Fetal glucocorticoid exposure and cardiovascular risk

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Submitted in satisfaction of the requirements for the degree of Ph.D. in the University of Edinburgh
1997

Declaration

I declare that this thesis and the work presented here are entirely the result of my own independent investigation. Where I received assistance this is acknowledged in the text and in the acknowledgements. This work has not been and is not currently submitted for any other degree.

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Abstract

Epidemiological evidence suggests that factors within the intra-uterine environment programme the propensity to high blood pressure, diabetes and cardiovascular disease in the adult. In this thesis I examine the hypothesis that exposure to glucocorticoids in utero alters birth weight and might act to determine later cardiovascular risk.

I present evidence that exposure of the rat fetus to exogenous glucocorticoids in utero, by means of administration of dexamethasone to female Wistar rats during pregnancy, results in reduction in birth weight and later rises in blood pressure, when measured both by direct carotid cannulation and by indirect tail cuff measurement at 3 to 17 months of age. I further examine the observation that exposure of the fetus to maternal glucocorticoid is influenced in normal physiology by the placental glucocorticoid metabolising enzyme, 11 beta hydroxysteroid dehydrogenase, presenting evidence that inhibition of this enzyme by carbenoxolone increases fetal glucocorticoid exposure and also results in changes in birth weight and later changes in birth weight and blood pressure.

The mechanism of the rise in blood pressure induced by in utero glucocorticoid exposure is examined by: i) assessment of the plasma-renin-aldosterone axis *in vivo* in adult animals both basally and in response to acute and chronic infusion of angiotensin II, ii) examination of vascular structure and reactivity *in vitro* in vessels derived from adult animals and iii) examination of central expression of glucocorticoid receptors in areas of the brain known to influence the control of blood pressure.

Finally I present evidence that exposure the fetus to glucocorticoids also influence glucose metabolism in the adult, examining potential mechanisms for this including alteration in insulin sensitivity, insulin secretion and hepatic glucose output.

Publications arising from this thesis

Papers

Benediktsson R, **Lindsay RS**, Noble J, Seckl JR and Edwards CRW. (1993) Glucocorticoid exposure in utero: a new model for adult hypertension. Lancet 341: 339-341.

Edwards CRW, Benediktsson R, **Lindsay RS** and Seckl JR. (1993) Dysfunction of the placental glucocorticoid barrier: a link between fetal environment and adult hypertension? Lancet 341: 355-357.

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Levitt NS, **Lindsay RS**, Holmes MC and Seckl JR (1996) Prenatal dexamethasone programmes blood pressure, basal corticosterone and hipocampal glucocorticoid receptor gene expression in the rat Neuroendocrinology 64, 412-418.

Acknowledgements

Immunohistochemistry of vessels was performed by Dr P. McEwan (Chapter 3) while the digital imaging and statistical analysis was carried out by myself. Measurement of central expression of mineralocorticoid and glucocorticoid receptors was carried out by Dr N.S.Levitt. All other work was entirely carried out by myself with technical advice and help from J.M. Noble (carotid cannulation), S.Rossiter (mesenteric perfusion), Dr C.Kenyon (corticosterone assay, indirect measurement of blood pressure), Dr B Williams (renin assay).

I would also like to acknowledge my debt to two others: Professor C.R.W. Edwards, who introduced me to this area of science, and Jonathan Seckl, whose continued patience, advice and good humour has supported me since.

Abbreviations

A	11-dehydrocorticosterone
B	Corticosterone
11ß-OHSD	11 ß-hydroxysteroid dehydrogenase
GDM	Gestational diabetes mellitus
HPA	Hypothalamic- pituitary- adrenal
ICD	International Classification of Diseases
IDDM	Insulin-dependent diabetes mellitus
NAD	nicotine adenine dinucleotide
NADH	nicotine adenine dinucleotide (reduced form)
NIDDM	Non insulin-dependent diabetes mellitus
SAME	Syndrome of Apparent Mineralocorticoid Excess
SMR	Standardised Mortality Ratio

Introduction

In this thesis I present experimental work examining the hypothesis that exposure of the fetus to glucocorticoids in utero leads to lifelong programming of cardiovascular risk, in particular high blood pressure and impaired glucose tolerance. The introduction concerns previous observations which underpin this hypothesis. In the first part I will review the epidemiological evidence supporting a role of intra-uterine influences in the later development of cardiovascular risk, work chiefly arising from the MRC Environmental Epidemiology Unit in Southampton. In the second part I will discuss the potential mechanisms for this influence, reviewing the known influences on birth weight and later diabetes mellitus and hypertension, I will also examine the hypothesis that under nutrition in utero might underlie the observed epidemiological phenomena. The third part concerns the known effects of glucocorticoids on the fetus as well as considering the normal physiological control of glucocorticoid exposure of the fetus, concentrating upon the role of the placental glucocorticoid metabolising enzyme 11 ß-hydroxysteroid dehydrogenase type 2(11ß-OHSD 2).

The prenatal environment and cardiovascular disease

a) The relationship of cardiovascular risk factors in childhood to cardiovascular risk as an adult

Cardiovascular disease occurs almost exclusively in middle and old age, but it is clear that the pathogenesis of the disease starts many years earlier. Prospective studies in adulthood highlight well described risk factors for cardiovascular disease including diabetes mellitus, impaired glucose tolerance, hypertension, hyperlipidaemia and smoking[1]. Such studies, conducted entirely in adult life, necessarily emphasise associations with current risk factors or current environmental factors but it is apparent that the origins of cardiovascular disease and of its major risk factors lie well before the time that the disease is symptomatic.

Autopsy studies of young adults show that, while symptomatic cardiovascular

disease is rarely present at these ages, there is evidence of the early development of atherosclerosis from as early as 15 years in a significant percentage of the population[2]. Risk factors also seem to be established early. Familial influences on blood pressure are present in the first decade of life[3] and, when ranked in terms of cardiovascular risk factors, individuals tend to maintain that rank order within a population from early childhood[4-6], the phenomenon known as "tracking". This suggests that while cardiovascular risk may be influenced by factors in the immediate environment (smoking, salt intake, caloric intake) there are also longstanding influences from either genetic or early environmental factors[7]. Tracking has been observed for blood pressure [5, 6] as well as other variables such as cholesterol [4, 6], height[6] and weight[6] and is established early, being observed for blood pressure and cholesterol from as early as 6 months to 1 year [8, 4]. In the study of Gillman et al[5] the observed tracking meant that 44% of the individuals with systolic blood pressure at age 10 greater than the 95th centile had systolic blood pressure greater than the 90th centile at age 20, leading to the observation that the best predictor of adult blood pressure is blood pressure level in childhood[9]. Tracking is often used as evidence of genetic influences in these risk factors and while such influences may play a part it also raises the possibility that factors in the early environment might be important.

b) Birth weight and cardiovascular risk

There is now extensive evidence for a relationship between birth weight and later cardiovascular risk. These studies are detailed below along with possible additional and confounding factors, in particular whether the changes observed by Barker et al are explained by factors relating to current and previous social class and whether other factors such as placental weight or fetal morphology (for example ponderal index, head circumference or abdominal circumference) are important indicators. The potential mechanisms for the association between birth weight and later risk will be examined in the next section.

The design of most of these studies is similar, involving retrospective data on gestation length, birth weight, placental weight and newborn morphology which are compared to current data regarding a variety of cardiovascular risk factors. It should be noted that data regarding offspring morphology are, in the main, only available for the studies based in the Sheffield population, while placental weight was available for the Preston and Sheffield studies only.

i) Cardiovascular mortality and disease

Epidemiological studies from the Southampton group display a graded relationship between birth weight and later cardiovascular death, in studies of the population of Hertfordshire[10, 11], Sheffield [12] and in Mysore in India[13], in both men [10, 12, 11, 13] and women [11, 13]. Importantly, the differences predicted by birth weight are large enough to be biologically important. The larger of the Hertfordshire studies included 5585 women and 10141 men[11] and found a fall in SMR (Standardised Mortality Ratio) for cardiovascular disease from 80 for birth weight ≤5.5 lb. to 46 if birth weight 9-9.5 lb. in women and from 96 to 61 for men in the same weight categories. A doubling of the risk of non-fatal coronary heart disease across the range of birth weights has also been suggested from a similar large study in the United States[14].

The studies conducted in the UK usually consider all cardiovascular disease together taking as their range International Classification of Diseases(ICD) codes 390-459, including coronary heart disease (ICD codes 410-414) and stroke (ICD codes 431-438)[11]. In considering the underlying factors in the relation of lower birth weight to cardiovascular risk, it may be interesting to consider whether diagnoses within this classification behave differently. World-wide, there is a large range in the ratio of stroke deaths to coronary heart disease deaths in different populations[15]. This suggests that either other risk factors are involved or that the classical risk factors vary in their propensity to cause these conditions. Comparisons of international populations

suggest the latter, with hyperlipidaemia more important as a risk factor for coronary heart disease than stroke, and hypertension most closely related to stroke[16, 17].

In the original studies which displayed the relationship of cardiovascular disease to lower birth weight[12, 11], similar trends were observed for coronary heart disease(ICD codes 410-414)[11] as all cardiovascular disease, but trends for stroke were not reported, presumably due to the smaller total number of stroke deaths compared to coronary heart disease deaths[12, 11]. In populations in India and the United States it is again comparisons of birth weight with coronary heart disease alone that have been reported[14, 13]. More recently however an analysis of the combined Sheffield and Hertfordshire populations have suggested a very similar inverse relationship between birth weight and both stroke and coronary heart disease. The populations were divided into quintiles and SMR for stroke was found to fall by 12% as birth weight rose in successive quintiles, while SMR for coronary heart disease showed a similar 10% fall for each quintile[18]. The similar relationship between birth weight and both stroke and coronary heart disease death might suggest that factors such as blood pressure and impaired glucose tolerance are potentially important underlying factors in the genesis of this risk. The evidence for the influence of birth weight on these various cardiovascular risk factors is described below.

ii) Hypertension

In 1985 Wadsworth et al reported data from the Medical Research Council National Study of Health and Development, examining a cohort of subjects aged 36. In examining blood pressure, multiple regression analysis revealed that as well as a range of less surprising factors (father's prevalence of cardiovascular disease, current BMI, smoking in males) systolic blood pressure was also inversely associated with birth weight in both sexes[19]. These data were later re analysed by Barker and Wadsworth, leading to the "fetal origins of adult disease hypothesis"[20]. Further studies by this and other groups are summarised in Tables 1 and 2.

Lower birth weight babies have higher blood pressure in the majority of these studies (Table 1) with the differences between groups becoming more prominent in older age groups, both in comparisons of cross sectional studies[21] and in longitudinal studies[22]. The negative studies in the main involve very low birth weight infants (<1.5 kg [23] or < 10th centile [24]) or have rather small numbers [25]. Those studies that showed a very small[26] or no [27, 24, 28] difference in subjects aged from 15-19 may reflect the known disruption of tracking of blood pressure during the adolescent growth spurt [9].

While the magnitude of such changes may on the face of it appear small, this should be placed in context with the powerful effect of blood pressure on disease. Meta-analysis of the available epidemiological studies suggest that a 5 mmHg reduction in diastolic blood pressure is associated with a 34% reduction in strokes and 21% less coronary heart disease[29]. Clearly numerically modest changes in blood pressure when exerted lifelong may have great importance to later disease.

Few of the studies have examined how much of the variance of adult blood pressure might be explicable by birth weight. One such study examining blood pressure in 3332 individuals aged 36 suggested that 4% of the adult variance of blood pressure was attributable to birth weight, compared to 12% of the variance being attributed to adult BMI in the same population[30]. Again, although small, such an effect across a population might be expected to produce a significant effect upon later disease. No study has modelled whether the changes observed in cardiovascular disease could be attributable to this size of change in blood pressure, it seems likely that they are not and that effects on other risk factors are of importance.

Table 1: Low birth weight is associated with later raised blood pressure

Population	Sex	Age	n		ref
Holland(1993)	M+F	3 months +4 years	476	negative association between birth weight and BP	31
Salisbury(1991)	M+F	4		2.6mmg increase in blood pressure between birth weight ≤3,000 g and birth weight >3,600 g.	32
9 U.K. towns (1989)	M+F	5-7	3591	1.83 mmHg fall in BP for each kg rise in birth weight	33
10 U.K. towns (1992)	M+F	5-7	3360	2.9mmHg difference SBP between the highest and lowest quintiles	34
New Zealand(1992)	M+F	7	1200	1mmHg rise SBP in growth retarded infants (birth weight <10th centile)	24
Oxford U.K. (1985)	M+F	7.5	216	negative assoc birth weight and blood pressure in males	35
Aberdeen (1984)	M+F			negative assoc. birth weight and blood pressure	36
Guildford and Carlisle, U.K. (1995)	M+F	5-7 and 9-11	1511	birth weight inversely assoc. with SBP, (-2.8 mmHg.kg-1) and DBP (-1.4 mmHg.kg-1)	22
Kingston, Jamaica (1996)	M+F	6-16	2337	SBP inversely related to birth weight (-2.6 mmHg.kg-1)	37
MRC national(1989)	M	10		fall SBP 0.38mmHg lowest to the highest tertile	
	M	36		SBP fell by 2.57 mmHg (0.98-4.16)from the lowest to the highest tertile	
MRC national (1989)	F	10		SBP fell 1.32mmHg from the lowest to the highest tertile	
	F	36		SBP fell 1.83 mmHg (0.28-3.39) from lowest to highest tertile	20
Israel (1991)	M	17	19,734	"small effect"	
Israel (1991)	F	17	12,846	"small effect"	26
Croatia(1988)	M	19.9	214	5 mmHg difference SBP between the highest and the lowest quartile of blood pressure	
Croatia(1988)	F	19.6	251	SBP 1mmHg difference	38
Sweden (1988)	M	28	77	significant increase in hypertension (DDBP≥90) if birth weight <2.5 kg	39
San Antonio Heart Study(1994)	M+F	25-39	447+ 135	1kg fall birth weight assoc. 2.1mm increase SBP and 1.7mm increase DBP	40
England, Scotland, Wales(1993)	M+F	36	3332	inverse linear relationship between birth weight and BP aged 36	30
Mysore (1996)	M +F	38-60	517	significant association birth weight with hypertension	41
Preston (1990)	M+F	46-54	449	SBP 11 mmHg rise from birth weight ≤5.5 lb. to >7.5 lb.	42
Sweden	M	50	1333	2.2 mmHg reduction in blood pressure for every 1000g increase in birth weight	43
Hertfordshire (1991)	M	64	468	SBP fell by 11 mmHg from the group with birth weight ≤5.5 lb. to >9.5 lb.	44
Hertfordshire (1995)	F	60-71(64)	297	NS trend SBP fell by 2.1 mmHg per lb. increase in birth weight	45

Table 2: Blood pressure and birth weight: negative studies

Population	Sex	Age	n		Ref
Cleveland, US(1993)	M+F	8	490	very low birth weight (<1.5 kg) infants	23
Glasgow,U.K. (1991)	M+F	15	959	maternal recall of birth weight	27
Wales (1994)	M+F	15	660	low birth weight <2.5 kg matched to 3-3.8 kg infants: no difference in SBP	28
New Zealand(1992)	M+F	18	1200	no difference in growth retarded infants (birth weight <10th centile)	24
Tecumseh, Michigan, US(1980)	M+F	20	4500	No consistent association between BP and birth weight	46
Oxfordshire (1993)	M+F	43	101	1st degree relatives of NIDDM	25

iii) Impaired glucose tolerance

In 1991 Hales et al reported the association of lower birth weight with an increased risk of impaired glucose tolerance and diabetes mellitus[44]. This has now been supported by several studies in adult populations examining impaired glucose tolerance or diabetes[47-49], syndrome X [50, 51], and in younger populations alterations in glucose metabolism short of diabetes[52, 53](Table 3). Hales and Barker have suggested the "thrifty phenotype hypothesis" in explanation of these associations, proposing that under-nutrition in early life might lead to the programming of various metabolic processes later expressed as hyperglycaemia and hypertension[44]. The title "thrifty phenotype" alludes to the previous "thrifty genotype hypothesis" of Neel[54], which had suggested that a genetic make up selected for in circumstances of periodic nutritional deprivation might be maladaptive in modern circumstances of over nutrition, explaining the rising prevalence of non insulin-dependent diabetes, most notably in ethnic groups experiencing relative over nutrition for the first time.

The studies examining the thrifty phenotype hypothesis have ranged over a variety of populations and ethnic groups including those in the United Kingdom (Hertfordshire [44, 50] Preston[50, 47], Southampton [52]) as well as the United States [48, 51], Sweden [49]and India [53]. I have summarised the findings in Table 3 and the influence of birth weight is striking. In the Preston Study the odds ratio for the risk of diabetes or impaired glucose tolerance fell from 3.5 for birth weight less than 2.5 lb. to 1.0 for birth weight greater than 3.4 lb. in men, and 12.1 for birth weight less than 2.5 lb. to 1.0 for birth weight greater than 3.4 lb. in women. In the combined data from the Preston and Hertfordshire studies there is an even more marked effect for Syndrome X (defined as 2 hr plasma glucose greater than 7.8 $mmol.l^{-1}$, fasting triglycerides greater than 1.4 $mmol.l^{-1}$ systolic blood pressure greater than 160 mmHg) with a fall from 22% of the population positive for Syndrome X if birth weight had been less than 6.5 lb. to under 2% if birth weight had been greater than 9.5 lb..

The only negative study examining birth weight and glucose tolerance is from Salisbury where children previously studied for blood pressure at the age of 4 years were examined with a modified oral glucose tolerance test at 7 years [55]. The authors report no influence of birth weight on plasma glucose or insulin either at 0 or 30 minutes (because the subjects were children only a 30 minute oral glucose tolerance test was performed) .

There are, therefore, several studies supporting an association in populations between lower birth weight and alteration in glucose tolerance. Perhaps one weakness of this data is that most of the studies, while differing in populations and age of participants use a broadly similar study design, perhaps with similar inbuilt bias[56]. It is important then that other confirmatory evidence is now appearing. Poulsen et al have recently examined birth weight in monozygotic and dizygotic twins discordant for non-insulin dependent diabetes and found that in both sets of twins birth weight was significantly lower in those with later NIDDM[57].

