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Epigenetic modification of the renin-angiotensin system in the fetal programming of hypertension

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Abstract

Hypertension is a major risk factor for cardiovascular and cerebrovascular disease. Lifelong environmental factors (e.g. 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 modelled 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 (RAS) in this process. Here we show that in this model, expression of the AT1b 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 AT1b gene in the adrenal is significantly undermethylated, and that *in vitro*, AT1b 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.

Keywords

Hypertension; Angiotensin receptors; Fetal programming; DNA methylation

INTRODUCTION

Although hypertension is recognised as one of the major contributing factors to cerebrovascular and cardiovascular disease [1], its pathogenesis remains incompletely understood. Genetic and environmental factors clearly contribute to this [2], but on current models fail to account entirely for the disease. There is now substantial epidemiological evidence that intra-uterine stress may 'programme' the later development of the disease [3]. The phenomenon of fetal programming can be modelled effectively in several mammalian

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DISCLOSURES

None

species [4]. 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 [4].

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 [5]. 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 [6,7]. This is highly suggestive of a role of the renin-angiotensin system 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 [8]. Following embryo implantation, the majority of the genome is demethylated [9]. 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 post-natal development, it is a good candidate for disturbance by environmental interference, and thus provides a potential mechanism for fetal programming. Whilst methylation patterns are generally considered to be established in early post-natal life and persist thereafter, there is evidence from human monozygotic twin studies that methylation patterns can change with ageing [10]

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, e.g. glycine, required to generate methyl donors, that underlies such changes [11]. Supplementation of low protein diets with glycine or folate reverses the programming effect of those diets [12,13]. However such a mechanism may lack gene specificity, whereas other data argues that it is specific genes that are susceptible to this effect [14 - 17].

Thus in this study we set out to test the hypothesis that alteration of DNA methylation of one 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 [4]. 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°C until further analysis. Up to three 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: $95^{\circ}\text{C} \times 10 \text{ min}$, then 40 cycles of $95^{\circ}\text{C} \times 30 \text{ s}$ and $59^{\circ}\text{C} \times 1 \text{ min}$. 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 analysed using MX4000 software version 3.0 (Stratagene). RNA expression data were given as copy number of gene of interest / μg of RNA. Standards used were PCR fragments purified from polyacrylamide gel electrophoresis. All PCR reactions were carried out in triplicate. Gene specific primer and probe sequences are shown in the online Table 1.

Immunoblotting

Rat adrenals were homogenised 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 min at 55°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 [17] and as described [18]. DNA was ethanol precipitated, dissolved in $50\mu\text{l}$ water and used immediately for PCR or stored at -20°C . PCR conditions were: $94^{\circ}\text{C} \times 12 \text{ min}$, then 40 cycles of $94^{\circ}\text{C} \times 30 \text{ s}$, $52^{\circ}\text{C} \times 1 \text{ min}$, $72^{\circ}\text{C} \times 30 \text{ s}$ and finally 1 cycle of $7 \text{ min} \times 72^{\circ}\text{C}$. The reaction mixture contained $1 \times$ Ampli Gold PCR buffer, 0.2mM dNTPs, 2mM MgCl_2 , $1\mu\text{M}$ primers, 1.25 units of Ampli *Taq* Gold DNA polymerase (Applied Biosystems, Warrington, UK) and $2-5 \mu\text{l}$ DNA template. Primer sequences are shown in online Table 2.

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 PSQTM 96MA 2.1 instrument (Biotage AB). The primer sequences are listed in online Table 2. 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 AT1b 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.

AT1b promoter analysis

A 1.2kb fragment (positions 277 – 1611 from Genbank U01033) containing the rat AT1b receptor promoter was cloned into pGL3 basic (Promega). The promoter was methylated *in vitro* with 10 units of *SssI* or *HhaI* (NEB) according to the manufacturers instructions. Controls included a mock methylated construct. 150 ng of each plasmid were then co-transfected 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 Victor² 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 1-3 in the modified AT1b promoter (positions 277 - 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 two-tailed t test and methylation density in control and MLP adrenals was compared using χ^2 .

