated site,  $\times$ =mutagenised site.

ylation remains to be determined. The data obtained using in vitro methylation shown in Figure 5 argue that CpG site 3 has a particularly potent influence on promoter activity in that when mutated or methylated the promoter is essentially inactive. This base is at the center of an Sp1 consensus site, suggesting that failure to bind this, or a related transcription factor may have a crucial impact. However it is generally considered that Sp1 binding is not influenced by DNA methylation<sup>32</sup> although there are exceptions to this<sup>33</sup> and it is possible that another methylation sensitive transcription factor binds here.

The human genome only contains a single AT<sub>1</sub> receptor gene which is widely expressed in a pattern similar to that of the AT<sub>1a</sub> receptor.<sup>34</sup> There is no human equivalent to the relatively adrenal-specific AT<sub>1b</sub> receptor. Thus these findings may be difficult to translate directly into the human hypertensive scenario. Furthermore there is also data derived from human monozygotic twin studies that challenges the view that methylation patterns are established in early life and remain unchanged thereafter.<sup>10</sup> Nevertheless, the possibility that maternal undernutrition may result in highly specific altered methylation patterns in the offspring provides increased mechanistic understanding worthy of investigation in man.

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### Disclosures

None.

### References

- He FJ, MacGregor GA. Cost of poor blood pressure control in the UK: 62,000 unnecessary deaths per year. *J Hum Hypertens.* 2003;17:455–457.
- Staessen JA, Wang J, Bianchi G, Birkenhager WH. Essential hypertension. *Lancet.* 2003;361:1629–1641.
- Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ.* 1989;298:564–567.
- Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. *Br Med Bull.* 2001;60:103–121.
- Langley-Evans SC. Critical differences between two low protein diet protocols in the programming of hypertension in the rat. *Int J Food Sci Nutr.* 2000;51:11–17.
- Sherman RC, Langley-Evans SC. Early administration of angiotensin-converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low-protein diet in the rat. *Clin Sci (Lond).* 1998;94:373–381.
- Sherman RC, Langley-Evans SC. Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. *Clin Sci (Lond).* 2000;98:269–275.
- Burns SP, Desai M, Cohen RD, Hales CN, Iles RA, Germain JP, Going TC, Bailey RA. Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *J Clin Invest.* 1997;100:1768–1774.
- Cedar H. DNA methylation and gene activity. *Cell.* 1988;53:3–4.
- Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A.* 2005;102:10604–10609.
- Rees WD, Hay SM, Brown DS, Antipatis C, Palmer RM. Maternal protein deficiency causes hypermethylation of DNA in the livers of rat fetuses. *J Nutr.* 2000;130:1821–1826.
- Jackson AA, Dunn RL, Marchand MC, Langley-Evans SC. Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine. *Clin Sci (Lond).* 2002; 103:633–639.
- Brawley L, Torrens C, Anthony FW, Itoh S, Wheeler T, Jackson AA, Clough GF, Poston L, Hanson MA. Glycine rectifies vascular dysfunction induced by dietary protein imbalance during pregnancy. *J Physiol.* 2004; 554:497–504.
- Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. Epigenetic programming by maternal behavior. *Nat Neurosci.* 2004;7:847–854.
- Lillicrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr.* 2005;135:1382–1386.
- Pham TD, MacLennan NK, Chiu CT, Laksana GS, Hsu JL, Lane RH. Uteroplacental insufficiency increases apoptosis and alters p53 gene