Table 3: Birth Weight and Later Glucose Tolerance

Population	Age	n		ref
Hertfordshire (1991)	64 (59-70)	468 men	≤2.5 kg 30% IGTT, 10% DM >4.3 kg 14% IGTT, 0% DM	44
Preston(1993)	50(46-54)	266 men and women	IGTT or DM , ≤2.5 kg 27% >3.41 kg 6%	47
Sweden(1994)	60	1000	relative risk NIDDM 1.4 for every 1 SD fall in birth weight	49
Pima Indians(1994)	20-29	1464	<2.5 kg 34% DM 2.5-4.4 kg 15% DM	48
Syndrome X				
Hertfordshire + Preston(1993)		407+266	≤ 6.5 lb. 22% Syndrome X >9.5 lb.<2.2% Syndrome X	50
San Antonio Heart Study(1994)	(25-39)	582	1 kg decrease in birth weight assoc. 3.7μU/ml increase in fasting insulin, increase SBP and DBP	51
Younger subjects				
India(1995)	4	379	no trend for fasting glucose, 30 minute glucose falls as birth weight rises	53
Southampton(1992)	21	40 men	decrease of 1 kg birth weight assoc. with 1.5 mmol rise in 30 min pg	52

iv) Birth weight and Insulin

Both ß-cell failure and insulin resistance are present when non-insulin dependent diabetes is clinically apparent[58] and have been implicated as early aetiological factors in non-insulin dependent diabetes[59-62] and in the development of obesity[63]. Influences in early life might affect either one or both of these factors. The large epidemiological studies rely upon oral glucose tolerance tests which, while showing rises in both glucose and insulin in men[44, 47] and women[51, 45] at two hours, are difficult to interpret with regard to insulin resistance or insulinopenia. Some information might be gleaned from fasting measures of insulin and incompletely processed forms of insulin (pro insulin and 32-33 split pro insulin) which are shown to correlate with more detailed measures of insulin resistance in population studies[64]. The results are inconsistent however, with a relationship of higher fasting insulin in keeping with insulin resistance in those of lower birth weight in some[51, 45], but not all [47, 51] female populations and not in male populations[44, 47, 51]. Fasting pro-insulin shows a relationship in some

studies[47], but not others[44, 45] as does fasting split pro-insulin (negative[47] and positive[44, 45]). The more recent twin study has also been interpreted as favouring a role of insulin resistance as a consequence of lower birth weight, in this case due to an inverse relationship between birth weight and 120 minute plasma insulin concentrations after an oral glucose tolerance test[57]. More detailed studies of much smaller groups of non-diabetics have supported an association of low birth weight with insulin resistance (assessed by intravenous insulin injection) [65]. Such detailed metabolic studies in twins of birth weights are awaited.

Lower birth weight might also act by affecting either B-cell mass or B-cell function. Some support for this might be inferred form the observation that lower birth weight and small for gestational age infants have been reported as having an earlier onset of insulin dependent diabetes mellitus compared to other diabetic children in retrospective analysis[66]. In studies examining birth weight in non-diabetic subjects evidence of impaired insulin secretion has been present in some[25], but not all [67] populations.

As yet the evidence of a relationship between lower birth weight and insulin secretion or resistance are at a rather early stage, some of the evidence is contradictory, and in addition almost all of the studies use indirect measures of insulin action and secretion: the interpretation levels of insulin and incompletely processed forms of insulin during glucose tolerance tests as being indicative of either insulinopenia or insulin resistance must be dealt with caution.

v) Other factors

Other factors potentially relating to cardiovascular risk have been examined, raised plasma fibrinogen has been associated with lower birth weight in some[42] but not all studies[68], while plasma lipids have shown trends short of significance [69]. Cholesterol has shown a relationship to abdominal circumference[69] and length at birth[37].

c) Placental weight and other morphological factors

Placental weight

In the studies of Barker et al, where such data are available, importance has also been given to placental weight in determining fetal outcomes. In particular, higher placental weight has been associated with higher blood pressure in children[32] and in adults[42]. This is a slightly curious finding, given the data on birth weight, as there is a linear relationship between placental and fetal weight in human populations and in terms of perinatal outcome, it is reduced placental size, and a high fetal weight to placental weight ratio which appears to be detrimental[70].

The change in blood pressure that has been related to placental weight is certainly large enough to be biologically important, however, with a rise in mean systolic pressure from 148 mmHg in those with placental weight ≤ 1.5 lb. to 159 mmHg in those with placental weight >1.5 lb.[42]. Data for other outcomes and risk factors are less clear: no independent association for placental weight has been apparent for cardiovascular mortality [12] or insulin resistance [65], while placental: birth weight ratio appears to be predictive of cardiovascular mortality [12] and impaired glucose tolerance[47] (the data for the relationship to placental weight alone are not given).

In the majority of the studies relating low birth weight to hypertension[45], diabetes mellitus[44, 48] or cardiovascular disease[10, 11, 71] placental weight is not available . The data on the relation of placental weight to blood pressure are not uniform, however, with other workers reporting an inverse relationship (i.e. babies with heavier placentae having lower blood pressure in later life)[24, 22, 37] or no relationship of birth to placental weight ratio[24, 22, 37]. In addition, stroke mortality, thought to be strongly related to high blood pressure, appears to fall with higher placental weight[18], making one more cautious about the interpretation of the positive studies.

Ponderal Index

Other studies have suggested that a number of patterns of fetal morphology might

be of importance, potentially representing the impact of different intra uterine insults on growth patterns and later disease. If corroborated, these different patterns might give important clues as to the factors leading to the growth retardation. Of all of these patterns, most data are available regarding ponderal index, derived from birth weight divided by the cube of newborn length. In studies of the Preston population, Barker et al [72] defined two at risk groups for hypertension:

1) babies with placental weight <1.25 lb., thin (ponderal index ≤12) , below average birth weight, below average head circumference babies and

2) if placental weight >1.25 lb. babies with above average birth weight, above average head circumference, but below average length were at risk. i.e. babies short in relation to their head size.

While the cause of these different patterns remains uncertain, thinness at birth has been implicated in a number of other studies where the detailed morphological information to allow this assessment are available. Low ponderal index appears to be more strongly related than birth weight to hypertension later in life both in children[32] and in adults[72], and to impaired glucose tolerance[47, 65, 49] and cardiovascular death [12] in adults. When different causes of cardiovascular death are considered, ponderal index appears to relate to coronary heart disease mortality but not stroke mortality[18], perhaps a surprising result given the stronger relationship of hypertension to stroke mortality[16, 17]. In the majority of studies ponderal index is not examined (hypertension [42, 26, 45] diabetes [44, 48] cardiovascular mortality/ prevalence [11, 71]) as the detailed morphological data on newborn length was not available. The association of lower ponderal index to cardiovascular risk is not universal with some studies showing no relationship of ponderal index to higher blood pressure[24] and to cardiovascular mortality[13].

The relationship of systolic blood pressure to small head circumference relative to length[72] has been observed in other populations [32, 12] and also for diabetes and cardiovascular mortality[12] by the Southampton group. By contrast death from stroke

has been related to higher head circumference in relation to birth weight[18]. Other studies have related length at birth to Haemoglobin A1 in childhood[37]

The epidemiological studies suggest a variety of associations; with low birth weight, with thinness, with babies of small head circumference and with babies short in relation to their weight. Currently the greatest consistency in these results appears for the relation of birth weight to cardiovascular risk. While some other patterns may emerge as important in the future it would be reassuring to see them repeated in different populations in the first instance.

Birth weight vs. weight at 1 year

The studies of Barker have studied both birth weight and weight at 1 year. The hypothesis that early nutrition is of central importance would allow both influences in the intrauterine period and in the early postnatal period and therefore both measures might be related to later cardiovascular disease. At first sight the hypothesis that fetal glucocorticoid exposure is the underlying mechanism would seem to predict a greater influence of birth weight than weight at 1 year. Clearly however an adverse intrauterine milieu might still be reflected in weight at 1 year. The hypothesis would only be challenged if weight at 1 year were a much stronger predictor than weight at birth. This does not seem to be the case.

In the studies of cardiovascular mortality both birth weight and weight at 1 year predicted mortality in men[10, 11], but only birth weight in women[11]. Only the study of the subgroup of 290 individuals assessed for current cardiovascular disease showed a relationship with weight at 1 year but not birth weight [71].

Individual cardiovascular risk factors seem to vary in their association perhaps reflecting different patterns of early growth [12]. Thus blood pressure has been associated chiefly with low birth weight[44], an exception being the study of Kolacek et al [38], while plasma fibrinogen and factor VII concentrations correlate with weight at 1 year but not birth weight [68].

Conclusion

The epidemiological studies cited above present good evidence of an association between birth weight and later cardiovascular risk, high blood pressure and impaired glucose tolerance. The strength of the data on birth weight is that such associations now appear in several studies and in different population groups. The data on birth weight are provocative, leading to a novel and exciting hypothesis in cardiovascular disease: that influences exerted in utero many years before might alter cardiovascular risk as an adult. In the next section I review what is known of the control of birth weight and address those influences which might act in utero both to affect birth weight and later cardiovascular risk.

In considering the above studies it is also important to note that the presence of a statistical association does not infer causation, even with a great weight of evidence. There are many ways in which low birth weight might be linked to later cardiovascular risk, not all of which involve concepts of metabolic programming, for example a given factor may lead independently to both lower birth weight and later disease. To this end I will also consider factors such as social class which might both point to aetiological links between low birth weight and later cardiovascular disease but might also exert a confounding influence due to co-associations of poverty with low birth weight and cardiovascular disease independently.

A role in the prediction of cardiovascular risk has also been suggested for other factors, including placental weight, ponderal index, newborn length and head circumference. Such associations, when available, hold out the intriguing possibility of giving clues as to the potential insult which might be acting *in utero* to alter cardiovascular risk later. In the next section I will also discuss the factors which, in particular, might affect ponderal index. As a note of caution, however it is reasonable to note that the evidence for these other morphological associations is more sparse and there are contradictory studies. For this reason I have concentrated upon birth weight and await further, confirmatory studies with regard to some of the other factors.

Another relevant question is to the extent of the influence of birth weight upon later disease or risk factors. Where it attempted, the analysis of how much of the variance of blood pressure or glucose tolerance is explained by birth weight appears to be rather small, being 4% for blood pressure[30] and 5% for blood glucose concentrations[57]. It is important to point out however that much of the importance of birth weight may be lost in such analyses. The study of Poulsen et al in twins[57] shows that it is not being of low birth weight *per se* but rather being of a lower weight than expected that appears to be important. Thus, the co-twins who later developed diabetes were by no means small, but they were smaller than their fellows who did not later develop diabetes. It is impossible in the large studies to estimate the degree of reduction in "expected" birth weight versus later disease and as such the raw comparison of birth weight to later disease might be expected to underestimate this potential effect.

Finally in the studies relation birth weight to later mortality, it is observed that known risk factors do not explain all of the associations[13], highlighting the possibility that influences on birth weight may act via as yet undescribed risk factors.

2) Early influences: potential mechanisms for the association between birth weight and later cardiovascular risk

a) The control of birth weight

Birth weight has been the subject to extensive epidemiological investigation partly as it has proven a powerful predictor of perinatal mortality. The weight of the newborn might be influenced by many factors: both the products of fetal genes and the fetal environment may be of importance and the latter will in turn be determined by maternal genotype, phenotype and influences in the maternal environment. One of the features of the epidemiological data linking birth weight to cardiovascular risk is that it appears to act in a graded fashion across the population and is not an effect of shorter length of gestation but lower weight in full term infants. While it remains possible that genetic factors in the fetus might be the common factor in both the control of birth weight and later cardiovascular disease, it is the hypothesis of the Southampton group that the link between birth weight and cardiovascular risk is determined by environmental factors *in utero*. In attempting to explain the connection between birth weight and cardiovascular risk then, it is important to consider the contribution of genetic and environmental influences upon the variance of birth weight in the normal population. If birth weight were almost entirely controlled by genetic factors then it would be difficult to interpret the data of Barker et al as reflecting environmental effects. Further, it is necessary to look for factors within the fetal environment that might influence birth weight across the range of the normal population.

If one examines certain specialised situations then both environmental and genetic factors clearly have some influence. Effects of the fetal environment are seen in pattern of growth in multiple births. The growth curves of triplets are similar to those of singleton births until the 32nd week and diverge thereafter. For twin births the divergence is not until the 36th week[73]. This difference in birth weight between multiple and single births is generally attributed to the physical constraint of the uterus apparent from animal studies[74]. Effects of fetal genes are seen in the growth

inhibiting effects of chromosomal abnormalities[75], and of influences of the sex chromosomes: males are observed to grow faster after the 28th week and are born on average 150g heavier[73].

In part reflecting such effects, examination of the extremes of the distribution curve for birth weight reveals a number of causes. Intra-uterine growth retardation is said to affect 3-10% of all pregnancies depending on diagnostic criteria used: arising secondary to causes such as chronic utero-placental insufficiency, drug exposure, congenital infection and genetic abnormalities (reviewed in [76]). In the UK population, obstetric studies pick out maternal height, smoking in pregnancy, parity and history of pre-eclampsia as the most important determinants of birth weight in surveys[77]. While some of these factors (smoking, pre-eclampsia, congenital abnormalities) are estimated to account for up to 60% of the occurrence of very low birth weight (below 2 standard deviations)[78], their influence on weight away from the extreme appears to be less marked.

For whatever reason there is a strong familial aggregation of birth weight[79]. Studies examining the importance of the genetic and environmental influences on birth weight across the whole population vary widely in their estimation[80-86] but in the main favour a low influence of the fetal genotype[80, 84-86] and stress the importance of environmental factors derived from the mother[80, 84, 86]. It should be pointed out that all such studies are poor at picking out environmental factors which might be shared across generations from genetic factors[83] and as such will tend to underestimate environmental factors. Further caution is necessary as the relative influences of genetic and environmental factors may differ depending on the population studied and with time. There is a large international variation in birth weight with frequency of births weighing less than 2500g varying between 3 and 43% in different populations[87] with undoubted effects of poverty and under nutrition at the extremes of these figures[87].

While bearing these caveats in mind, in Western populations that factors such as parity, pre-pregnancy weight, weight gain in pregnancy, maternal and paternal birth

weights, maternal smoking and fetal gender are all associated with birth weight but account for only 10-20% of the variance[83, 85], with gestational age accounting for a further 10%[85]. Even in those studies which add measures of maternal glycaemia, only 30% of the total variance in birth weight is explicable[88]. The available data then, would leave much of the variance of birth weight unexplained.

In considering the fetal origins hypothesis, the most important point is the low influence on birth weight attributed by most studies to the fetal genome. It is of interest is that much of the variance in birth weight is unexplained, even in populations highly defined for maternal and paternal factors. It is entirely plausible, if unproven, that environmental factors not represented in the various measures previously studied might influence birth weight.

Given the data derived by Barker on aspects of fetal morphology such as ponderal index, it is worthwhile at this point considering possible influences on this and other morphological factors. Different pathological causes of intrauterine growth retardation lead to different patterns of growth retardation. As peak velocity for length growth occurs in the 20th week and peak velocity for weight growth occurs in the 33rd week, insults occurring at different times in pregnancy may partly underlie these different patterns[89]. In particular, insults late in pregnancy would be expected to have a greater effect on weight than length, resulting in low ponderal index (weight/ length^3) Malnutrition throughout pregnancy, smoking, alcohol, drugs, pancreatic agenesis, chromosomal abnormalities and chronic intrauterine infections result in growth retarded infants of appropriate ponderal index, while insults in the third trimester result in low ponderal index (disproportional, non-symmetrical or wasted growth retardation)[76]. Low ponderal index has been observed in offspring of well nourished mothers with low weight gain through pregnancy, in association with preeclampsia and in utero-placental insufficiency[90, 76]. Interestingly, follow up of such infants shows that the appropriate ponderal index group of growth retarded infants continue to grow poorly,

whereas the low ponderal index group show a rapid catch up growth[90, 91]. The influence of nutrition on ponderal index is considered below.

b) Environmental influences on birth weight and cardiovascular risk factors

In this section I will consider some of the various adverse environmental factors which might impact upon fetal growth and later cardiovascular risk.

It is possible that the association of lower birth weight and cardiovascular risk is not cause specific: that any growth restraining insult might result in later risk, perhaps as has been proposed for high blood pressure, as a result of catch up growth[7]. This, however, does not seem to be the case. When one considers twin births, the physical restraints of the uterus results in a reduction in birth weight[73] but this does not seem to be reflected in a later increase in cardiovascular mortality compared to the singleton population[92]. All insults then, do not appear to have the same long term impact on cardiovascular risk and it is important to consider whether any of the factors currently known to influence birth weight have been studied with regard to effects on such risk.

i) Hyperglycaemia

Glucose crosses the placenta by a carrier-mediated facilitated diffusion and is an essential source of fuel for both fetus and placenta, with fetal tissues such as brain, kidney and the adrenals relying almost entirely upon glucose for their energy needs (reviewed in [93]). Given the important role of glucose as a fuel for fetal growth it has been proposed that the intra uterine environment of a diabetic pregnancy might have many long term effects on the fetus[94]. Further evidence for the potential effects of diabetic pregnancy on the offspring come from studies of the inheritance of non-insulin-dependent diabetes. In insulin-dependent diabetes there appears to be a higher rate of paternal than maternal transmission. Thus, the offspring of insulin dependent diabetic fathers are two to four times more likely to develop insulin dependent diabetes

than the offspring of insulin dependent diabetic mothers[95, 96]. In non-insulin dependent diabetes the converse is true, with several studies suggesting a greater maternal than paternal transmission[97-101]. While there many potential explanations for this (effects of mitochondrial DNA[102], genomic imprinting and confounding effects of the long-term diabetogenic effects of pregnancy itself[103]) this has led to the suggestion that maternal hyperglycaemia might itself be diabetogenic for the fetus[98]. Does maternal hyperglycaemia explain the epidemiological association of lower birth weight and diabetes?

Maternal diabetes clearly does affect the fetus, with rates of congenital malformation of between 4 and 12%, spontaneous abortion rates ranging from 9 to 45% (reviewed in [93]). Changes in fetal growth are complex; hyperglycaemia has been proposed to lead to some inhibition of growth early in pregnancy[104, 105], although this is disputed[106], but clearly promotes growth late in the pregnancy resulting in macrosomia (defined as birth weight greater than the 90th centile) in 25-42% of pregnancy in diabetic women (reviewed in [93]). In 1954, Pedersen proposed a central role for maternal hyperglycaemia in promotion of the typical overgrowth and adiposity seen in the newborn offspring of diabetic mothers: maternal hyperglycaemia leads to fetal hyperglycaemia and stimulation of the fetal pancreas to produce insulin resulting in fetal overgrowth (The Pedersen Hypothesis[107]), later modified to include amino acids as a co-stimulus to fetal insulin production[94]. With some exceptions[108-110], the majority of studies in diabetic pregnancy support the relationship of maternal hyperglycaemia to fetal macrosomia with an emphasis on hyperglycaemia early in the third trimester[111, 112].