RESULTS

Programmed and control animals from 10 control (5 male, 5 female) and 12 MLP (7 male, 5 female) offspring from three litters in each group were obtained as described and were harvested for liver, lung, kidney, brain, heart and adrenal either at one or twelve weeks after birth. Real-time RT-PCR assays for angiotensinogen, angiotensin converting enzyme (ACE), renin, and the AT1a, AT1b and AT2 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 three angiotensin receptors in the adrenal are shown in Figure 1. Detailed results of all analyses are shown in online Table 3. The only genes which showed a significant increase in expression in maternal low protein offspring (MLP) at both 1 and 12 weeks were the AT1a angiotensin receptor in the female, but not the male kidney, and the AT1b 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 AT2 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 AT1a and b 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 AT1b expression in the rat is the adrenal zona glomerulosa (ZG) [20 21] 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 three CpG sites in the proximal AT1b 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 AT1b promoter ($\chi^2 = 7.37$; $p < 0.01$) (Figure 4a,b). The

alteration of AT1b 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. These results are shown in Figure 4d and are significantly different when assessed as a group (all three 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 AT1a promoter and first exon using Pyrosequencing showed only occasional methylation of site 13 (position 3278 in rat At1a 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 AT1b promoter on gene expression was demonstrated by transfection of 1.2 Kbp of the AT1b promoter coupled to a luciferase reporter gene into mouse adrenocortical Y1 cells. Treatment of this promoter with the site-specific *HhaI* methylase or the non-specific CpG methylase *SssI* prior to transfection results in methylation of either one or all six CpG sites respectively in this fragment, and in the latter case is associated with markedly reduced promoter activity. When the three 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 AT1b receptor mRNA and protein in the adrenal. The antibody used in immunoblotting will detect the AT1a receptor equally well, but as the relative expression of AT1b at the mRNA level in these adrenal glands is much greater we believe that the majority of receptor protein identified on immunoblotting is the AT1b form. Similar findings of increased AT1 receptor expression have been reported in sheep following maternal protein deprivation [22]. Moreover it has also been shown that the MLP rat adrenal is more responsive to angiotensin II (Ang II) [23]. 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 AT1a receptor in kidney and AT1a and AT2 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 AT1a 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 AT1a 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 AT1b receptor is similar or reduced in the spontaneously hypertensive rat when compared to control Wistar Kyoto rats, probably as an adaptive response [24]. Since 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 AT1 receptor using a receptor antagonist will prevent development of hypertension in MLP offspring in contrast to nifedipene [6,7]. These findings confirm an important, if not unique, role for this receptor in this model. Investigation of the role of the AT1b receptor in mice in which one or the other, or both AT1 receptors have been deleted suggests the AT1b receptor contributes to blood pressure maintenance and in the absence of AT1a receptors mediates a pressor response to angiotensin [25,-27]

Two possible explanations for increased receptor expression are conceivable. Expression of the AT1b 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 AT1b mRNA expressed in the adrenal. Differentiation of the rat ZG cells is first apparent just before birth [28,29] 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 AT1b receptor gene expression is that the fetal insult has induced a persistent modification of AT1b gene expression. Altered DNA methylation has long been proposed as a candidate mechanism for fetal programming, although there are few examples in which this has been directly demonstrated by bisulphate sequencing [14,16] or inferred from methylation sensitive PCR analysis [15,17], and at least one case in which increased gene expression appears to be independent of DNA methylation [19]. We have shown here that the AT1b 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 AT1b 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 AT1b methylation pattern has been set [30]. 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 [8]. 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 [31]. Whether such an occurrence can influence the normal process of DNA methylation 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 centre 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 [32] although there are exceptions to this [33] and it is possible that another methylation sensitive transcription factor binds here.