- methylation in the full-term IUGR rat kidney. *Am J Physiol Regul Integr Comp Physiol*. 2003;285:R962–R970.
17. Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect*. 2006;114:567–572.
  18. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl AcadSci USA*. 1992;89:1827–1831.
  19. Bogdarina I, Murphy HC, Burns SP, Clark AJ. Investigation of the role of epigenetic modification of the rat glucokinase gene in fetal programming. *Life Sci*. 2004;74:1407–1415.
  20. Sandberg K, Ji H, Clark AJ, Shapira H, Catt KJ. Cloning and expression of a novel angiotensin II receptor subtype. *J Biol Chem*. 1992;267:9455–9458.
  21. Gasc JM, Shanmugam S, Sibony M, Corvol P. Tissue-specific expression of type 1 angiotensin II receptor subtypes. An in situ hybridization study. *Hypertension*. 1994;24:531–537.
  22. Whorwood CB, Firth KM, Budge H, Symonds ME. Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. *Endocrinol*. 2001;142:2854–2864.
  23. McMullen S, Gardner DS, Langley-Evans SC. Prenatal programming of angiotensin II type 2 receptor expression in the rat. *Br J Nutr*. 2004;91:133–140.
  24. Jhren O, Golsch C, Dendorfer A, Qadri F, Hauser W, Dominiak P. Differential expression of AT1 receptors in the pituitary and adrenal gland of SHR and WKY. *Hypertension*. 2003;41:984–990.
  25. Chen X, Li W, Yoshida H, Tsuchida S, Nishimura H, Takemoto F, Okubo S, Fogo A, Matsusaka T, Ichikawa I. Targeting deletion of angiotensin type 1B receptor gene in the mouse. *Am J Physiol*. 1997;272:F299–F304.
  26. Oliverio MI, Best CF, Kim HS, Arendshorst WJ, Smithies O, Coffman TM. Angiotensin II responses in AT1A receptor-deficient mice: a role for AT1B receptors in blood pressure regulation. *Am J Physiol*. 1997;272:F515–F520.
  27. Audoly LP, Oliverio MI, Coffman TM. Insights into the functions of type 1 (AT<sub>1</sub>) angiotensin receptors provided by gene targeting. *Trends Endocrinol Metab*. 2000;11:263–269.
  28. Mitani F, Mukai K, Ogawa T, Miyamoto H, Ishimura Y. Expression of cytochromes P450aldo and P45011 beta in rat adrenal gland during late gestational and neonatal stages. *Steroids*. 1997;62:57–61.
  29. Wotus C, Levay-Young BK, Rogers LM, Gomez-Sanchez CE, Engeland WC. Development of adrenal zonation in fetal rats defined by expression of aldosterone synthase and 11beta-hydroxylase. *Endocrinol*. 1998;139:4397–4403.
  30. Hammer GD, Parker KL, Schimmer BP. Minireview: transcriptional regulation of adrenocortical development. *Endocrinol*. 2005;146:1018–1024.
  31. Seckl JR, Meaney MJ. Glucocorticoid programming. *Ann N Y Acad Sci*. 2004;1032:63–84.
  32. Tate PH, Bird AP. Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr Opin Genet Dev*. 1993;3:226–231.
  33. Alikhani-Koopaei R, Fouladkou F, Frey FJ, Frey BM. Epigenetic regulation of 11 beta-hydroxysteroid dehydrogenase type 2 expression. *J Clin Invest*. 2004;114:1146–1157.
  34. Inagami T. Recent progress in molecular and cell biological studies of angiotensin receptors. *Curr Opin Nephrol Hypertens*. 1995;4:47–54.

**Online Table 1**

Sequence of DNA oligonucleotide primers and probes used in RNA quantitation experiments

<b>GENE</b>	<b>PRIMERS and PROBES</b>
<b>RENIN</b> NM_012642	5' (F) GTAAGTGTGGGTGGAATCATTGTG 5' (R) TGGGAGAGAATGTGGTCTGAAGA probe TTGGAGAGGTCACCGAGCTGCCCC
<b>AT1A</b> M86912	5' (F) GGAGAGGATTCGTGGCTTGAG 5' (R) CTTTCTGGGAGGGTTGTGTGAT probe TTCCACCCGATCACCGATCACCGG
<b>AT1B</b> M90065	5' (F) TTGTCCACCCAATGAAGTCTCG 5' (R) CGCAAAGTGTGATATTGGTGTCT probe CCGCCGCACGATGCTGGTAGCC
<b>AT2</b> D16840	5' (F) CATCACCAGCAGTCTTCCTTTTG 5' (R) AAAACAGTGAGACCACAACAATGT probe CGCAAAGTGGCACCAATGAGTCCGC
<b>ACE</b> NM_12544	5' (F) CGGGTCGCAGAGGAATTCTT 5' (R) CCTGAAGTCCTTCCTGTTGTAGA probe CACGCAGAGGCATGGCACACCACC
<b>Angiotensinogen</b> NM_134432	5' (F) AGAACCCAGTGTGGAGACG 5' (R) AGCCAACCTTTGAGCCTGTGCCCA probe AGCCAACCTTTGAGCCTGTGCCCA

**Online Table 2**

Primers used for AT1a (top) and AT1b (bottom) bisulphate sequencing and pyrosequencing

GENE	PRIMERS
<b>AT1A</b> Amplification	F1A 5'- TGTTAGTATTTTGATTTAGATGTGG R1A 5'- CATATAACCTTATACCCACCTC- <b>biotin</b> F2A 5'- TGTTTGGAGGTTAGTAGTTAG R2A 5'- AACCTAAAATTTAAACCTACAAAACAA- <b>biotin</b>
<b>AT1A</b> Pyrosequencing	SeqF(6-12) 5'- AATGTGGTAGAGTTGAGAGA SeqF(13-14) 5'- GGGTGAATAGGATTTAAG SeqF(15-16) 5'- TTTATATTTATAGTTTAGGG SeqF(17-19) 5'- GTTTTGTAGGTTTTAATTTT SeqF(20-22) 5'- TTTGAGATTTTTTGTTTAAT

GENE	PRIMERS
<b>AT1B</b> Amplification	F <sub>B</sub> -5' GATTTTTTTTTTATAATTTTTTTAAGGTGG R <sub>B</sub> - 5'CAAATAAACCTATATCAAATAAATAACAC.
<b>AT1B</b> Pyrosequencing	Fp - 5'ATTTTTTGTGTTGTTGGGATTTAGG Rp - 5'CATTCCAACCCAAATAAACCTAT- <b>biotin</b> SeqF(1-3) - 5'TTAATTTATTTAGTAAAGGG