Freinkel had proposed a series of potential sequelae for such offspring of diabetic pregnancies termed 'fuel mediated teratogenesis' by him[94]. Thus maternal fuels were proposed to influence the development of the fetus, modifying phenotype especially in those tissues made up of terminally differentiated cells, with both ß cells of the pancreas and adipose tissue proposed as tissues vulnerable to such manipulations in

early life[94]. It had been suggested historically that the offspring of diabetic pregnancies were more likely to be obese in childhood[113], with conflicting data reflecting various methodological problems (references in [114]). Studies in the Pima Indians, notable for their careful metabolic data on the mothers before, during and after pregnancy, support a role for maternal hyperglycaemia both in the promotion of offspring obesity[115] and later non-insulin dependent diabetes[98, 116]. Such studies, carried out in populations highly prone to non-insulin dependent diabetes, are interpreted by the authors as showing the diabetogenic effect on the offspring of maternal hyperglycaemia, but might equally be explained by graded genetic effects within this population, an argument with any study involving non-insulin dependent diabetic mothers. Similar effects of offspring obesity[117, 118] and teenage impaired glucose tolerance[119] have been reported in the offspring of a mixed population of mothers with both previous IDDM and gestatational diabetes supporting an environmental role of maternal hyperglycaemia and, more specifically in these studies, fetal hyperinsulinaemia in these effects[117, 119]. Again there is a potential confounding role of genetic influences which predispose to glucose intolerance, leading to gestational diabetes in the mothers and NIDDM in the offspring. More than half of the mothers in this population had gestational diabetes and studies of the offspring of mothers with only insulin-dependent diabetes might serve to clarify this point.

Animal studies have supported a role in maternal hyperglycaemia in the generation of offspring hyperglycaemia[120-125], with effects on both offspring insulin resistance[120, 121, 126, 127, 123] and insulinopenia[121, 124, 125] in a variety of models (streptozozotocin[126, 127, 123, 125] and glucose infusion[120, 124]) and species.

While the relationship of maternal hyperglycaemia to offspring diabetes risk is an intriguing one, these data clearly do not explain the relationship of low birth weight to cardiovascular risk. This is perhaps unsurprising. Firstly, due to the high failure rate and mortality of frankly diabetic pregnancy in the first half of this century, such pregnancies will have had little if any impact on the populations studied by Barker et al.

Secondly, since even within the reference range of maternal plasma glucose there appears to be a positive relationship between glucose and birth weight [88], it seems unlikely that maternal glycaemia will be part of the interaction between lower birth weight and cardiovascular risk. It seems likely then, that different mechanisms might act to lead to the association between low and high birth weight and diabetes. To that end it is interesting to note that in the highly diabetes prone populations studied by Pettitt et al there appears to be a U shaped relationship between offspring diabetes risk and birth weight[48], with increased risk both in low and high birth weight infants, a relationship not present in the populations less prone to diabetes studied by Barker et al[44, 47, 45]. This infers that while low and high birth weight infants may be at risk, the aetiology of their later diabetes may be very different. This concept is supported by observations in the offspring of various diabetes prone groups in New Zealand. Simmons noted that offspring of Indian parents tended to be thinner and have lower cord insulin levels, in keeping with the low birth weight groups of Barker whereas the offspring of Polynesian parents tended to be heavier and have higher cord insulin levels, in keeping with the high birth weight Pima Indian model[128]. Thus, different mechanisms may operate in different ethnic groups and different birth weights to create a U shaped relationship between diabetes risk and birth weight. Such a U-shaped relationship, with high risk at the extremes of birth weight, has also been suggested for blood pressure[31] but not observed by other workers [129, 22].

Overall then, although potentially an important aetiological factor both in the later development of obesity and non-insulin dependent diabetes, maternal hyperglycaemia is strongly linked to higher birth weight and as such is a poor explanation for the observations of Barker et al. In considering other risk factors, maternal hyperglycaemia has also been linked to offspring high blood pressure both in neonates[130] and children[131], but in both cases this effect seems to be explicable by the influence of offspring obesity[131, 130], and in animal studies, hyperglycaemia of the dam has been suggested to worsen offspring hypertension in the Spontaneously

Hypertensive Rat (SHR) model of genetic hypertension in rats[132]. While of interest, the application to the human epidemiological studies is again limited.

While it seems less likely that direct effects of maternal hyperglycaemia underpin the fetal origins hypothesis, the possibility of a more complex relationship with maternal glucose metabolism exists. An association between maternal hyperinsulinaemia and low birth weight, independent of maternal glucose has been reported in a single study[88]. This is an intriguing observation as it raises that possibility that maternal insulin resistance itself might change the intra uterine environment and lead to lower birth weight, a possibility for which other experimental evidence is not yet available. It is also important as it suggests an alternative explanation for the connection between birth weight and later cardiovascular risk, which does not involve metabolic programming. Genetic predisposition to insulin resistance might be expressed both as cardiovascular risk in adulthood and impaired growth in fetal life. Transgenic mouse models disrupting insulin receptor substrate-1 (IRS-1), part of the second messenger pathway of insulin action, lead to both fetal growth retardation and later insulin resistance[133]. Polymorphisms in this gene are also associated with insulin resistance[134] and later non-insulin dependent diabetes in human populations[135] and insulin resistance has been proposed as an aetiological factor in cardiovascular disease in human populations[136].

Other evidence would appear to argue against this model, however. In human populations growth retardation is, in general, associated with lower insulin levels in cord blood[137]. Moreover, ethnic groups with the conjunction of a high later risk of NIDDM and lower birth weight, also have lower insulin levels in cord blood[128]. While fetal hyperinsulinaemia as measured by raised amniotic fluid insulin levels is predictive of later obesity[117, 118] and impaired glucose tolerance[119] in human populations and insulin infusion predisposes to later diabetes in animal models[124], these situations are again associated with maternal hyperglycaemia and fetal macrosomia[117, 118, 138]. It is difficult, then, to easily support a model of a genetically

programmed insulin resistance both affecting birth weight and later cardiovascular risk.

Impaired insulin secretion might also be of importance in both lower birth weight and predisposition to later diabetes mellitus and again this might be under environmental or genetic control. Knowledge of genes affecting pancreatic development and limiting insulin secretion is at an early stage, but clearly such a genetic influence might predispose to both impaired intrauterine growth and later non-insulin dependent diabetes as a co-association. The *PAX4* gene is involved in determining differentiation of β and σ cells in the pancreas[139]. Disruption of this gene results in both lower fetal size and in the absence of development of mature β and σ cells in the murine pancreas[139]. More subtle abnormalities of pancreatic development might conceivably underlie the connection of lower birth weight to later non-insulin dependent diabetes. Some of the evidence cited above, such as the lower birth weight and lower cord blood insulin levels in ethnic groups with a high later risk of NIDDM, would be in keeping with such a mechanism[128]. That lower birth weight acts in identical twins discordant for non-insulin dependent diabetes mellitus to predispose to later diabetes would argue against such a genetic effect, at least in these individuals.

ii) Maternal high blood pressure

Maternal high blood pressure might be associated with an increase in offspring blood pressure both as a direct aetiological factor or appear because of the shared genetic inheritance of mother and offspring. Genetic studies of the inheritance of high blood pressure estimate that 30-60% of variation in blood pressure is accountable by genetic factors[140] but have difficulty in estimating early environmental influences[141].

Some familial studies suggest an increased similarity between maternal and offspring blood pressure than paternal blood pressure[142, 143, 32, 131] but this is by no means a universal finding[144]. Hypertensive disorders in pregnancy such as eclampsia, preeclampsia and pregnancy-induced hypertension are clearly associated with preterm delivery and reduced birth weight [88, 145, 146], but the outcome for the child remains

controversial with some studies suggesting an increase[147, 148] and others no effect[35] on offspring blood pressure. The study of Ounsted et al[35] is of interest as it demonstrated no effect on offspring blood pressure after treatment of hypertension in pregnancy. This argues against a simple mechanism of maternal high blood pressure in pregnancy leading to offspring high blood pressure by influences of maternal high blood pressure directly on the fetal environment[35].

Co-association of raised maternal and offspring blood pressure may also arise because of shared detrimental genes. The example of preeclampsia is of interest as it has been associated with mutations of the angiotensinogen gene[149] also shown by linkage analysis to have an association with essential hypertension[150]. The connection to essential hypertension is not entirely clear, however, in that, despite this potential for a shared genetic cause preeclampsia does not predict later hypertension in the mother[151, 152]. The potential for such observations to explain the data of Barker et al are further weakened by the rarity of hypertensive conditions of pregnancy. Such disorders affect a minority of pregnancies (216 of 17,000 pregnancies- 1.3%- in the study of Himmelman et al[148]) and therefore may be of limited relevance to the populations studied with relation to birth weight and high blood pressure.

While the data of Barker et al are unlikely to be explained by the influence of the specific hypertensive disorders of pregnancy, it remains possible that lesser degrees of high blood pressure might lead to this association. If maternal blood pressure acted as an environmental factor to cause lower birth weight, then as offspring blood pressure might be expected to resemble maternal blood pressure secondary to shared genes, leading to a non-causal association of lower birth weight and high offspring blood pressure. Such a mechanism has been proposed following a recent study examining maternal ambulatory blood pressure and birth weight[153]. The authors report a negative association between maternal blood pressure and birth weight, with each 5 mmHg rise in mean diastolic blood pressure at 36 weeks being associated with a 76g fall in birth weight[153]. This is an important potential confounder to the birth weight hypothesis.

There is, however, an older literature a positive correlation of systolic blood pressure with birth weight at levels of blood pressure short of hypertensive disorders of pregnancy[154, 155]. In these studies birth weight only begins to show a negative correlation with higher blood pressure than most women exhibit(>80-90mmHg DBP) or when proteinuria is present[154, 155]. Further, those studies examining mothers with only mild degrees of high blood pressure have suggested a rather weak association between maternal and offspring blood pressure[156, 157]. Increases in offspring blood pressure, given mild degrees of maternal high blood pressure were only described in males[156, 157] or females[147] and disappeared after correction for offspring body mass[156, 157]. Such data would appear to argue against a significant environmental effect of moderate degrees of maternal high blood pressure on low birth weight and subsequent high blood pressure.

iii) Under nutrition

Barker et al have proposed that fetal under nutrition is the link between low birth weight and later cardiovascular risk. In this section I will consider the evidence for the effect of maternal under nutrition on birth weight in animal and human populations, the effects that this might have on morphology and the evidence for an effect on later cardiovascular risk.

Extreme degrees of under nutrition have been shown both in wartime[158-160] and developing world[161] populations to lead to reductions in birth weight, while supplemental feeding results in an increase in birth weight in such populations[161]. It is difficult to assess the full relevance of this to the populations studied by Barker et al where the degree of under nutrition may have been far more subtle, especially as the effects of under nutrition on birth weight often appear relatively modest. Famine in Holland during the Second World War resulted in maternal dietary restriction to 600 to 900 calories per day for six months but only a 240g fall in birth weight[162]. In reviewing the available literature in 1991, Susser[163] estimated that while nutrition will

influence birth weight in famine, outside this special situation the influence of maternal nutrition on birth weight is far less clear. Support for such a threshold effect has come from more recent studies[164]. The influence of maternal dietary supplementation is also only clearly beneficial in the case of famine, and food supplementation even in deprived parts of the developed world have produced less clear results(reviewed in[163] and also[165]); protein supplementation in one study being associated with a reduction in birth weight[163]. Such a degree of fetal under nutrition was calculated to affect 2 to 3% of newborns in the United States and 8-10% in the developing world in 1967[87] and although of vital importance to this minority it is difficult to explain the graded relationship of birth weight to later cardiovascular risk reported by Barker if only 2% of the newborn population are affected.

Barker et al detected a series of morphological changes associated with cardiovascular risk, including increased placental weight[42, 32], reduction in ponderal index[32, 72, 12, 47, 65] and relative sparing of the head[32, 72, 12]. Barker has proposed that reduction in birth weight to placental weight ratio might reflect under nutrition[166], but both the relation of placental weight to later blood pressure[24, 22] and the relation of under nutrition to altered placental: birth weight ratio have been disputed[167]. Do patterns of increased placental to birth weight ration and reduced ponderal index relate to underlying undernutrition and, as such, support the role of nutrition in the fetal origins hypothesis?

While maternal malnutrition is associated with a reduction in placental weight (references in[70]), there appears to be a greater decline in placental weight than fetal weight[159]. The study of Smith et al[159] does, however support effects on ponderal index. Under nutrition in the third trimester resulted in a reduction in birth weight with relative sparing of head and brain, and while this is not calculated in this study, one would expect ponderal index to be reduced[159], a finding supported by others[89, 164]. In examining growth of the placenta and the fetus, the picture is made more complicated when one considers the effects not of combined protein and calorie

malnutrition, however, but isolated deficiencies of one or other of these factors. Animal models of purely protein malnutrition do support greater effects of fetal than placental size[168], and recent studies from Southampton, examining the effects of nutrition on placental and fetal size, suggest complex and differing effects of protein, the type of dietary protein and carbohydrate acting at different times in human pregnancy[169]. In this study higher carbohydrate intake in early pregnancy and lower protein intake in late pregnancy were associated with lower birth and placental weights[169]. The effects of protein intake appeared to be confined to late pregnancy, however, and placental weight to be affected by only dairy protein intake and birth weight by meat protein intake. Given this degree of complexity, with competing effects of different nutrients at different times, it is certainly possible to imagine nutrition leading to the patterns observed by Barker et al but difficult to be certain of their role without prospective data. If nutrition is key, however, it is likely that it will be operating at the level of relative protein malnutrition or loss of other micro nutrients. The data on the extremes of protein and calorie under nutrition do not seem supportive: both in terms of the effects on placental to birth weight ratios and because the numbers of mothers at such extremes of under nutrition would seem to be small.

The importance of nutrition to the fetal origins hypothesis can also be examined by considering whether under nutrition leads to alteration in cardiovascular risk factors and cardiovascular disease.

Population subjected to under nutrition in utero have also been studied with regard to later cardiovascular risk. Clearly under nutrition in early life alone may not increase cardiovascular risk: low birth weight is commoner in Third World populations where the incidence of cardiovascular disease later is very low [170] and in a similar way under nutrition would have been commoner in Western Societies in the past at a time when cardiovascular disease was rare. The combination of under nutrition early with repletion later has been postulated to increase cardiovascular risk, however, with the

high degrees of vascular risk in migrant populations to the developed world cited as evidence of this.

Certain populations subjected to deprivation in early life have been studied with regard to later cardiovascular risk. Ravelli et al [171] examined the later influence of under nutrition in utero to later obesity. They found an increase in obesity in those individuals exposed to famine in the first two trimesters, whereas exposure to famine in the last trimester and the first 3 to 5 months postnatally resulted in a reduction in the frequency of obesity. They conclude that factors in early life affect adiposity later in life, a hypothesis previously proposed, with some experimental evidence, by C.G.D.Brook[172]. In examining the relationship of this data to the Barker hypothesis it is interesting to look at the birth weights of these cohorts. The group exposed to famine in the first two trimesters in the original study had no reduction in birth weight but did have an increase in later obesity. In contrast the group with exposure to famine in the last trimester and the first 3 to 5 months postnatally suffered both a reduction in birth weight and a reduction in later obesity. Thus the data of Ravelli may support a effect of maternal under nutrition in programming later obesity in the fetus, but it is difficult to relate this directly to lower birth weight.

In a large study of the St Petersburg population, no influence of under nutrition on later diabetes or blood pressure was observed in the groups exposed to famine[173]. Increases in levels of pro-insulin and lower levels of insulin in response to an oral glucose tolerance test in famine-exposed groups have been interpreted as showing possible evidence of pancreatic beta-cell dysfunction, but given that no overall changes in glucose tolerance were observed this might be interpreted with caution[173]. No information on birth weight was available in these studies and the possibility that the pregnant mothers in this population may not have been subjected to the same degree of deprivation as the rest of the population has been raised as a potential confounder and explanation for the lack of marked differences between the groups.

Lucas and Morley[174] investigated the relationship between fetal nutrition and

blood pressure at age 7.5-8 years in a cohort of children included in a large multicentre trial of supplemental feeding to preterm infants. This group have previously demonstrated the importance of this period of nutrition to developmental status[175] and allergy and atopy [176] but found no differences in later blood pressure in any of the randomised groups. It could be argued that under nutrition out of utero is not necessarily the same as under nutrition in utero and that these premature infants were a selected and unusual cohort to begin with. Finally, one of the effects of maternal under nutrition might be to promote maternal anaemia, proposed to lead to both changes in the placental to birth weight ratio[177, 32] and of childhood blood pressure[32]. The relation of maternal anaemia to both childhood blood pressure[178] and placental to birth weight ratio[167] have been disputed by other workers, however.

In animal models of under nutrition, both caloric deprivation and protein malnutrition during pregnancy result in reduction in birth weight. In addition the studies of McCance and Widdowson display that caloric deprivation in early life can produce permanent alterations in body composition at least in terms of relative organ weights[179, 180], with the effects of such under nutrition appearing to be less reversible the earlier that they occur in life[180]. Protein malnutrition during pregnancy has been associated both with reduced birth weight and an increase in blood pressure (15-22 mmHg increase versus controls)[181].

There is a wealth of data relating changes in glucose tolerance to under nutrition in early life. In the 1950s workers in the tropics described tropical or Type J diabetes, different from the described patterns in the developed world [182]. These patients had a mixture of the features of IDDM and NIDDM: diabetes developed at a young age, patients were insulin requiring but not insulin dependent, being resistant to ketoacidosis after insulin withdrawal, and in all cases previous malnutrition was involved[182]. Insulin secretory responses were found to be acutely reduced by severe malnutrition in childhood[183] and the study of some populations after severe, childhood under nutrition support a continued effect on glucose tolerance[184] and on the incidence

of NIDDM[185]. Ethiopian Jews migrating after famine from Ethiopia to Israel experienced a 9% rate of diabetes in those under the age of thirty within 4 years of migration[185]. Clearly dissecting the effects of under nutrition from pre-existing genetic risks in a population experiencing a large change in adult environment is difficult. Animal models suggest both that the pancreas is sensitive to protein malnutrition in development and that the effects of this under nutrition vary depending on the timing of restriction. Malnourishment of dams results in lower offspring birth weight[186] along with impaired development of the fetal endocrine pancreas[186]. Protein malnutrition during pregnancy alone[187] and in the first postnatal weeks of life alone[188, 189] have been shown to result in permanent deficits in insulin secretion in such animal models, while protein malnutrition during pregnancy and up to weaning has been associated with evidence of muscle insulin resistance in the absence of insulin secretory defects[190]. In recent years the place of malnutrition as an aetiological factor in tropical diabetes has weakened however, with evidence of tropical diabetes in individuals never exposed to malnutrition and low rates of such diabetes in previously malnourished African populations[191] (reviewed in [192]). Malnutrition may, however, play a role as a contributory factor in some individuals[192].