The human genome only contains a single AT1 receptor gene which is widely expressed in a pattern similar to that of the AT1a receptor [34]. There is no human equivalent to the relatively adrenal-specific AT1b 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 [10]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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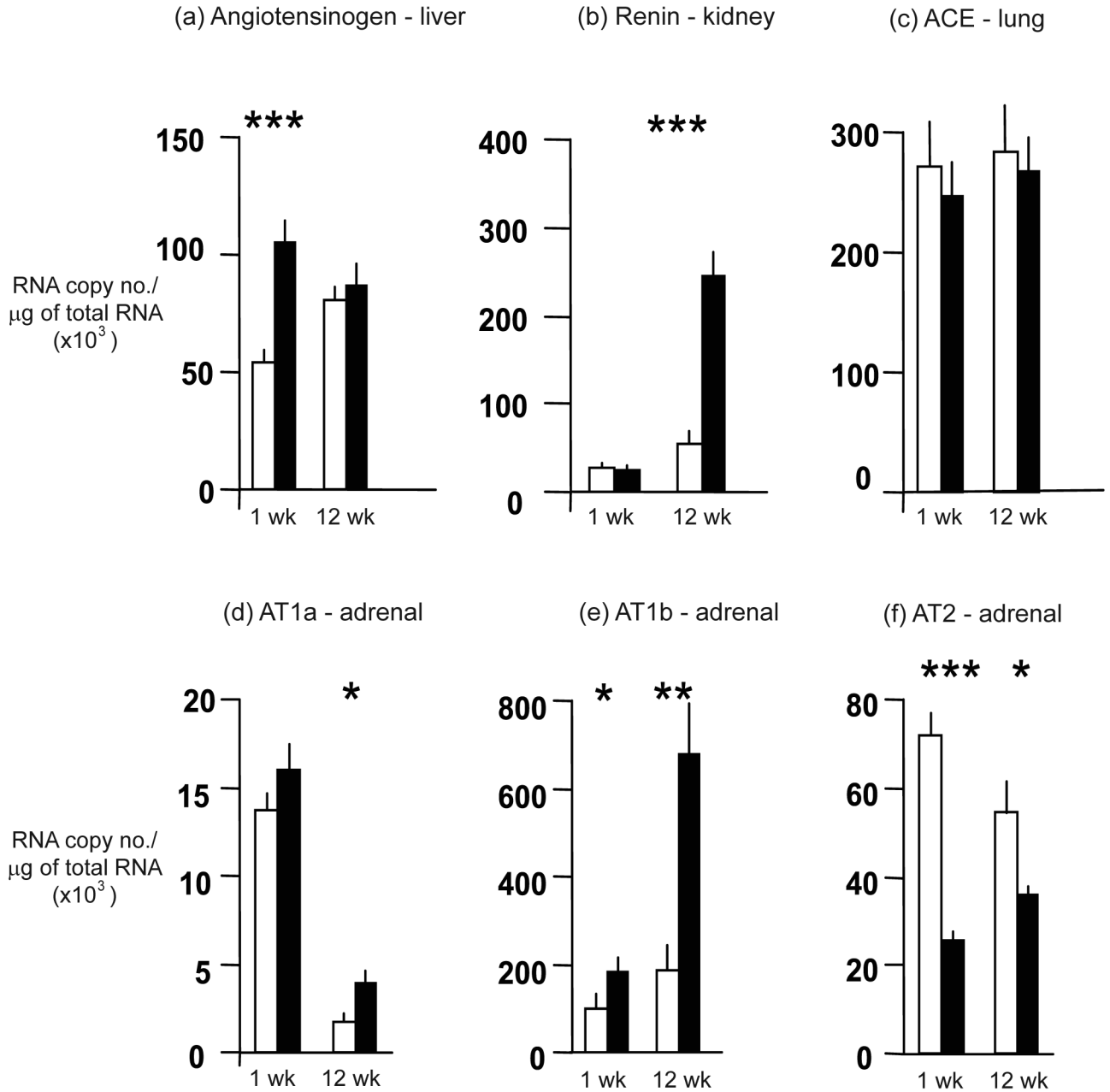