**Online Table 3**Values of RNA copy number/ $\mu\text{g}$  RNA of all genes analysed in this study at 1 and 12 weeks of age**Angiotensinogen**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>				
Mean	54060	105000	80570	87060
SD	5657	10010	4574	10940
N	4	4	4	4
	P<0.001		NS	
<b>Kidney</b>				
Mean	Low values		1688	5058
SD			316	188
N			4	4
			P<0.001	
<b>Lung</b>				
Mean	Low values		Low values	
SD				
N				
<b>Brain</b>				
Mean	1501	934	4445	1839
SD	535	579	3322	1116
N	3	3	6	6
	NS		NS	
<b>Heart</b>				
Mean	Not done		Not done	
SD				
n				
<b>Adrenal</b>				
Mean	969	522	242	256
SD	169	103	14	47
n	4	4	4	4
	p = 0.004		NS	

**Renin**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>	Not done		Not done	
Mean				
SD				
N				
<b>Kidney</b>				
Mean	27170	25640	55770	244800
SD	6250	7469	28440	76450
N	8	8	8	8
	NS		p<0.001	
<b>Kidney (Male)</b>				
Mean	26820	24980	80640	305100
SD	2529	6525	13300	45990
N	4	4	4	4
	NS		p<0.001	
<b>Kidney (Female)</b>				
Mean	27510	26300	30900	184400
SD	9189	9297	7850	42650
N	4	4	4	4
	NS		p<0.001	
<b>Lung</b>	Not done		Not done	
Mean				
SD				
N				
<b>Brain</b>	Low values		Low values	
Mean				
SD				
N				
<b>Heart</b>	Not done		Not done	
Mean				
SD				
N				
<b>Adrenal</b>	Low values		Low values	
Mean				
SD				
N				

**ACE**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>	Not done		Not done	
Mean				
SD				
N				
<b>Kidney</b>				
Mean	349300	328500	340500	333000
SD	81700	44300	78700	37500
N	4	4	4	4
	NS		NS	
<b>Lung</b>				
Mean	2708000	2464000	2832000	2664000
SD	747700	519300	834800	553700
N	5	5	5	5
	NS		NS	
<b>Brain</b>	Not done		Not done	
Mean				
SD				
N				
<b>Heart</b>	Not done		Not done	
Mean				
SD				
N				
<b>Adrenal</b>	Not done		Not done	
Mean				
SD				
N				

**AT1a receptor**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>				
Mean	19410	38020	87830	118100
SD	2792	5042	8841	19880
N	3	3	3	3
	p = 0.005		NS	
<b>Kidney</b>				
Mean	16160	16780	8035	17360
SD	1067	5111	5608	4455
N	6	6	9	9
	NS		p = 0.001	
<b>Kidney (Male)</b>				
Mean	16250	12470	12170	18480
SD	985	2481	3736	4645
N	3	3	5	5
	NS		p = 0.045	
<b>Kidney (Female)</b>				
Mean	16080	21090	2863	15960
SD	1361	1862	1027	4412
N	3	3	4	4
	P = 0.02		p = 0.001	
<b>Lung</b>				
Mean	Low values		Low values	
SD				
N				
<b>Brain</b>				
Mean	Not done		Low values	
SD				
N				
<b>Heart</b>				
Mean	Not done		Not done	
SD				
N				
<b>Adrenal</b>				
Mean	13730	15990	1700	3885
SD	2273	2986	317	878
N	4	4	3	3
	NS		p = 0.015	

**AT1b receptor**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>	Low values		Low values	
Mean				
SD				
N				
<b>Kidney</b>	Low values			
Mean			18790	16480
SD			19410	11880
N			6	6
<b>Lung</b>	Low values		Low values	
Mean				
SD				
N				
<b>Brain</b>	Low values		Low values	
Mean				
SD				
N				
<b>Heart</b>	Low values		Low values	
Mean				
SD				
N				
<b>Adrenal</b>				
Mean	99430	182000	186000	678800
SD	4410	4593	12800	224000
N	4	4	4	4
	p = 0.04		p = 0.004	



**AT2 receptor**

	1 week		12 weeks	
	Control	MLP	Control	MLP
<b>Liver</b>	Low values		Low values	
Mean				
SD				
N				
<b>Kidney</b>			Not done	
Mean	13810	17710		
SD	5888	1079		
N	6	6		
	NS			
<b>Lung</b>	Not done		Not done	
Mean				
SD				
N				
<b>Brain</b>	Not done		Not done	
Mean				
SD				
N				
<b>Heart</b>	Low values		Low values	
Mean				
SD				
N				
<b>Adrenal</b>				
Mean	71730	25350	54380	36100
SD	10560	2700	15980	2934
N	6	6	6	6
	p<0.001		p = 0.02	