In summary then, the human data from historical studies suggest that only severe degrees of protein-calorie under nutrition of mothers leads to reduced birth weight of offspring but this would appear to affect too small a proportion of the population to explain the epidemiological data. This does not exclude the influence of under nutrition on birth weight and later blood pressure or diabetes by other mechanisms: micro nutrients might affect birth weight and blood pressure, with the role of purely protein under nutrition being stressed in animal studies[186, 188, 187, 181, 190]. The potential importance of purely protein under nutrition in late pregnancy is supported by the results of Godfrey et al in human pregnancy[169]. As discussed above the results are complex with differential effects on birth and placental weight by meat and dairy protein intake respectively, making extrapolation to the epidemiological studies more

difficult.

Further problems in extracting the potential effects of under nutrition lie in the potential role of inter-generational effects. Malnourishment of mothers during their own childhood to impact upon maternal phenotype and therefore offspring birth weight was proposed in 1956 by Tanner et al, considering the experience of birth weight and deprivation in pregnancy in Aberdeen[193] . These authors suggested that such effects might be mediated through reduction in maternal size and thus restriction in her babies birth weight. Some of the animal data examining protein under nutrition in early life might support such effects. Protein malnutrition in the first three weeks of life of the dams has been shown to result in hyperinsulinism in the offspring of these animals as adults, albeit in association with higher dam glucose and offspring macrosomia[189], while perhaps more pertinently Holness et al displayed a persisting impairment of muscle glucose utilisation without deficits in insulin secretion persisting for two generations after protein restriction during pregnancy and until weaning of the first generation and in association with reductions in fetal at least in the second generation[190]. A further interesting feature of this study is that the third generation fetuses also appeared to have reduced glucose utilisation *in utero* although the mechanism of this potential insulin resistance of the fetus is unknown.

iv) Social Class

The interaction of social deprivation with vascular disease may occur in several ways. In terms of the birth weight hypothesis it is first important to consider social deprivation as a potential simple confounder. Poverty is associated with low birth weight[194], adult cardiovascular death[194] and adult risk factors such as non-insulin dependent diabetes[195] and strong correlations exist between previous deprivation and current deprivation[196]. While this observation might be due to a causal chain reflecting the influence of poverty on the ante-natal environment, birth weight and thence adult disease, it is also possible that it reflects an independent association of poverty with

low birth weight and some environmental factor in adult life conferring cardiovascular risk, with the most obvious candidates being smoking or diet. This means that an apparent association between lower birth weight and adult cardiovascular disease might arise due to the co-association of social class with low birth weight and adult cardiovascular disease.

This does not seem to be the full explanation, however, for while the previously observed association of lower birth weight with lower social class is seen either significantly[71] or as a trend[12] in most, but not all [11] studies, the relationship of birth weight and adult blood pressure[42, 44], cardiovascular mortality/ prevalence [11, 71], diabetes[44] and insulin resistance[65] is present within groups subdivided either for social class at birth or for current social class. In other words, while there may be effects of social class on birth weight, the risk associated with being a lower birth weight baby is present in each social class. This would not support the interpretation that adult high blood pressure and low birth weight are linked simply because of a shared association with poverty. Similarly, the results also do not seem to be easily explicable simply because of independent association of social class with lower birth weight and later risk factors such as smoking. Other smoking related disease have been examined and show for example no association of low birth weight with later lung cancer in either men [12, 11] or women[11].

The role of social class is still of interest: the hypothesis that deprivation in early life might lead to adult cardiovascular disease has a long history[197] and the above evidence, while not supporting social class as the entire explanation for connections between birth weight and later cardiovascular death, do leave potential for social class to impact upon later cardiovascular disease either via effects on birth weight or by other mechanisms. Recent evidence suggests that paternal social class does influence ischaemic heart disease risk in later life[41]. Wannamethee et al produced evidence from the British Regional Heart Study population to suggest that even after adjustment for current social class, lower paternal social class is associated with offspring ischaemic

heart disease and both lower height and increased obesity in adulthood. While other risk factors were associated with paternal social class these associations seemed explicable by current social class. This would suggest that deprivation in early life may well influence later cardiovascular risk, with the mechanism of this unknown. In this context it is interesting to note that the previously observed negative effect of socio-economic grouping on adult blood pressure is observed in childhood populations in some[198, 32], if not all[34, 37] studies. This would suggest that some of the negative influence of lower socio-economic group on blood pressure may be acting from a very early stage.

Effects of social class do not seem to explain the associations seen in the birth weight hypothesis, indeed the available evidence supports social class as a marker for environmental effects which are themselves active in early life to increase cardiovascular risk.

v) Maternal anaemia, smoking and living at altitude

Barker et al have noted a relationship between increased placental to birth weight ratio and later cardiovascular risk[12], high blood pressure[42] and diabetes[47]. They propose that such an increased ratio might reflect adaptive changes of the placenta to increase oxygen extraction from the maternal circulation. Such an increase in placental weight relative to birth weight, along with an increase in the number of low birth weight infants is well described in pregnancies at altitude, presumably secondary to the lower oxygen tension[199]), but there are no studies to determine the later cardiovascular implications of this.

Maternal anaemia and smoking might potentially exert similar effects on oxygen delivery to the fetus. Maternal smoking certainly leads to an increase in the numbers of low birth weight babies[77, 78, 73] and to an increase in the placental to birth weight ratio[177], but there appears to be no effect on offspring blood pressure, at least in childhood [32, 178]. Maternal smoking would also seem an unlikely mechanism as it has

been pointed out that smoking among women was still unusual at the time of birth of the subjects reported in most of the older studies[200]. Markers of maternal anaemia are negatively correlated with birth weight and placental weight[177, 178] but with a larger effect on placental weight, so that while maternal anaemia is associated with both a heavier placenta and birth weight, the calculated placental to birth weight ratio is higher. Maternal anaemia has also been related to a 2.9 mmHg rise in blood pressure in children at age 4 years[32], but this result was not repeated in other studies[131, 178].

vi) Other Factors

Blood pressure is susceptible to various other environmental factors in adult life but their relationship to exposure in fetal life is less clear.

Blood pressure shows a positive relationship to salt intake both across[201] and within[202] populations and falls with salt restriction[203]. High salt diet induces the onset of high blood pressure in genetically susceptible animal populations[204]. This effect is more pronounced young animals, with life long influences on blood pressure of salt diet early in life[205]. The role of salt diet in humans is less clear, however. Salt restriction has been shown to have a short term effect on blood pressure, leading to a 2.1 mmHg fall pressure by six months of life[206], but differences in salt intake early in life have not been shown to influence blood pressure into childhood[207, 208].

In population studies, higher potassium and calcium intake are associated with lower blood pressure [209] although addition of these agent have a variable effect on blood pressure[210, 211]. Maternal potassium intake during pregnancy has been inversely related to offspring blood pressure[212] and there is limited evidence from animal models to suggest an influence of low potassium diet in pregnancy and high sodium intake to influence later blood pressure[213].

Conclusion

Many factors influence birth weight and any or all of these might be connected to the expression of later cardiovascular risk. I have tried to summarise some of this information and, as detailed, there is much of the variance of birth weight that is unexplained and none of the proposed mechanisms explain all of the findings. It is the hypothesis of this work that another factor might underlie the epidemiological findings: exposure of the fetus to glucocorticoids. As an introduction to this I now consider what is known about the physiological exposure of the fetus to glucocorticoids, the pathological effects of excessive exposure and the role of the steroid metabolising enzyme 11ß-hydroxysteroid dehydrogenase (11ß-OHSD) in the placenta.

11beta-OHSD, Glucocorticoids and the fetus

Introduction

In this section I review the importance of glucocorticoids in the fetus and the role of 11ß-OHSD in metabolism of glucocorticoids in the feto-placental unit. In the first instance, I review the known biology of 11ß-OHSD with regard to the different isoforms of the enzyme, the function of 11ß-OHSD in the kidney in protecting the mineralocorticoid or type 1 corticosteroid receptor, the role of inhibitors such as carbenoxolone and glycyrrhetinic acid, and finally the clinical syndromes where deficiency of 11ß-OHSD is of importance (e.g. the Syndromes of Apparent Mineralocorticoid Excess and liquorice abuse). In the second section, I consider the normal development of the HPA axis in the fetus, in the human and in the rat, and review the current evidence for the role of placental 11ß-OHSD in modulating access of glucocorticoids to the fetus. Finally, I consider the developmental role of glucocorticoids, both normally and the effects of excessive glucocorticoid exposure.

11beta-Hydroxysteroid dehydrogenase

11ß-hydroxysteroid dehydrogenase exists in two known isoforms and catalyses the oxidation/reduction of steroid hormones in the 11 position (Figure 1). The two isoforms of the enzyme differ greatly in their tissue localisation, substrate specificity and indeed in the preferred direction of the enzyme. For both, however, their biological importance arises because of key differences in the actions of the 11 keto and 11hydroxy glucocorticoids that they interconvert. 11-hydroxy forms (cortisol in man and corticosterone in the rat) are biologically active and bind corticosteroid receptors with far greater affinity than the 11 keto forms. Thus the 11ß-hydroxysteroid dehydrogenases, in the interconversion of 11 keto and 11 hydroxy steroids potentially control the access of active forms of glucocorticoids to receptors and to tissues[214].

The type 1 isoform of the enzyme (11β-OHSD) has been purified[215], cloned[216]

and expressed[217] and although present to some degree in a variety of tissues is mainly expressed in the liver[218]. It is NADP dependent[215] and has a low affinity for cortisol and corticosterone (Km for corticosterone 1.83± 0.06μM and for cortisol 17.3±2.24μM[215]. Indeed, although bi-directional in homogenised tissues, 11β-OHSD appears to mainly act as a reductase in intact hepatocytes, that is catalysing the conversion of inert cortisone to cortisol (or 11-dehydrocorticosterone to corticosterone)[219]. At the time of the cloning of 11ß-OHSD 1, most scientific interest centred upon the role of 11ß-OHSD in controlling access of glucocorticoids to mineralocorticoid receptors, especially in the distal convoluted tubule of the kidney[220, 214]. After the cloning of 11ß-OHSD 1 it became apparent that this isoform was not the active isoform in either the distal convoluted tubule or in the placenta and interest eventually turned to the second isoform. The physiological role of 11ß- OHSD 1 is as yet unknown, but the enzyme has proposed roles in the supply of glucocorticoid to the liver[221].

The type 2 enzyme (11β-OHSD 2) has also been recently cloned; from sheep kidney[222], rat kidney[223] and in the human form from placenta[224] and kidney[225] and is a member of the short chain alcohol dehydrogenase superfamily[226]. The rat form shows an 83% and 77% homology to human and sheep respectively[223]. This type 2 isoform is only 14% homologous to the type 1 form and is present in classical mineralocorticoid responsive tissues, being highly expressed in the human colon, kidney (distal convoluted tubule, cortical collecting duct and medullary collecting ducts[224]) and parotids[225] as well as being expressed in brain, placenta and human fetal tissues[224]. The enzyme is NAD dependent, showing a 10 fold difference in activity with NAD *vs* NADP in human[225] and rat[223] and a 4 fold difference in the sheep[222] and has a high affinity for classical glucocorticoids (Km cortisol 47 nM, corticosterone 5 nM in[225]), in keeping with its role in these tissues of protecting mineralocorticoid receptor. Dexamethasone is metabolised to a far smaller extent (Km 119 dexamethasone) and aldosterone not at all[224]. The expressed 11ß-OHSD 2 isoform

Figure 1

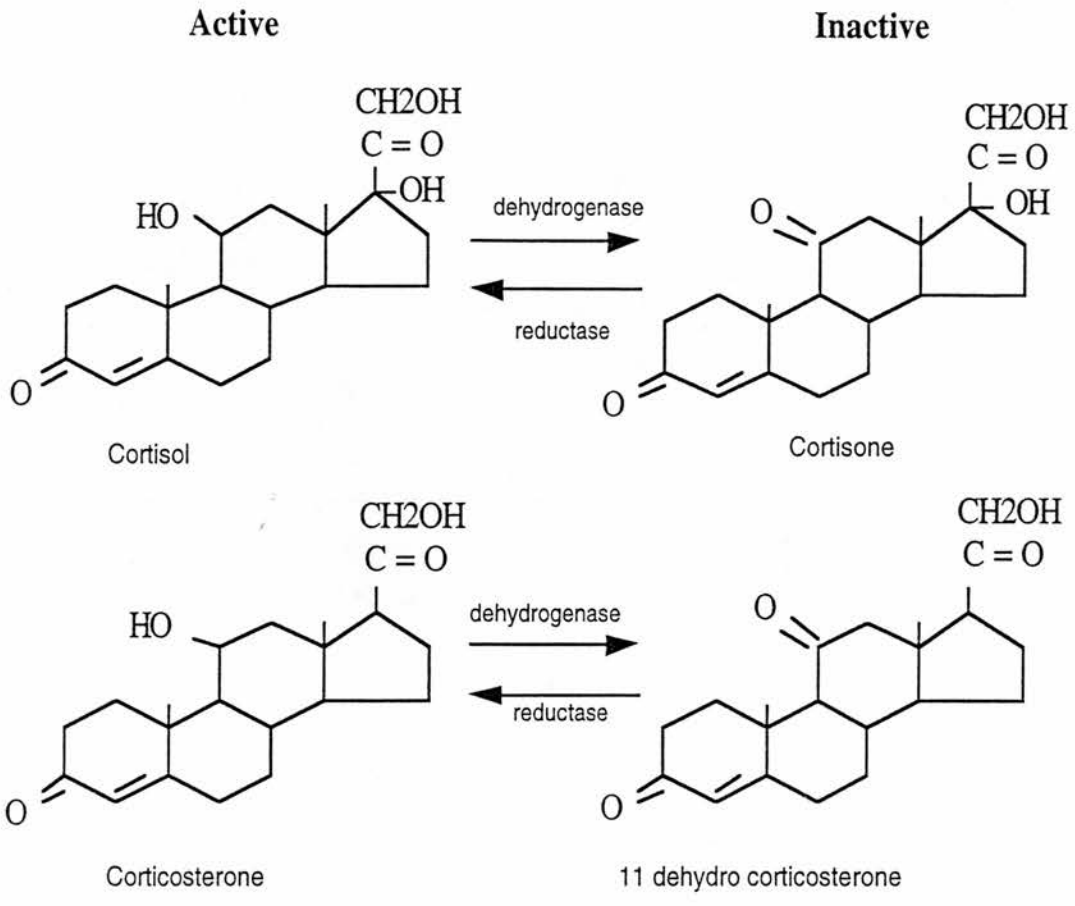


Figure 1: Inter conversion of glucocorticoids by 11 ß hydroxysteroid dehydrogenase

acts almost entirely in the dehydrogenase direction, with little reductase activity in either intact cells or homogenates[224] and this activity is potently inhibited by carbenoxolone (IC_{50} 18 nM) and glycyrrhetinic acid (IC_{50} 11 nM)[224]. Developmentally, 11ß-OHSD 2 enzyme activity is present in the developing human [227] and rat, as is the mRNA encoding for the type 2 enzyme in human[228] and rat, but its functions in normal development are as yet still speculative. While this isoform is key to the protection of mineralocorticod receptor in the kidney, colon and parotid it is also present in less clearly mineralocorticoid responsive tissues. For example, 11ß-OHSD 2 is highly expressed in the pancreas[225] and present in the ovary, testis, prostate[225] and brain[224]. Its function in these tissues remains speculative. This is also the

isoform present in placenta[224] and its role in this tissue is discussed below.

The Syndromes of Apparent Mineralocorticoid Excess :

11§-hydroxysteroid dehydrogenase and the kidney

Clinically interest in the 11ß-hydroxysteroid dehydrogenases developed because of the syndrome of apparent mineralocorticoid excess (SAME), first described in the late 1970 s by New and Ulick[229, 230]. Around 20 patients with this syndrome have been reported[221], presenting as a clinical paradox where clinical and biochemical findings in keeping with mineralocorticoid excess (hypertension, hypokalaemic alkalosis, kaliuresis and renal sodium retention, suppression of plasma renin activity) are associated with no elevation of any known mineralocorticoid[230, 231]. Other features of the syndrome include reversal of the clinical and biochemical features by administration of dexamethasone; mildly elevated urinary free cortisol concentrations; evidence of inhibition of cortisol dehydrogenation in the kidney with elevation of the ratio of urinary metabolites of cortisol versus cortisone (THF+ allo-THF :THE ratio, see Figure 2) and prolonged half life of (11α^3H)-cortisol; and impaired 5ß-reductase activity(decreased THF: allo THF ratio, see Figure 2)[232].

In 1988 it was proposed that apparent mineralocorticoid excess resulted from a deficiency in the normal action of 11ß-OHSD in the kidney[220, 214]. This hypothesis resulted from a series of observations. Firstly it was known that the mineralocorticoid receptor, which had been cloned in 1987, had an equal affinity, at least in vitro, for both classical glucocorticoids (cortisol, corticosterone) and mineralocorticoids (aldosterone)[233]. Since glucocorticoid circulate at a 10^4 fold excess over mineralocorticoids in both man and the rat, this created a paradox. Either the in vitro results were misleading or other factors in whole tissue were controlling the access of glucocorticoids to receptors. It was suggested that 11ß-OHSD functioning as a dehydrogenase in the kidney might fulfil this role and this would explain both the lack

of any clear excess of known mineralocorticoids in the syndrome and the reversibility of SAME with other glucocorticoids such as dexamethasone, as these suppressed the HPA axis and did not bind MR. Following the cloning of the 11ß-OHSD 2 gene[222, 225, 223, 224] it has been demonstrated that SAME is indeed associated with mutations in this gene resulting in defects in glucocorticoid metabolism[234, 235].