Figure 1. Altered expression of RAS genes in MLP offspring. Real-time RT-PCR was used to quantitate angiotensinogen, renin, ACE and the AT1a, AT1b and AT2 angiotensin receptor mRNAs in various control and MLP offspring rat tissues. Notable shifts in expression at either 1 or 12 weeks of age, or both, were demonstrated (a) for angiotensinogen in liver, (b) renin in kidney, and (d, e and f) the three 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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

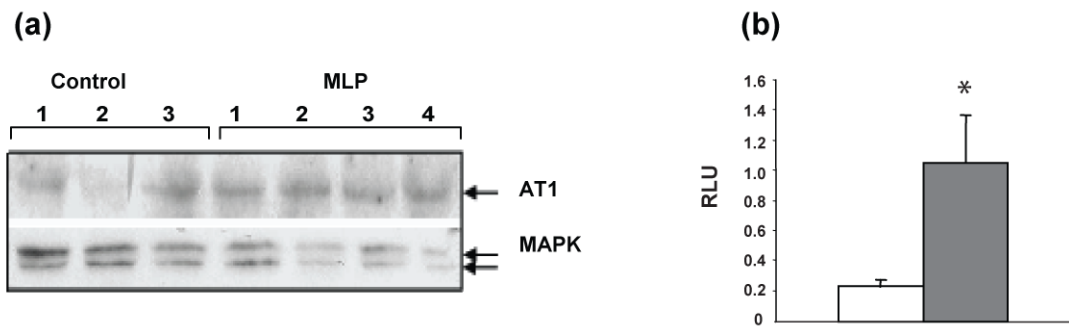


Figure 2.

(a) Immunoblot of AT1a 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. *, $p < 0.05$