Figure 2

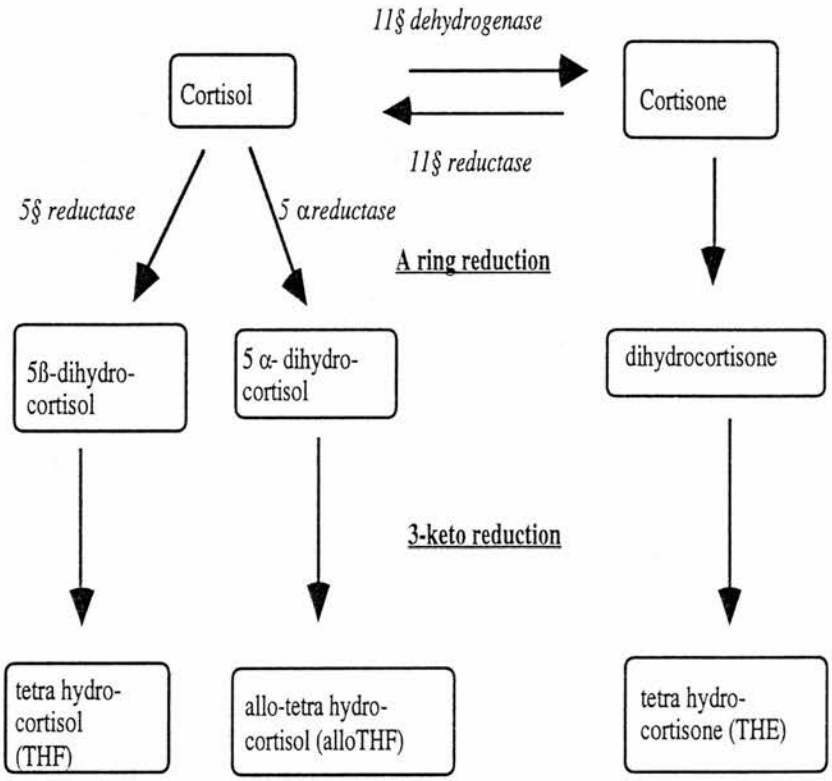


Figure 2: Principal urinary metabolites of cortisol and cortisone, after Walker [221]

Ulick et al have reported a group of patients with slightly different biochemical findings(SAME 2)[236, 237]. These patients again have clinical findings in keeping with SAME (biochemical findings in keeping with mineralocorticoid excess without elevation of any known mineralocorticoid and reversed by administration of dexamethasone) but ratios of THF+ allo-THF :THE ratio are normal[236, 237]. It has been proposed that such patients will have a primary abnormality comprising either

defective A ring reduction[237] (see Figure 2) or alternatively a combined defect in both dehydrogenase and reductase activities in vivo[238] (a situation analogous to the differences in the *in vivo* actions of glycyrrhizic acid and carbenoxolone, see below) with observations of reduced half life of oral cortisone to cortisol and prolonged half life of (11a^{3}H)-cortisol favouring the latter interpretation[221]. Genetic studies on these patients may help to clarify some of this puzzle, but no mutations in 11ß-OHSD 2 in SAME 2 have yet been demonstrated.

Inhibitors of 11beta-OHSD

While it is only recently that the physiology of 11ß-OHSD has begun to be discovered, use of its inhibitors has a long medical history. Confectionery liquorice is derived from the root of the plant *Glycyrrhiza glabra* and use of liquorice or liquorice root as an agent against mouth and stomach ulcers dates back for two thousand years[239]. The principal active constituents of liquorice are the triterpenes glycyrrhetinic acid and its glycosylated form glycyrrhizic acid[240]. Both glycyrrhizic acid and the artificial derivative carbenoxolone have additional groups at C3 allowing greater water solubility[240]. Carbenoxolone was used until the 1970 s in the treatment of peptic ulcer. It was appreciated for some time that the use of liquorice was associated with both clinical and biochemical features of mineralocorticoid excess[241, 242]. Known mineralocorticoids are suppressed with use of these agents and therefore it was proposed that the biological effects were secondary to direct binding to the mineralocorticoid receptor[243, 244]. The relatively low affinity of glycyrrhetinic acid for the mineralocorticoid receptor (Kd 2x 10^{-6} M versus 5x 10^{-10}M for aldosterone) made this unlikely and this along with the older observations that liquorice was not associated with mineralocorticoid effects in the absence of circulating glucocorticoids[245, 246] led to the proposal that liquorice exerted its mineralocorticoid actions by inhibition of 11ß-OHSD[247]. Liquorice was shown to lead to an increased ratio of THF+ allo-THF :THE in urine and lengthened half life of (11α^3H)-cortisol *in*

vivo [247] and to potently inhibit 11ß-OHSD *in vitro* [248, 249].

Both carbenoxolone and glycyrrhetinic acid also inhibit 11ß-OHSD *in vitro* but their actions *in vivo* in humans are not absolutely equivalent. While liquorice[247], glycyrrhetinic acid[250] and carbenoxolone[249] prolong the half life of (11α^3H)-cortisol *in vivo*, liquorice[247] and glycyrrhetinic acid[250] are associated with an increase in THF+ allo-THF :THE ratio in urine and a fall in plasma cortisone concentration but carbenoxolone is not[249]. As well as inhibiting dehydrogenase activity, carbenoxolone also appears to inhibit reductase activity *in vivo* [249]. Thus, while intra renal dehydrogenase is inhibited by carbenoxolone resulting in mineralocorticoid effects, inhibition of hepatic reductase activity results in no overall difference in circulating levels of cortisol and cortisone and since A-ring reduction leading to the generation of the tetra hydro metabolites takes place in the liver and presumably reflects metabolism of circulating F and E this also accounts for the lack of change in THF+ allo-THF :THE ratios in urine[221]. In keeping with this the ratio of urinary free cortisol to cortisone, believed to more closely approximate the intra renal concentrations of F and E, is increased[251].

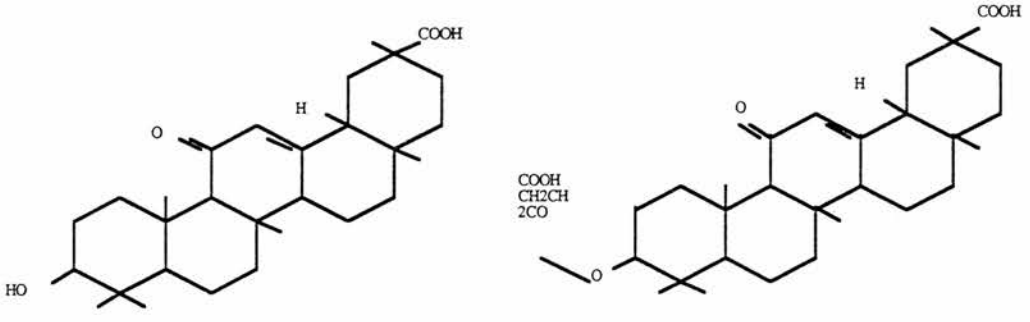
Clearly differential effects of these inhibitors on the different isoforms of 11ß-OHSD might explain these findings and lead to alteration in their actions on different tissues and in different species. Caution in the interpretation of such results is necessary as the effects of such inhibitors may be dependent upon the conditions of the particular assay used: in expressed systems of cloned rat 11ß-OHSD 1 glycyrrhetinic acid inhibits dehydrogenase but not reductase activity in intact cells but inhibits both in cell lysates[217]. Further, the inhibition of reductase activity by carbenoxolone *in vivo* in man[249] is not readily apparent *in vitro* in rat tissues[248] and probably does not occur in vivo in the rat[252].

Liquorice has other actions on glucocorticoid metabolism of less certain influence on health. Liquorice and its derivative glycyrrhetinic acid inhibits 5ß-reductase activity *in vitro* in the rat[253] and *in vivo* in humans, reflected in changes in

the THF: allo THF ratio[247]. This effect of glycyrrhetinic acid does not seem to be shared by carbenoxolone which is thought not to inhibit 5ß-reductase activity *in vivo* in humans[249] although again inhibition is observed in vitro in the rat[254].

Finally these agents may act via mechanisms independent of inhibition of 11ß-OHSD. This include inhibition of prostaglandin synthesis[255], alteration in hepatic aldosterone metabolism[253], direct effects on sodium and potassium transport[256], potentiation of the effects of steroids not subject to 11-OHSD metabolism (aldosterone, 11-deoxycorticosterone and synthetic glucocorticoid agonists)[257, 258] and direct activation of mineralocorticoid receptors[243, 244]. Carbenoxolone has been noted to enhance vasoconstrictor action in ex vivo preparations and to potentially exert damaging effects on endothelium in vivo[259]. The physiological importance of such observations remains uncertain especially as such actions usually require concentrations not usually occurring in vitro[220, 260]. For example, carbenoxolone inhibits both 15-hydroxyprostaglandin dehydrogenase and 13-prostaglandin reductase in the micromolar range[261].

Figure 3



Glycyrrhetinic acid

Carbenoxolone

Figure 3: Structure of glycyrrhizic acid and carbenoxolone

11beta-OHSD in the placenta: the glucocorticoid "barrier"

Cortisol metabolising activity was first demonstrated in the placenta by Osinski in 1960[262], while further studies in the 1970s by the group of Beverly Murphy led to the proposal that cortisol metabolism in the placenta might be important in protecting the fetus from circulating maternal glucocorticoids. Much of the interest at this time arose from analogies of the sheep model, where rises in cortisol towards the end of term had been proposed to initiate parturition[263]. Murphy made several observations supporting the placenta in the active oxidation of cortisol, allowing it to act as a "barrier" to maternal cortisol. She demonstrated a lower ratio of cortisol to cortisone at all times in pregnancy in the fetal compared to maternal compartment. From the earliest point she measured (8 weeks), F to E ratio was 0.2 rising only to 0.6 after the onset of labour and suggesting a net conversion of F to E in the fetus or placenta[264]. Oxidation of cortisol to cortisone in vivo in passage across the placenta was also demonstrated: C^{14} cortisol was injected to mothers about to undergo abortion with detection of mainly C^{14} cortisol in maternal plasma but C^{14} cortisone in placental tissue and fetal plasma[265]. They concluded that in early pregnancy (18 weeks in this study) there was active conversion of cortisol to cortisone in the placenta[265], later estimating around 80% conversion of circulating cortisol to cortisone in passage across the placenta, creating an 8-10 fold gradient[266]. Murphy concluded a potential for 11ß-OHSD in the placenta to be acting to protect the fetus from maternally derived glucocorticoid [267]. Other workers had also demonstrated a high level of 11ß-OHSD activity in human placenta from the 7th to the 40th week of pregnancy[268, 269] and predicted that the oxidative capacity of the placenta would always exceed the delivery of cortisol by the maternal circulation[269]. While almost exclusively oxidative activity has now been demonstrated both for whole human placenta[270] and for 11ß-OHSD 2 cloned from human placenta[224], the ontogeny of enzyme expression and activity in human placenta remains to be fully demonstrated. Further complexity may exist, as while the *in vitro* studies have also demonstrated only oxidative activity in the placenta[268], there has been

evidence of both oxidative and reductive activity in the human chorion[271, 268] and decidua[268], giving the potential for regeneration of cortisol on the fetal side of the placenta.

11ß-OHSD is also present in the rat placenta[272] with evidence of a both dehydrogenase and reductase activity present early and a rise in dehydrogenase activity towards term[273]. Activity has been shown to be sensitive to inhibition by glycyrrhetinic acid[274].

The baboon is an extensively studied model of steroid metabolism in pregnancy, albeit one which appears to have certain key differences form the human situation. As in the human, higher levels of cortisone (E) than cortisol (F) are found throughout pregnancy in the baboon fetus and there are higher concentrations of cortisol in the maternal than the fetal circulation[275]. Infusion of F and E into the maternal compartment, however, suggests more reductive activity (cortisone to cortisol) in the placenta at mid gestation changing to a preponderance of oxidative activity at term[275], possibly under control of rising maternal estrogen levels[276, 277]. This creates a situation where production of cortisol by the fetal adrenal at mid term is negligible; minimised by negative feedback effects on the fetus of maternal cortisol until there is a rise in placental cortisol oxidising ability, to protect the fetus from the effects of maternal glucocorticoid[275, 278]. In keeping with this, treatment with androstenedione (proposed to act by increasing maternal estrogen levels) leads to an increase in oxidative activity in the placenta and an increase in fetal adrenal cortisol production at mid gestation[278].

Clearly parts of this model do not agree with the data of Murphy. Firstly, Pepe et al favour a predominant reductive capacity of the placenta at mid term whereas the studies of Murphy favoured oxidation. Secondly, Pepe et al would favour low fetal cortisol production at mid gestation[278]. While these differences may simply reflect true species differences, equally the studies of Murphy on placental metabolism may be criticised for not being conducted at steady state and as such might artefactually over-

represent oxidation. The steady state experiments of Pepe and Albrecht are unlikely to be performed in human pregnancy, *in vitro* studies of human placenta have shown that the type 2 isoform is the only form in placenta mid and late gestation and is overwhelmingly oxidative[227], although there is expression of the type 1 form and reductive activity in the decidua and chorion[279, 227] and this activity has been associated with high levels of cortisol in the uterine wall[267]. Studies of the HPA in human fetuses are few, but in the main do not support a suppression of the fetal adrenal by maternal glucocorticoid at mid gestation: fetal ACTH levels are higher at mid than late gestation[280], the fetal adrenal attains its largest size relative to body weight at mid gestation, peaking at around twenty weeks[281] and shows a rapid increase in size in absolute terms from the twelfth week on[282]. Clearly these changes may represent a variety of suppressive and trophic stimuli other than cortisol, however cortisol levels themselves seem to be at a nadir around 18 weeks[283] and most studies support derivation of cortisol from the fetal rather than maternal circulation unless the mother is stressed, even at these stages of pregnancy[284, 285, 283].

Finally, 11ß-OHSD may not be the only element in the placental glucocorticoid "barrier". While dexamethasone is not appreciably metabolised in vitro by human[286] or rat [287]placenta and less well metabolised by 11ß-OHSD 2 than cortisol[224], pharmacokinetic studies in the rat show that after maternal injection of dexamethasone there is a ratio of dexamethasone in plasma of 8.5 to 1 from maternal to fetal compartments[288]. The ratio is maintained by transport by the placenta of dexamethasone from the fetus to the mother[288]. The same workers have shown that there are higher levels of dexamethasone in the fetuses of mothers after protein calorie malnutrition[287], while maternal streptozotocin diabetes resulted in a *reduction* of fetal dexamethasone levels[289] perhaps reflecting increased clearance of drug by the fetal kidney into amniotic fluid[289]. Whether such transport mechanisms are also active for other glucocorticoids is not known.

The role of glucocorticoids in the fetus

Glucocorticioids may be active at a variety of levels in the fetus. Firstly, there are clear toxic effects on both the fetus and the placenta. Secondly, both the regulation of glucocorticoid exposure of the fetus and the wide number of genes regulated by glucocorticoids suggest that they may be pivotal in the maturational events in a variety of tissues. I will first consider the teratogenic and toxic effects observed with glucocorticoids before considering these more important physiological effects.

i) Toxic effects

Exogenous glucocorticoids are associated with both fetal loss and teratogenic effects in animals. Dexamethasone has been extensively studied being associated with reduced fetal weight, placental weight and fetal death in a variety of species [290-292]. All other glucocorticoids are associated with such effects including hydrocortisone[293], although hydrocortisone appears to be a weaker teratogen [293]. The effect of dexamethasone is dose dependent (in the rat at doses greater than 200μg/kg/day[294]) and associated with a range of organ malformations, of which cleft palate appears to be one of the most frequent[294]. Maternal stress has also been found to reduce birth weight in animal studies[295], while in human populations the role of psychosocial stress to reduce birth weight remains controversial (reviewed in[296]). Glucocorticoids in high doses also exert toxic effects, observable as ultrastructural changes in the placenta[297-299], giving a potential for effects on the fetus secondary to changes in placental function[299].

The teratogenic role of glucocorticoids in human pregnancy are less clear. Successful pregnancy can occur both after administration of exogenous glucocorticoids and in Cushing's syndrome, but a high incidence of spontaneous abortion is noted. Effects of the underlying condition confound analysis as to whether glucocorticoid use leads to reduced birth weight but it appears that in sufficient dosage both glucocorticoids[300] and Cushing's syndrome[301] are at least anecdotally associated with retarded fetal growth in humans[302]. Most authors considering the medical uses of

glucocorticoids in pregnancy report fewer teratogenic complications than might be expected from the animal studies[303, 304] and a very low incidence of adrenocortical insufficiency in the newborn[303, 304], while not commenting on effects on birth weight. Historically, most of the glucocorticoids used in pregnancy were those extensively metabolised by 11ß- OHSD, such as cortisol or prednisolone[303, 304], and one might speculate that this might have led fortuitously to fewer side effects on the fetus. By contrast, in the treatment of congenital adrenal hyperplasia, dexamethasone is used precisely because it crosses the placenta, to deliver a glucocorticoid dose to the fetus. I am not aware on longer term studies investigating the effects of such exposure, but again such studies would be confounded by the underlying genetic condition (e.g. congenital adrenal hyperplasia).

ii) Development of the HPA axis

In the human, primordial adrenal structures are visible from 4 weeks of gestation(reviewed in[305]) with a clearly identified adrenal at 6 weeks[282]. Hormone production also begins early in life: there is evidence of cortisol production from as early as the 8th week[264, 306] and of corticosterone and aldosterone at 16-20 weeks[307] although at this age adrenal steroids are derived at least partly from placental precursors[307]. Development of basophilic cells in the adenohypophysis is visible at 8 weeks of life(reviewed in[305]) and ACTH is measurable in human fetal plasma from 12 weeks[308]. Cultured human fetal adrenal cells show cortisol and DHEA-S production in response to ACTH from 10 weeks[308]. The dynamics of cortisol levels in the human fetus remain controversial, but most evidence supports a rise in cortisol levels through gestation and towards term[309, 310], while there is more limited evidence that the HPA axis is responsive to stresses such as intrauterine needling from as early as the 23rd week in utero[311].

The human fetal adrenal is relatively large, comprising at its peak at mid gestation 0.38% of body weight compared to 0.13% of body weight in a 1 year old

infant and 0.01% of adult weight[281]. The adrenal in fetal life has a different morphology form the adult, the cortex being divided into fetal and definitive zones with the former being 80-88% of total adrenal weight[281]. The fetal zone produces DHEA-S, while the definitive zone is cortisol producing[308]. The fetal zone involutes in the first few weeks of extra-uterine life[281]. The purpose of this large production of DHEA-S has been speculated to be as a substrate for the production of estrogens by the placenta[305] and this unique pathway has been proposed as a marker for fetal adrenal activity[305].

In the rat vascular connections between the neurohypophysis and adenohypophysis exist from as early as the 15th day of life[312] and there is an upsurge of glucocorticoid levels in the 2nd to 3rd post-partum[313]. The circadian rhythm of corticosterone is absent at birth, but established at between 21 and 32 days of life (refs in[314]) and chiefly dependent on feeding pattern rather than the light-dark cycle[314]. Low CBG levels in the immediately postnatal period lead to very low total levels of corticosterone, but measurable responses to stress[315] and adult patterns of response to adrenalectomy and corticosterone replacement[316] are observed in pups even in the first few days of life if sufficiently sensitive assays are used. The amplitude of the response to stress increases with age in the first few weeks[315].

iii) Physiological effects

Towards term a variety of species show a rise in glucocorticoid levels [317] and this rise, reflecting maturation of the fetal HPA axis, is pivotal initiating normal parturition in the sheep[317, 318]. The role of glucocorticoids in other species remains less clear: dexamethasone in high doses(500µg/ day) delays the onset of labour in the rat[319]. While glucocorticoids appear to rise before parturition in the human[320] and other primates, their place in initiating delivery remains controversial[321]. The rise in glucocorticoid levels is implicated, however, in a variety of maturational events, especially in the foregut and liver, and are proposed, in general, to promote

competence for extrauterine life[317, 321].

Maturational effects are best described in the lung where glucocorticoid exposure leads to expression of surfactant and other changes leading to therapeutic use in pre term delivery in human pregnancy[322]. The lungs are not alone in such effects. Glucocorticoids have well known effects on a range of gluconeogenic enzymes in adult life[323] and are involved in induction of these enzymes either in late gestation in a range of species (the sheep[324], horse[325, 326], mouse[327] and rat[328, 329]) or in the first day of life[330]. In the pig they are also implicated in increases in pancreatic enzymes [331] and maturation of gastric function [332]. The majority of such studies involve pharmacological doses of glucocorticoid, but glucocorticoids are also proposed to promote maturational changes in normal physiology, to allow maturation of gut and liver related gluconeogenic enzyme activities in the rat both pre-natally and post-natally[333, 334].

Exogenous glucocorticoids have also been associated with maturational effects in late intrauterine life on the kidney in the rat[335, 336]and sheep [337]. This includes promotion of tubular function without effect on glomerular filtration rate[336], alteration of responses to beta adrenergic agonists[338] and induction of rat renal Na/K ATPase activity [339]. Importantly, such effects occur at doses less than those expected to lead to teratogenic effects of fetal death (0.05-0.2 mg/kg days 17-19 for effects on tubular function[336]) and are dose-dependent[338]. In the experiments of Bian et al, dexamethasone 0.2 mg/kg (gestational days 17-19) enhanced the response of the kidney (cAMP) to a beta agonist (isoproterenol) in young adult life, but larger doses (0.8 mg/kg) reduced the response to beta agonist. Thus, there may be complex effects based upon different doses and times of exposure. Similar effects to promote adrenergic responses in the heart have been reported by the same group [340-342]. Structural changes, with an increase in the prevalence of polycystic kidneys, have also been reported with large doses of cortisol in mice[343].

The effects of glucocorticoids on brain development have also been studied.

Again there is a wide range of possible effects with some groups recording evidence of accelerated maturation of motor ability[344], synaptic connections [345] and catecholaminergic pathways [346]. Other groups report evidence of damage in the primate brain with degenerative changes in the hippocampus in macaques following dexamethasone exposure, even at doses similar to those used in lung maturation in human pregnancy[347]. A variety of groups have recorded the possibility of late effects on HPA axis reactivity after intrauterine glucocorticoid exposure albeit with conflicting results[348-350].

Conclusion

We hypothesised that glucocorticoid exposure in utero might be involved in the long term programming of blood pressure and glucose tolerance. It is clear that glucocorticoid exposure leads to dose dependent reductions in birth weight and further, that concentrations of glucocorticoids in utero are of importance in promoting a series of developmental changes especially of the gut, liver and lung. Alterations in the timing and extent of such maturation could conceivably alter blood pressure and glucose tolerance in the mature animal. I now discuss experimental evidence that glucocorticoid exposure in utero does indeed have such effects.

Materials

Chemicals

General Drugs and Chemicals

Acetylcholine	Sigma Chemical Company, Poole, Dorset, England
Angiotensin II(Hypertensin)	Ciba Geigy, Basle, Switzerland
Ascorbic acid	BDH Chemicals Ltd, Poole, England.
Biorad protein assay	Biorad Ltd
bovine serum albumin	Sigma Chemical Company, Poole, Dorset, England
5-bromo-2'-deoxyuridine (BrdUrd)	Sigma Chemical Company, Poole, Dorset, England
Calcium Chloride ($CaCl_2$)-	BDH Chemicals Ltd, Poole, England.
Cocktail T	Sigma Chemical Company, Poole, Dorset, England
Corticosterone	Sigma Chemical Company, Poole, Dorset, England
11-dehydrocorticosterone	Sigma Chemical Company, Poole, Dorset, England
Dexamethasone	Sigma Chemical Company, Poole, Dorset, England
Dimethyl sulfoxide	Sigma Chemical Company, Poole, Dorset, England
D-Glucose	BDH Chemicals Ltd, Poole, England.
Halothane(Fluothane)	Zeneca Pharmaceuticals,Macclesfield, England
Heparin	Leo Laboratories Ltd, U.K.
Magnesium Sulphate ($MgSO_4$)	BDH Chemicals Ltd, Poole, England.
nicotine adenine dinucleotide(NAD)	Sigma Chemical Company, Poole, Dorset, England
Noradrenaline	Sigma Chemical Company, Poole, Dorset, England
95% Oxygen:5% CO2	British Oxygen Company, Edinburgh, Scotland.
Potassium Chloride (KCl)	BDH Chemicals Ltd, Poole, England.
Potassium Phosphate (KH_2PO_4)	BDH Chemicals Ltd, Poole, England.
Sodium Bicarbonate ($NaHCO_3$)	BDH Chemicals Ltd, Poole, England.
Sodium Chloride (NaCl)	BDH Chemicals Ltd, Poole, England.
Sodium Pentobarbitone(Sagatal)	RMB Animal Health Ltd, Dagenham,Essex, England

TLC plate	BDH Chemicals Ltd, Poole, England.
tris buffer	Sigma Chemical Company, Poole, Dorset, England
Trypsin	ICN Biomedical, Bucks, England

Reagents for Immunocytochemistry

Anti-BrdUrd monoclonal antibody Europath Ltd, Bude, Cornwall, UK

rabbit anti-mouse antibody conjugated to alkaline phosphatase

Dako Ltd, Bucks, UK

Alkaline phosphatase substrate (fast BB salt)

Sigma Chemical Company, Poole, Dorset, England

Radiochemicals

corticosterone	Amersham Interational, Bucks, England.
dexamethasone	Amersham Interational, Bucks, England.

Equipment

Thin layer chromatography

Merck 20x20cm plate, aluminium sheets, Silica gel 60 F_{254}

BDH Chemicals Ltd, Poole, England.

High performance liquid chromatography

HPLC grade Methanol	Rathburn Chemicals, Walkerburn, Scotland
HPLC grade Water	Rathburn Chemicals, Walkerburn, Scotland
Liquid scintillant (Quicksafe Flow 2)	Zinsser Analytic UK Ltd
automatic sample injector (Waters 712 WISP)	-Millipore, Milford, MA, USA
HPLC pump (Waters 510)	Millipore, Milford, MA, USA
Absorbance detector (Waters Model 411)	Millipore, Milford, MA, USA
3.9x 300 mm C_{18} HPLC column (Waters Bondapak™)	Millipore, Milford, MA, USA

radioactivity monitor (Berthold LB 506 C-1)Berthold Analytical Ltd, Nashua, NH, USA

scintillant pump (Berthold LB 5035) Berthold Analytical Ltd, Nashua, NH, USA

11§-OHSD assay

ß- radiation detector (Minaxi, Tri-carb 4000)

Canberra Packard, Pangbourne, Berks, England

Direct Blood pressure measurement

pressure transducer (Gluck 470) Lectromed, Letchworth, England

chart recorder(Lectromed Multitrace 2) Lectromed, Letchworth, England

Indirect Blood pressure measurement

Incubator (Thermacage) Beta Medical and Scientific.,Sale, England

Air Pump Air Shields UK Ltd., Shoeburyness, Essex, England.

Mesenteric Perfusions

polythene PP50 tubing Portex, Hythe, Kent, England

peristaltic pump (Pharmacia Pump P3) Pharmacia Ltd., Milton Keynes, England

chart recorder (Elcomatic EM 720) Elcomatic Ltd., England.

Infusion of 3H Corticosterone

Syringe Driver B.Braun-Melsungen, Germany

Image analysis

image analyser Seescan Ltd, Cambridge, England.

Computing and data analysis

Statview TM

Animals

Wistar rats were purchased from Harlan Olac. In all experiments standards conforming to " The Principles of Animal Care" (NIH publication No.85-23, revised 1985) were followed.

Methods

1) Measurement of blood pressure

a) Direct measurement by carotid cannulation

Surgery

Animals were anaesthetised using halothane and an incision made in the left side of the neck. The carotid artery was isolated by blunt dissection and tied off (2G cotton suture-(Ethilon)) as far cranial as possible. The artery was then clamped proximal to the suture and a teflon cannula inserted and secured with two further ties. The free end of the cannula was tunnelled to the back of the neck and stitched to skin. The cannula had been prepared by flushing with heparin (1,000 units/ml) and after insertion and a further 30µl of heparin was introduced into the cannula and the cannula sealed with a metal pin. The area was sprayed with antiseptic and the animals left to recover on a heat pad.

Measurement of blood pressure

48 to 72 hours after cannulation blood pressure was measured. The cannula was connected to a 20 cm length of polythene tubing (Portex, Hythe, Kent) with a 22G steel needle as connector. Pressure was measured by directly by a pressure transducer (Gluck 470) connected to a chart recorder (Lectromed Multitrace 2, Letchworth, UK) which converted the signal to mm of mercury. The recorder was calibrated with an internal standard prior to and once during recording and with a mercury sphygmomanometer prior to each experiment. For blood pressure measurement the animals were placed at the level of the pressure transducer on a towel. Blood pressure was measured on for 10 min on three consecutive days and recorded as the mean of the 3 readings. The coefficient of variation for the repeated measures of blood pressure on separate days was 7.0% for systolic blood pressure and 9.1% for diastolic blood pressure.

Blood pressure measurement was successful in around 75% of animals,

problems mainly being due to eventual blockage of the cannulae. The heparin regimen maximised the number of animals with patent cannulae, while leading to no apparent bleeding problems as determined at post mortem.

b) Indirect measurement of blood pressure by tail cuff

Method

Blood pressure was measured as described by Lovenberg [351]. Animals were warmed to 38°C in a converted incubator. Individual animals were then restrained in a towel and the tail fitted with an inflatable cuff, distal to this a sensor was fitted to the tail to detect blood flow. The sensor incorporates a light source and piezo-electic crystal connected to a locally generated software programme controlling the rate of inflation and deflation and recording the return of blood flow. On deflation of the cuff, the resulting increase in electrical activity is represented as a diamond shaped pattern, where systolic blood pressure is represented by the onset of signal and diastolic from the point where the signal returns to baseline. Cuff size and the measuring programme had been previously validated. Daily recordings for each animal represent the mean of 5 distinct readings for each animal.

2) Mesenteric Perfusions

The mesentery was removed from an anaesthetised rat and perfused using a protocol very similar to that described by McGregor in 1965[352]. Animals were anaesthetised by injection of pentobarbitone and heparinised (heparin: 1000 $U.kg^{-1}$). A single midline incision was made and the intestine, aorta and subsequently superior mesenteric artery exposed using blunt dissection. Cotton sutures were passed around the aorta and superior mesenteric artery to allow control of the bleeding and the superior mesenteric artery cannulated using a length of polythene PP50 tubing (Portex, Hythe, Kent, UK) and perfused continuously oxygenated

Krebs-Ringer buffer solution (123 mM NaCl, 2.65 mM KCl, 1.29 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.5 mM $NaHCO_3$, 11.1 mM glucose and 18μM ascorbic acid continuously oxygenated by bubbling 95%O_2: 5%CO_2 through a reservoir)at a rate of 4 $ml.min^{-1}$ using a peristaltic pump (Pharmacia Pump P3).

The mesentery was dissected, and the gut removed from the duodenum to end of ileum using surgical scissors. After this dissection, the gut itself was removed and the isolated superior mesenteric arterial cascade maintained at between 36°C and 38°C (Solex digital thermometer) on a petri dish heated from below by a water bath. Tissues were protected from drying out by application of warm buffer and covering with the petri dish lid.

Pressure within the system was recorded continuously using a pressure transducer by means of a T-piece attached to the perfusion circuit close to the inflow to the mesenteric artery, and attached in turn to an chart recorder (Elcomatic EM 720) calibrated using a mercury sphygmomanometer. After a 30 minute equilibration period, preparations were administered a "wake up" bolus of 30 nmol of noradrenaline, after constriction and relaxation in response to this a further bolus of 30 nmol noradrenaline and 400 pmol acetylcholine was administered to assess the integrity of the endothelium dependent vasodilatation. The preparation was abandoned if it failed to give the expected responses (at least an 80 mmHg rise in pressure with the noradrenaline dose and a reduction in response to less than 80% of this with the combined noradrenaline and acetylcholine doses).

Noradrenaline, prepared in a 0.1% solution of ascorbic acid in buffer and diluted to concentrations between 0.2 and 20μM was then perfused in stepped concentrations (0.2, 0.5, 1, 2, 5, 10 and 20μM) at five minute intervals, with perfusion of buffer for five minutes between each step. Potassium was prepared by substitution of KCl for NaCl in buffer to a final potassium concentration of

between 25-125 mM and again perfused in a stepwise fashion (25, 45, 65, 85, 105, 125 mM).

This system has been extensively used within our department. Repeated perfusion with noradrenaline seemed to provoke a consistent response over time(Figure 4).

Figure 4

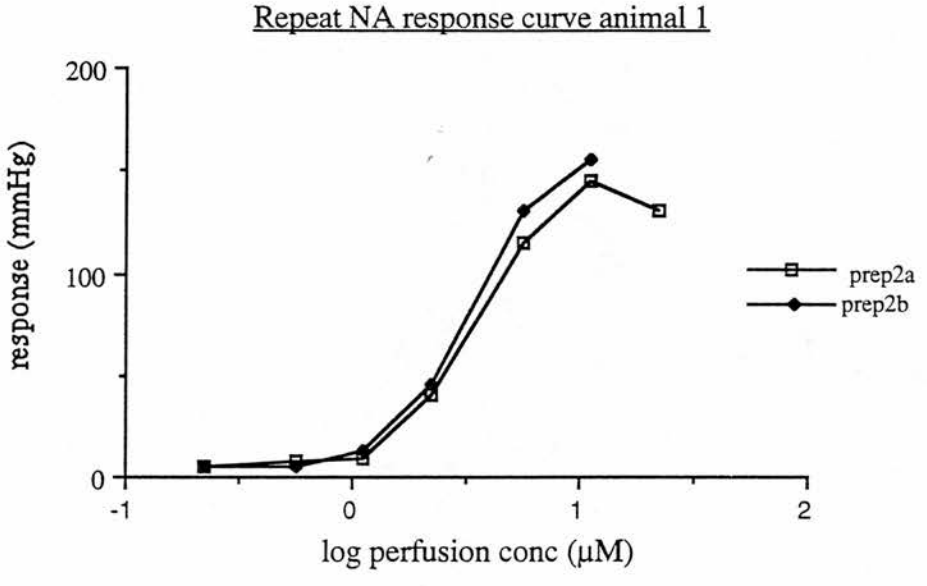


Figure 4: Mesenteric Perfusion. Rise in pressure after administrartion perfusion of noradrenaline 90 minutes apart in two separate preparations.

3) Immunocytochemistry

The technique for detecting BrdUrd in nuclei was modified from previously described methods. Mesenteric tissue was embedded in paraffin wax and cut in 3μm transverse sections which were then dewaxed, hydrated and immersed in 0.7N HCl for 1 hour to hydrolyse double stranded DNA. Sections were trypsinised for 2 minutes at 37^{O}C (0.03% trypsin) then washed in tris buffer at pH7.6 and treated with denatured rabbit serum to block endogenous enzyme activity. Sections were incubated overnight at 4^{O}C using a primary anti-BrdUrd monoclonal antibody and a secondary rabbit anti-mouse antibody conjugated to alkaline phosphatase. Alkaline

phosphatase substrate (fast BB salt) was used to visualise BrdUrd positive nuclei.

4) Oral Glucose tolerance test

Animals were fasted from 1600h the day before and 2 g/kg glucose (as a 0.5g/ml solution) was given by gavage between 0800 and 0900h the next morning. Blood was taken by tail tipping at 0, 30, 60, 90 and 120 min, centrifuged immediately and the plasma stored at -70C.

Glucose and insulin area under curves were calculated by the formula 15 a+ 30 b+ 30 c+ 30 d+ 15e where a-e are the values for glucose or insulin at times 0, 30, 60, 90 and 120 minutes respectively.

5) Insulin tolerance test

Insulin (100U/ml) was diluted in saline to a concentration of 1U/ ml in 0.9% saline. Insulin was administered in a dose of 0.1-0.75 U/kg by intraperitoneal injection. Blood glucose was assessed by tail tipping and using an Exactech meter at times 0, 15, 30, 45, 60 and 75 minutes.

Figure 5

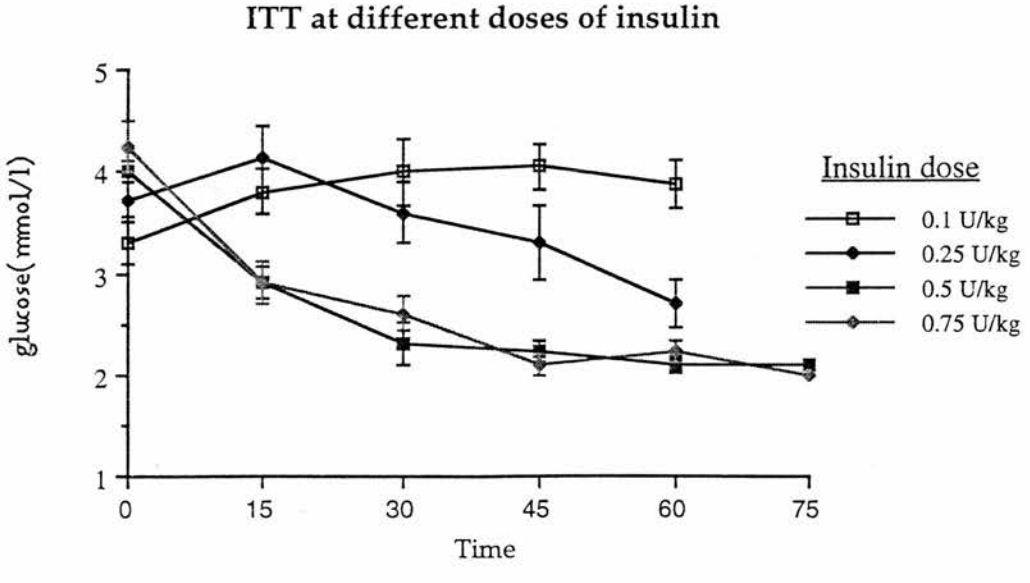


Figure 5: Blood glucose after intraperitoneal injection of insulin at various doses. Time points are mean ± SEM.