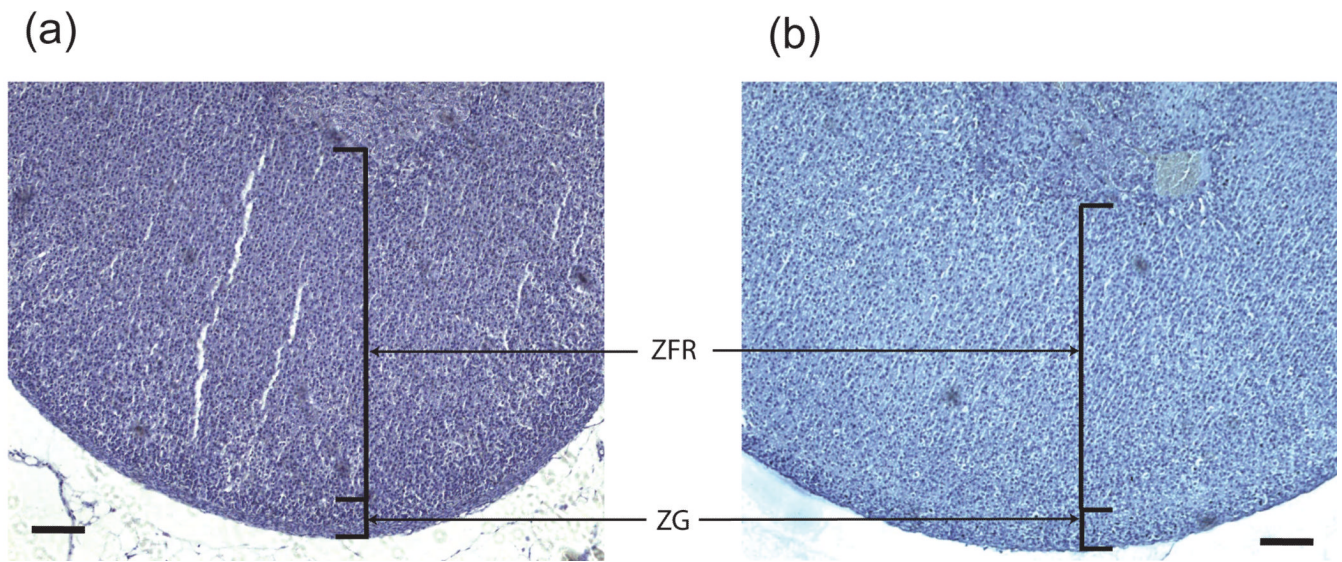


Figure 3. Adrenal histology from control (a) and MLP rat (b) at 10X magnification. ZG = zona glomerulosa; ZFR = zona fasciculata and reticularis. Scale bar represents 100 μm .