6) Assays

a) 11 beta hydroxysteroid dehydrogenase activity in vitro

i) Placental activity

The assay of this enzyme was well established in our department[353]. Assays were performed with 200μM NAD, 12nM ^{3}H corticosterone, 0.5 mg/l of protein (total volume 250μl) , at 37^{o}C for 10-20 minutes. The assay conditions were such that the concentration of NAD was not rate limiting while that of protein was rate limiting The time was chosen such that it was within the linear part of the relationship between time and the appearance of product.

Tissues were dissected and placed on ice immediately. Placentae were homogenised by hand in Krebs-Ringer bicarbonate buffer (144 mM Na^+, 126 mM Cl^-, 3.8 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 25 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 1.2mMSO_4^-, pH 7.4). Protein concentration was assessed by a colorimetric method (Biorad) against standard concentrations of bovine serum albumin. Assays were performed at 37^{o}C for 10 minutes with 0.5 mg.ml^{-1} protein, 200 mM nicotine adenine dinucleotide (NAD) and 12 nM ^{3}H corticosterone in Krebs-Ringer buffer with added 0.2% bovine serum albumin and glucose.

The reaction was stopped by mixing with 2.5 ml of ethyl acetate at 4^{o}C, and samples were then centrifuged at 2,000 rpm to allow separation of the ethyl acetate fraction which was then dried in a sample concentrator at 40^{o}C with blown air.

Separation was initially by thin layer chromatography. Samples were resuspended in 100μl of ethanol and pipetted onto a TLC plate (Merck 20x20cm plate, BDH Chemicals Ltd.,England), separation was at room temperature in chloroform and ether . Steroids were identified by viewing under ultraviolet light and sample areas scraped from the TLC plates and counted in Cocktail -T using a ß-radiation detector (Minaxi, Tri-carb 4000, Canberra Packard, England).

Subsequently this separation was by High Performance Liquid Chromatography in which case dried samples were resuspended in mobile phase (60% methanol and 40% water). Labelled peaks were detected by an on line radioactivity monitor (Berthold LB 506 C-1, Berthold Analytical Instruments Ltd, Nashua, USA) after the addition of liquid scintillant (Quicksafe Flow 2, Zinsser Analytic UK Ltd) and the identity of labelled peaks confirmed by non labelled standards (Sigma Chemical Company, England).

Rat placental 11-OHSD activity was shown to fall by 70% after freezing and therefore all assays were done on fresh tissue.

ii) Kidney 11beta OHSD activity

11ß OHSD activity in the kidney was assayed by a method similar to the above. In brief, kidneys were dissected and placed on ice immediately. Kidneys were minced and then homogenised by hand in Krebs-Ringer bicarbonate buffer (144 mM Na^+, 126 mM Cl^-, 3.8 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 25 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 1.2 mM SO_4^{2-}, pH 7.4). Protein concentration was assessed by a colorimetric method (Biorad) against standard concentrations of bovine serum albumin. Assays were performed at 37^{O}C for 10 minutes with 0.1 $mg.ml^{-1}$ protein, 200 mM nicotine adenine dinucleotide (NAD) and 12 nM ^3H corticosterone in Krebs-Ringer buffer with added 0.2% bovine serum albumin and glucose.

Assay conditions were maximised to ensure that the concentration of NAD was not rate limiting, that of protein was rate limiting and the time was such that there was still a linear relationship between time and the appearance of product.

b) Measurement of 11beta-OHSD activity in vivo

On days 17-21 of gestation animals (5 control, 3 treated with carbenoxolone 12.5 mg/day throughout pregnancy) were subjected to halothane

anaesthesia and the left carotid and right jugular vein cannulated. Animals were given a priming dose of ^{3}H corticosterone (3 μCi in 0.6 ml 0.9% NaCl, Amersham, UK) and followed by a constant infusion of 0.15 μCi/minute/30μl/min for a total of 80 min via the jugular catheter. Samples of arterial blood (300μl with replacement of volume with 0.9% saline) were obtained at 20, 40, 60 and 80 min of infusion to ensure steady state and blood pressure and pulse rate, measured as above (Lectromed Multitrace 2, UK). At 80 min, placental and fetal tissues were removed and frozen in liquid nitrogen. Steroids were extracted with ethyl acetate from 3 placentae and matched fetuses from each animal and separated by thin layer chromatography. Activity of 11ß-OHSD-2 was assessed by the increase in ^{3}H 11-dehydrocorticosterone from arterial blood to placental tissue.

c) Corticosterone Assay

Corticosterone was estimated by radio-immunoassay[354] using a locally generated antibody (gift of C.J. Kenyon). The intra-assay coefficient of variation was 3.8%.

Samples were diluted in buffer (135 mM sodium borate, 0.5% bovine serum albumin, 1% methanol, 0.1% ethylene glycol, pH 7.4) and heated to 80^{O}C to inactivate corticosterone binding globulin. Assessment of samples before and after heat treatment using the protocol for the CBG assay displayed no significant corticosterone binding activity in heat treated samples. Antibody specific to corticosterone (final titre 1:10,000) was added along with ^{3}H-corticosterone (Amersham International, Bucks., England) and samples incubated at 4^{O}C overnight. Unbound activity was precipitated by the addition of activated charcoal and the bound activity estimated in a ß radiation detector (Minaxi Tricarb 4000, Canberra Packard, Berks., England) after the addition of scintillant (Picofluoro 40- Canberra Packard, Berks. England). Corticosterone concentration

was estimated by comparison to cold corticosterone standards (Sigma Chemical Company, Poole, England).

d) Glucose, and insulin assays

Plasma glucose was determined by an enzymatic (glucose oxidase) method using a Beckman Synchron CX3 multichannel analyser (Beckman Instruments Ltd, High Wycombe, UK). The intra-assay and inter-assay coefficients of variation of variation were <1% and 2.2%, respectively. Plasma insulin was determined, as previously described [355] using rat insulin standards (Novo Nordisk, Copenhagen, Denmark) and iodinated insulin (Lifescreen, Watford, UK). The intra-assay and inter-assay coefficients of variation of this method are <10% throughout the range.

e) Renin Assay

Plasma renin activity was assessed by measurement of generation of angiotensin I at 4^{O}C and 37^{O}C. The protocol followed that of Dr B Williams (Department of Medicine, Western General Hospital, Edinburgh).

Samples were collected in Na EDTA on ice and later separated and plasma frozen at -80^{O}C. The assay was performed by incubation of plasma with an excess of angiotensinogen and inhibitors of the conversion of angiotensin I to angiotensin II. Generation of angiotensin I at 37^{O}C was assessed by measurement using a specific radio-immunoassay.

25 µl of sample was incubated with 175µl of reaction mix comprising 45.7% nephrectomised sheep plasma, 47.4% 0.1M phosphate buffer (pH7.4, with 0.1% Na Azide), 2.3% 270 mM EDTA, 2.3% British anti lewisite (comprising 1.5% dimercaptopropanol and 3.4% benzyl benzoate in arachis oil) and 2.3% 8-hydroxyquinilone. Duplicates were incubated either at 37^{O}C or on ice . A 25µl aliquot of the incubation mixture was then added to 100µl of antibody to angiotensin I (final antibody concentration of 1 in 5,000) and iodinated

angiotensin I in buffer(total counts 3-4,000) and incubated overnight at 4°C .

The bound and unbound fractions were separated by addition of activated charcoal (700µl 0.6% charcoal, 0.06% dextran, 0.05% gelatin at 4°C) and centrifugation at 3,000rpm for 20 minutes. The supernatant was discarded and the charcoal pellet counted in a gamma counter. The generation of angiotensin I was assessed by comparison with cold standards (angiotensin I 0.125-64 ng/ml, Sigma) as the difference between the concentrations at 4°C and 37°C and expressed as ng per ml of plasma per time

Initial assessment of antibody concentrations from 1: 1,000 to 1: 32,000 showed that an antibody concentration of 1,5,000 rendered B/ B0 of 40-50% on repeated curves and that generation of angiotensin I by sample was linear over 3 hours at rates of generation of 400 ng/ml/hour (Figure 6). Samples were therefore generated for 2 hours. The standard curve proved linear over a for concentrations between 0.125 ng/ml and 4 ng/ml corresponding to a rate of generation of between 20 and 640 ng/ml/hour for a standard 2 hour incubation. Concentrations in the samples incubated at 4°C were consistently below the limit of detection. The intra-assay coefficient of variation was 4.5%.

Figure 6

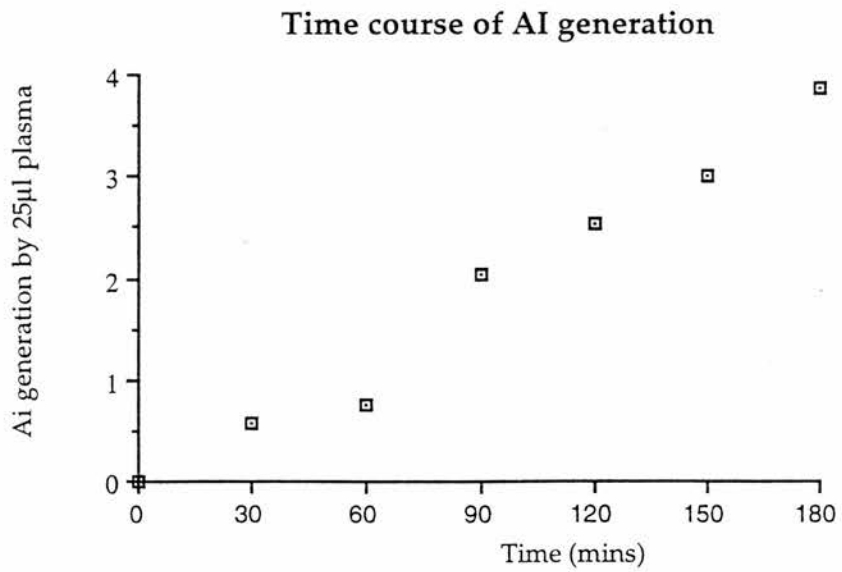


Figure 6: Time course of Angiotensin I generation. AI generated at 400ng/ml/hour.

f) Corticosteroid receptor mRNA levels

Levels of corticosteroid receptors were measured by Dr N.S.Levitt following the method outlined below.

GR and MR mRNA levels were determined by in situ hybridisation histochemistry. Rats were decapitated and brains removed and rapidly frozen on dry ice. Coronal cryosections (10μm) were cut at the level of the preoptic area/ organ vasculosum lamina terminalis, sub commisural organ, hippocampus, area postrema and nucleus tractus solitarius and thaw mounted onto gelatine and poly-l-lysine-subbed slides. Sections were post-fixed in 4% paraformaldehyde, washed and hybridised using a ^{35}S UTP-labelled cRNA antisense probe transcribed in vitro from rat cDNA clones encoding the 3' regions of MR and GR RNAs (513 and 674 bp respectively). Probes were denatured and added at a final concentration of 10x 10^6 cpm/ml to hybridisation buffer. 200-μl aliquots were pipetted onto sections and hybridised overnight at 50°C. Sections were treated with RNase A (30 mg/ml, 45 minutes at 37°C) and washed to a final stringency of 0.1 SSC at 60°C. Slides were dehydrated, dipped in photographic emulsion (Kodak, NTB2) and exposed in light tight boxes at 4°C for 21 days before being developed and counterstained with 1% pyronine. Hybridisation signal within hippocampal subregions was assessed by computer assisted grain counting using an image analysis system (Seescan plc, Cambridge, UK). Silver grains were counted, blind to treatment, under brightfield illumination over individual identified neurons in the hippocampal and other regions (8-12 cells per subfield for each of three replicate sections from each coronal level for each animal). In the dentate gyrus it was difficult to determine cell boundaries and therefore grains were counted over a fixed area approximating neuronal size. Results were calculated as mean grains/ neuron for each region after subtraction of background (counted over areas of white matter) subregions.

Results

Section 1: Administration of dexamethasone and glycyrrhizic acid in pregnancy: Effects on birth weight and later offspring blood pressure

Introduction

We hypothesised that exposure of the fetus to maternal glucocorticoid might have life long effects on offspring blood pressure. Exogenous glucocorticoids in pregnancy reduce birth weight but are also known in higher doses to reduce litter number and exert lethal effects on both the fetus and the dam[293, 290-292]. Slotkin et al [340] had highlighted the threshold for lethal or teratogenic effects on the fetus as greater than 800 µg/kg. We therefore studied two doses of dexamethasone below this threshold at 10 µg/kg and 100µg/kg. Dexamethasone is a synthetic glucocorticoid which is poorly metabolised by both the human[286] and the rat[287] placenta *in vitro* and by purified forms of 11ß-OHSD 2 derived from the placenta[225, 224].

There is little literature on the effects of glycyrrhizic acid in pregnancy. Therefore a dose of glycyrrhizic acid associated with sodium retention in vivo in animal models was selected.

Methods

Pregnant female Wistar rats were time mated and treated from the first day of pregnancy with 10 or 100 µg/kg of dexamethasone (DEX 10 and DEX 100) by subcutaneous injection, while controls were administered vehicle (4% ethanol, 0.9% NaCl, CONT). A further group were administered glycyrrhetinic acid orally (a concentration of 600 mg/l in the drinking water, GI) and this group was also administered vehicle injections. Eight pregnant females were entered into each group. To allow assessment of the glycyrrhetinic acid on enzyme activity, 3 controls and 2 GI treated animals were killed on day 20 of pregnancy with measurement of fetal and placental weights and 11ß-OHSD activity in placenta and maternal kidney, for comparison 1 animal in each of the dexamethasone groups was also sacrificed. Enzyme

activity was assessed with incubation of placenta and of kidney homogenates for 10 minutes.

Effects of treatment on litter size, litter number and birth weight were monitored in the remaining animals, which were subject to no treatment postnatally and blood pressure measured by direct carotid cannulation at 5 months of age.

Results

Pregnancy, placental enzyme activity and birth weight

Females tolerated treatment well. A female, in the DEX 100 group, was found not to be pregnant and excluded. Females were of equivalent weight at the beginning of pregnancy (CONT 226± 8.9g; DEX 10 228± 3.9 g; DEX 100 227± 7.8 g; GI 230± 8.4g) and all females had a net gain in weight through pregnancy. Weight gain in the DEX 10 and DEX 100 groups was significantly lower than control (Figure 7: repeated measures ANOVA, vs. DEX 10 $F=7.2$, $P<0.04$; Vs DEX 100 $F=71.9$, $P<0.01$). Thus, by day 19 the weight of the CONT and GI groups had increased by 35% above pre-pregnancy weight, while the DEX 100 and the DEX 10 groups had gained 20% and 31% respectively. The number in each litter was not affected by treatment (CONT 9.7± 0.4; GI 8.9± 1.3; DEX 10 8.3± 1.7; DEX 100 9.6± 0.5).

Water intake was increased in the GI treated animals ($P=0.02$) and led to a intake of drug of 112.5±9.6 mg/kg/day (range 91-156.3). There was no relationship between the drug dose and maternal weight gain across this range.

7 animals were sacrificed on day 19 (3 CONT, 2 GI, 1 DEX 10, 1 DEX 100) and 11ß-OHSD activity in placentae and dam kidney were assessed along with fetal and placental weights. There was no significant differences in fetal (CONT 2.23± 0.10g, n=30; GI 2.62±0.06g, n= 22; DEX 10 2.91± 0.05g, n= 10; DEX 100 2.45± 0.81g, n=10) or placental weights (CONT 0.52± 0.08g ; GI 0.51± 0.07g; DEX 10 0.48± 0.01g; DEX 100 0.43± 0.02g). Importantly we were unable to demonstrate a change in placental or renal 11ß-OHSD activity <u>*in vitro*</u> after GI treatment *in vivo* (Figure 8).

The remaining animals (5 CONT, 6 GI, 7 DEX 10, 6 DEX 100) littered with no difference in the length of gestation between groups (CONT 22± 0.3 days; GI 22.3± 0.2 days; DEX 10 22.4± 0.2 days; DEX 100 22.5± 0.2 days) or the ratio of males to females. 100μg/kg/day dexamethasone treatment resulted in a reduction in birth weight to 84% of the controls while the DEX 10 and GI groups were not significantly changed at 100% and 94% of control values, respectively (Figure 9)

Offspring weight gain and blood pressure

After birth, body weights increased such that by week 9 there were no significant differences between groups, by which time controls were 258± 8.1g in weight.

At 5 months offspring blood pressure was assessed by means of carotid cannulation. Systolic blood pressure was higher in the offspring of the DEX 100 dams by 13 mmHg in males and 8 mmHg in females (Figure 10: $F=5.1$, $P<0.01$,) There were no significant difference in the adult weights of the animals (Table 4).

Figure 7

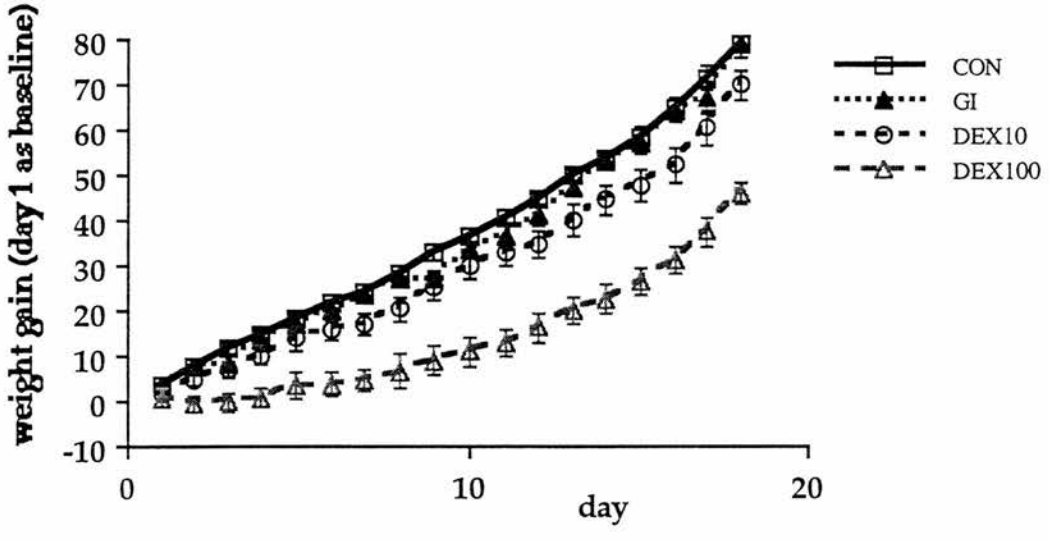


Figure 7: Weight gain (in grammes) of dams through pregnancy. Data are expressed as mean ± SEM.