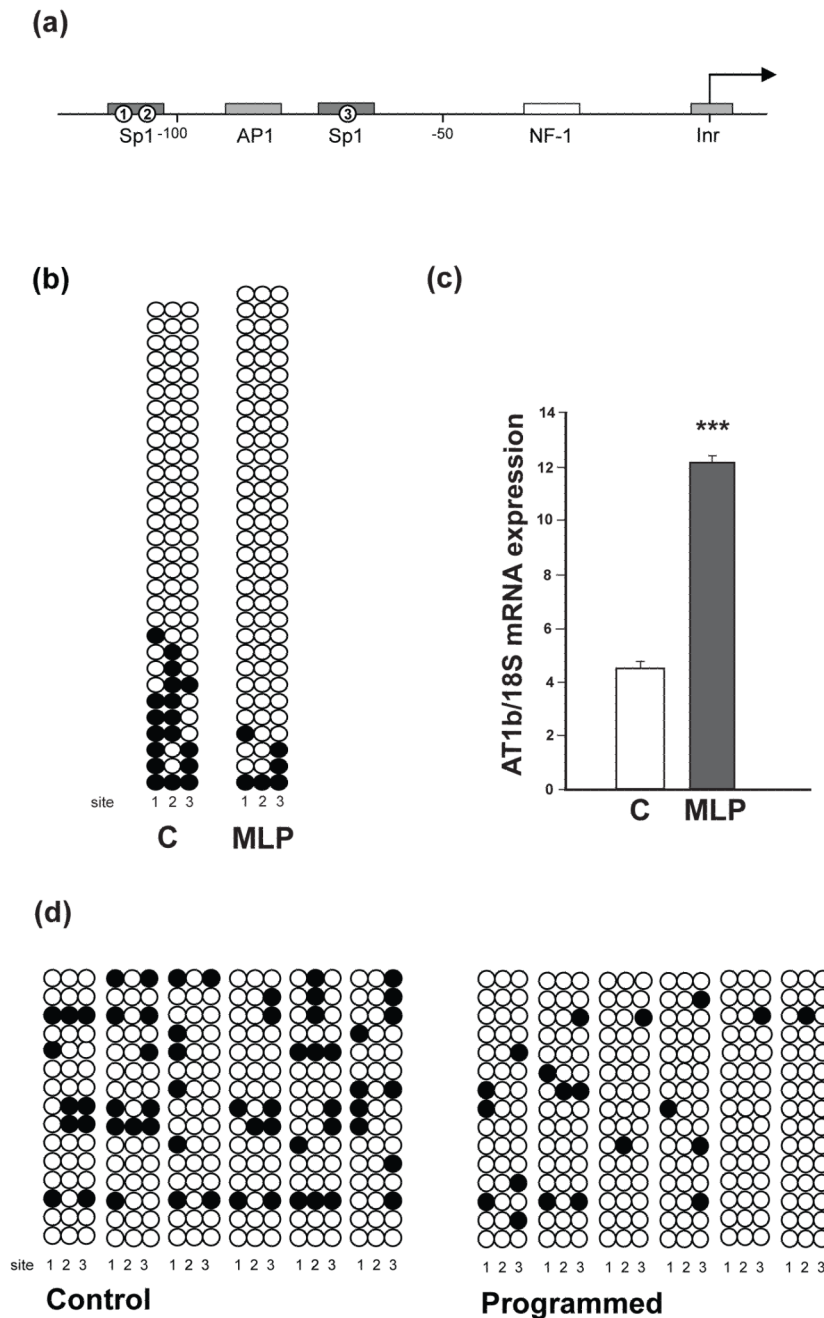


Figure 4. Methylation analysis of the AT1b promoter. (a) Structure of the At1b proximal promoter region showing location of putative transcription start site, consensus sequences for potential transcription factors and CpG methylation sites (open circles) analysed in this study numbered 1 - 3. Inr = Initiator element (b) Methylation of adrenal capsule AT1b gene in control and MLP animals. ○ = unmethylated; ● = methylated. One male and one female from each group had adrenal capsules harvested and used in this analysis. (c) Expression of AT1b 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 AT1b 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 three sites of interest. Fifteen clones were examined from each adrenal.

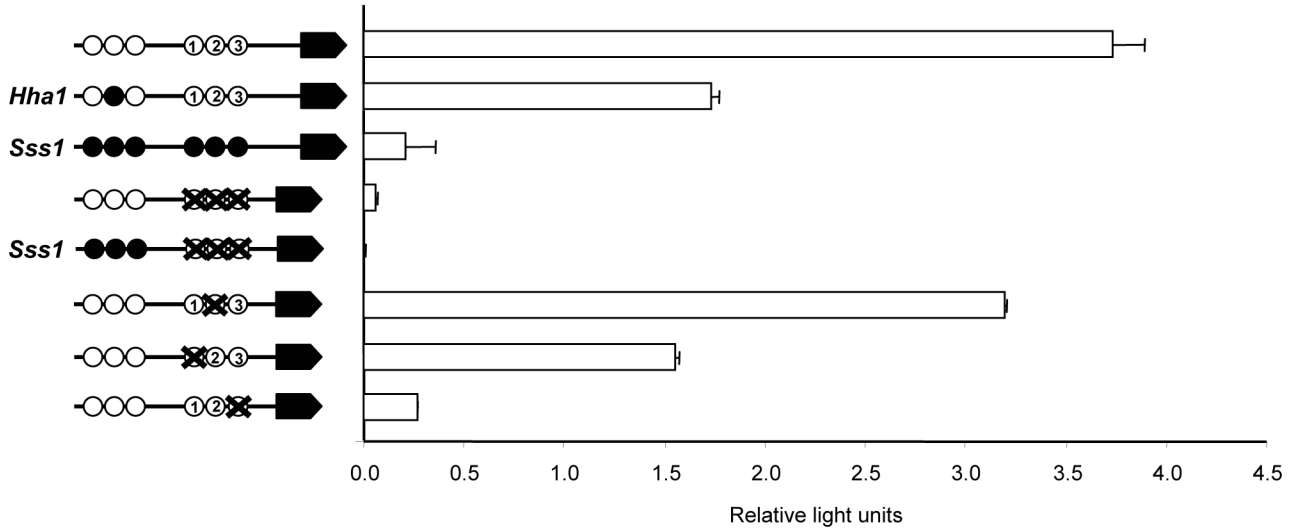


Figure 5. Influence of methylation on the AT1b promoter. Luciferase activity of AT1b-luc transiently transfected into mouse Y1 cells in the presence or absence of methylation of either one or six CpG sites in the proximal promoter using *Hha1* methylase (*Hha1*) or *Sss1* methylase (*Sss1*) respectively. Mutagenesis of the 3 proximal sites (1 – 3) to render them non-methylatable 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 co-transfected renilla luciferase activity (\pm SEM). n = 6. ○ = unmethylated site, ● = methylated site, X = mutagenised site.