Figure 8

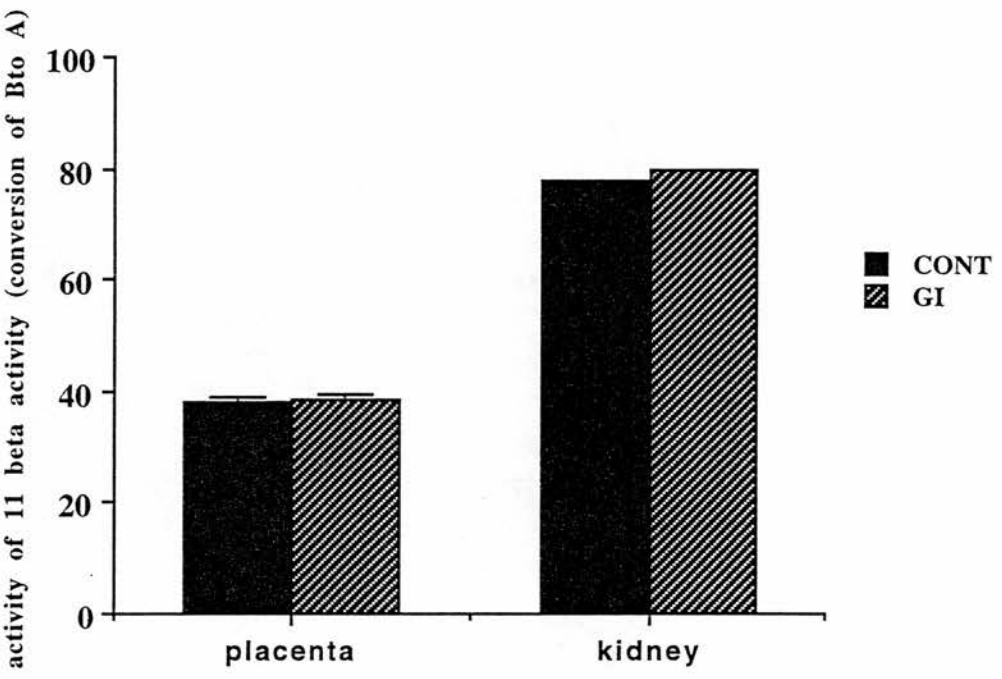


Figure 8: 11ß-OHSD activity *in vitro* after glycyrrhizic acid treatment *in vivo*. Data expressed as the percentage conversion corticosterone (B) to 11 dehydrocorticosterone (A), mean ± SEM. There are no significant differences.

Figure 9

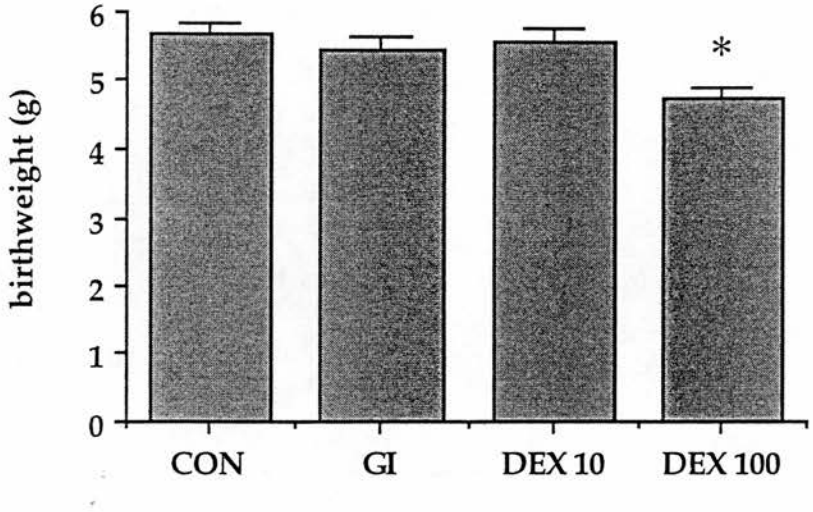


Figure 9: Dexamethasone reduces birth weight. Data expressed as mean ± SEM. ANOVA F=23.2, P<0.01 across all groups with significant difference in the DEX 100 group (*) vs control on post hoc testing.

Table 4: Body weights of treated animals

	CONT	DEX 10	DEX 100	GI
males				
body weight	453± 7.6g	415± 14.5g	424 ± 9.2g	438 ± 12.8g
heart weight (as % of body weight)	0.24 ± 0.004%	0.24± 0.005%	0.24 ± 0.003%	0.24 ±0.006%
left ventricle weight (as % of body weight)	0.21± 0.004%	0.21± 0.005%	0.21± 0.002%	0.21 ±0.005%
females				
body weight	277± 8.3g	268± 9.8g	254 ± 7.1g	261 ±3.9g
heart weight (as % of body weight)	0.26 ± 0.004%	0.24± 0.01%	0.26 ± 0.009%	0.25 ±0.004%
left ventricle weight (as % of body weight)	0.22± 0.005%	0.21± 0.005%	0.22± 0.008%	0.22 ±0.004%

Table 4: Body and organ weights of treated animals. All data are expressed as mean ± SEM and groups compared using ANOVA. There were no significant differences.

Figure 10

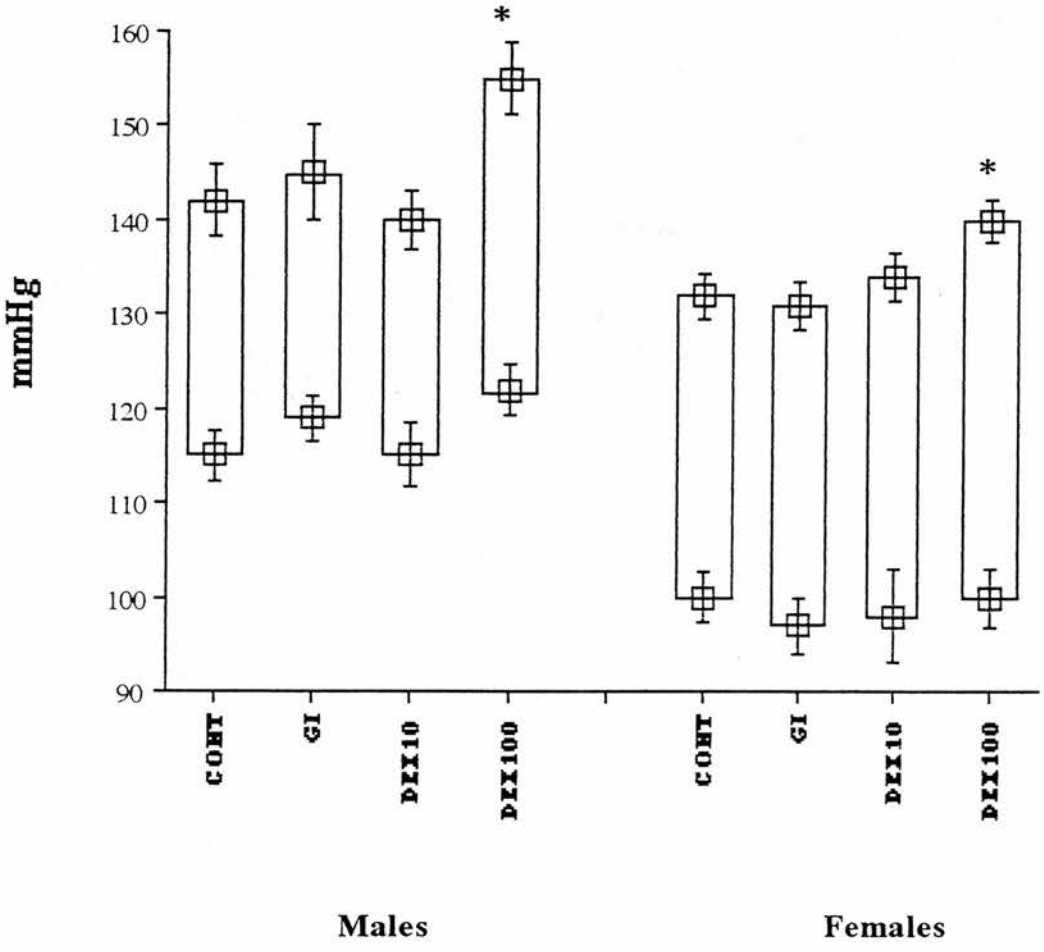


Figure 10: Systolic and Diastolic blood pressure in the offspring of treated animals. Data expressed as mean ± SEM. Systolic blood pressure higher in males and females in the DEX 100 group (ANOVA across all groups F=5.1, P<0.01). * denotes significant vs control at P<0.05.

Discussion

We have demonstrated a dose of dexamethasone which, without clear toxic effects on mother, litter size or litter number, has reduced birth weight to a modest degree (16%) and led to a rise in adult systolic blood pressure. While glucocorticoids are known to raise blood pressure in animals[356] and in man[357], such long lived effects, months after exposure have not been previously described and would represent the kind of "programming" effect proposed by Barker et al[200]. The term programming encompasses the idea that environmental insults or hormonal signals exerted at certain stages of development may lead to permanent alteration in the regulation of biological systems. Critically, such effects extend long after the removal of the programming influence. Is it feasible that hormones could exert such long lived effects?

There is an extensive literature on the role of androgen exposure in early life to alter neural development. It has been known since the 1960s that there is a "window" in the first ten days of life in which exposure of a newborn female rat to a single pulse of testosterone leads to reduced fertility in adult life[358]. This arises because androgen exposure or non-exposure at this stage of development permanently alters hypothalamic responses to a male or female pattern respectively[358, 359], a phenomenon the authors called "imprinting", borrowing the term from behavioural psychology[360]. Subsequent work has suggested that such phenomena arise from organisational effects on neural pathways by estrogens formed by aromatisation of injected or endogenous testosterone. Such sensitive time windows extend into late intrauterine life in the rat and may also exist in mid gestation in the human (reviewed in [361]).

Non steroid hormones have also been proposed to exert such effects. Towards the end of the 1970s Csaba, working in Budapest, described effects of exposure to a single injection of insulin[362] or vasopressin[363] in the first day of life to affect hormonal responses in adult life: increasing the hypoglycaemic response to injected insulin and increasing vasoconstriction to vasopressin in isolated aortic strips respectively, in animals at least two months after the initial treatment. Such effects were termed

"hormonal imprinting" and it was hypothesised that receptor number and response would be permanently altered by exposure in early life[364]. More recently, evidence has been forwarded for similar effects on the pituitary-thyroid axis, whereby exposure of dams to triiodothyronine(T3) late in pregnancy led to permanent alterations in the ratio of T3 to thyroid stimulating hormone[365].

The stress axis may also be subject to such programming effects. It is known that exposure of neonatal rats to handling early in life leads to permanent alterations in the pattern of response of the hypothalamic-pituitary-adrenal axis[366]. The mechanism is uncertain but shows features in keeping with programming: if exerted at the key time in early life, handling appears to alter the "hard-wiring" of the developing HPA axis with measurable differences both in the peripheral corticosterone response to stress and in the central expression of glucocorticoid receptors[367] and in subsequent propensity to disease[368].

The programming effects we have observed might be exerted by a variety of mechanisms. Firstly, the later rise in blood pressure might be inherent secondary to catch up growth and therefore a feature of any growth retarding insult early in life, perhaps mediated by the effects of such growth on vascular structure[7]. Alternatively, glucocorticoids might exert a number of more specific effects by altered expression of glucocorticoid regulated genes, changes in the levels of growth factors or by effects on organ growth and development.

Glucocorticoids are known to exert many influences in development and there are several ways in which glucocorticoids in utero might be expected to affect later blood pressure. Glucocorticoids infused in utero lead to rises in blood pressure, at least in the sheep fetus[369, 337] and dexamethasone given to preterm infants is also associated with raised blood pressure in the short term[370] . It is known in the adult that any primary cause of high blood pressure exerted for long enough will result in permanent rises in blood pressure even if the initial cause is removed with such effects having been attributed to alteration resistance vessels (reviewed in [7]). It may be then, that rises in

blood pressure early in life lead to structural alterations in the vasculature promoting higher blood pressure life long.

Secondly, glucocorticoids might exert programming effects on hormonal systems; we have discussed the influences of glucocorticoids on aspects of the developing sympathetic nervous system in the rat[340, 346] as well as known influences on the renin-angiotensin system[371, 372] and developing HPA axis[348-350]. Permanent alteration in the response of these hormonal axes would be expected to alter blood pressure life long. Finally, blood pressure may changed by long term alteration in organ function, most notably the kidney. Reductions in glomerular filtration area arising from fetal life have been proposed as an aetiological factor in the connection between low birth weight and later high blood pressure[373]. In the next section we begin to consider such potential mechanisms.

Section 2: Effect of dexamethasone in utero on vascular reactivity

Introduction

Data from human studies suggested that the differences in blood pressure associated with lower birth weight become more pronounced in older individuals[21, 22]. In addition changes in glucose tolerance had been observed in the older populations[44].

None of the human data suggested the aetiology of raised blood pressure in lower birth weight individuals. Essential hypertension is characterised by increases in peripheral vascular resistance[374], sympathetic tone[375] and eventually with structural changes in small vessels[376], and these changes are also found in animal models of hypertension[377]. In addition, but less consistently, several studies have suggested an increase in peripheral response to noradrenaline both in man[378-380] and animal models[381, 382].

Glucocorticoids raise blood pressure in both animals[356] and man[357] and are in turn associated with potentiation of responses to catecholamines (reviewed in[383]). Glucocorticoids had previously been shown to have a permissive role in allowing vasoconstriction to noradrenergic stimuli[384, 385] and in excess, to potentiate vasoconstriction in response to noradrenaline in whole animals[384] and in isolated mesenteric arterial beds[386]. Further, glucocorticoids are intimately involved in the development of noradrenergic and adrenergic systems in various organs in the developing fetus, including the brain[346], heart[342] and kidney[341]. Such effects are complex and dose related: doses as low as 0.05 $mg.kg^{-1}$ being associated with reductions in noradrenaline levels[342], enhanced cellular responses to ß-adrenergic stimuli[342] and increases in cellular adenylate cyclase[340], while higher doses (0.8 $mg.kg^{-1}$) are associated with promotion of noradrenergic activity in the brain[346]. Importantly, the *in utero* influences of glucocorticoids have also been postulated to lead to changes in adult function[387] with some evidence for permanent alteration in brain noradrenergic pathways[346].

Using animals from our initial cohort we decided to examine glucose tolerance,

blood pressure and vascular reactivity in isolated mesenteric beds.

Methods

Male animals from the cohort described in the previous section were examined at 17 months of age. In brief, pregnant female Wistar rats were time mated and treated from the first day of pregnancy with 10 or 100 μg/kg of dexamethasone (DEX 10 and DEX 100) by subcutaneous injection, while controls were administered vehicle (4% ethanol, 0.9% NaCl, CONT). A further group were administered glycyrrhetinic acid orally (a concentration of 600 mg/l in the drinking water, GI) and this group was also administered vehicle injections. Eight pregnant females were entered into each group.

At 17 months, male animals were subjected to an oral glucose tolerance test with 2 g/kg glucose (as a 0.5g/ml solution) given by gavage tube to animals fasted from 1600 the day before. Animals were left for 1 week to recover and then anaesthetised, the carotid artery cannulated and blood pressures measured directly at least two days after recovery from anaesthesia as previously described. Finally, the mesenteric bed was dissected and reactivity to noradrenaline and potassium examined as detailed in the methods section and the weights of a variety of organs recorded.

Results

Body weights, blood pressure and vascular reactivity

At seventeen months there were no significant differences in body weight or in organ weights (Table 5). Mean arterial pressure was 11 mmHg higher in the animals treated with DEX 100 in utero (CONT 112± 1.6, DEX 10 115± 3.7, DEX 100 123± 1.7, GI 110± 4.1: $F=4.73$, $P<0.05$)

Mesenteric perfusions displayed no difference in reactivity to noradrenaline (Figure 11) or to potassium (Figure 12). Preparations showing evidence of hypoxic damage with inadequate constrictor response to noradrenaline or dilator response to acetylcholine were excluded and the final numbers were CONT 3, DEX 10 4, DEX 100

6 and GI 5.

Offspring glucose tolerance

There were no significant differences on repeated measures ANOVA between groups. The error bars in the DEX 10 group are larger than the other groups due to a single outlier with fasting glucose of 7.7 and all subsequent glucoses greater than 11.5. There were no differences in fasting glucose (CONT, 5± 0.2 $mmol.l^{-1}$; DEX 10 6.2± 0.7 $mmol.l^{-1}$; DEX 100 5.6± 0.4 $mmol.l^{-1}$; GI 5.2± 0.4 $mmol.l^{-1}$: Figure 13) or insulin (CONT, 36.7± 4.4 $mU.l^{-1}$; DEX 10 49.9± 13 $mU.l^{-1}$; DEX 100 26.7± 1.05 $mU.l^{-1}$; GI 47± 10.6 $mU.l^{-1}$). The integrated area under the curve for insulin after the oral glucose tolerance test was significantly higher in the GI group at 162% of controls (Figure 14).

Table 5

	CONT	DEX 10	DEX 100	GI
n	6	4	6	5
weight	528 ± 33	548 ± 30.6	503 ± 31.4	546 ± 23.4
cardiac weight (as % body weight)	0.28 ± 0.006	0.27 ± 0.03	0.30 ± 0.03	0.29 ± 0.01
adrenal weight (as % body weight)	0.017 ± 0.004 %	0.014 ± 0.01 %	0.017 ± 0.002 %	0.012 ± 0.001 %
spleen weight (as % body weight)	0.32 ± 0.05 %	0.25 ± 0.09 %	0.28 ± 0.03 %	0.33 ± 0.03 %
brain weight (as % body weight)	0.39 ± 0.03 %	0.39 ± 0.03 %	0.40 ± 0.01 %	0.39 ± 0.02 %
thymus weight (as % body weight)	0.08 ± 0.012 %	0.07 ± 0.03 %	0.09 ± 0.02 %	0.08 ± 0.01 5

Table 5: Body and organ weights in male offspring at 17 months. Offspring of dams treated with either dexamethasone or glycyrrhizic acid. Data expressed as mean ± SEM and groups compared with ANOVA. No significant differences were observed.

Figure 11

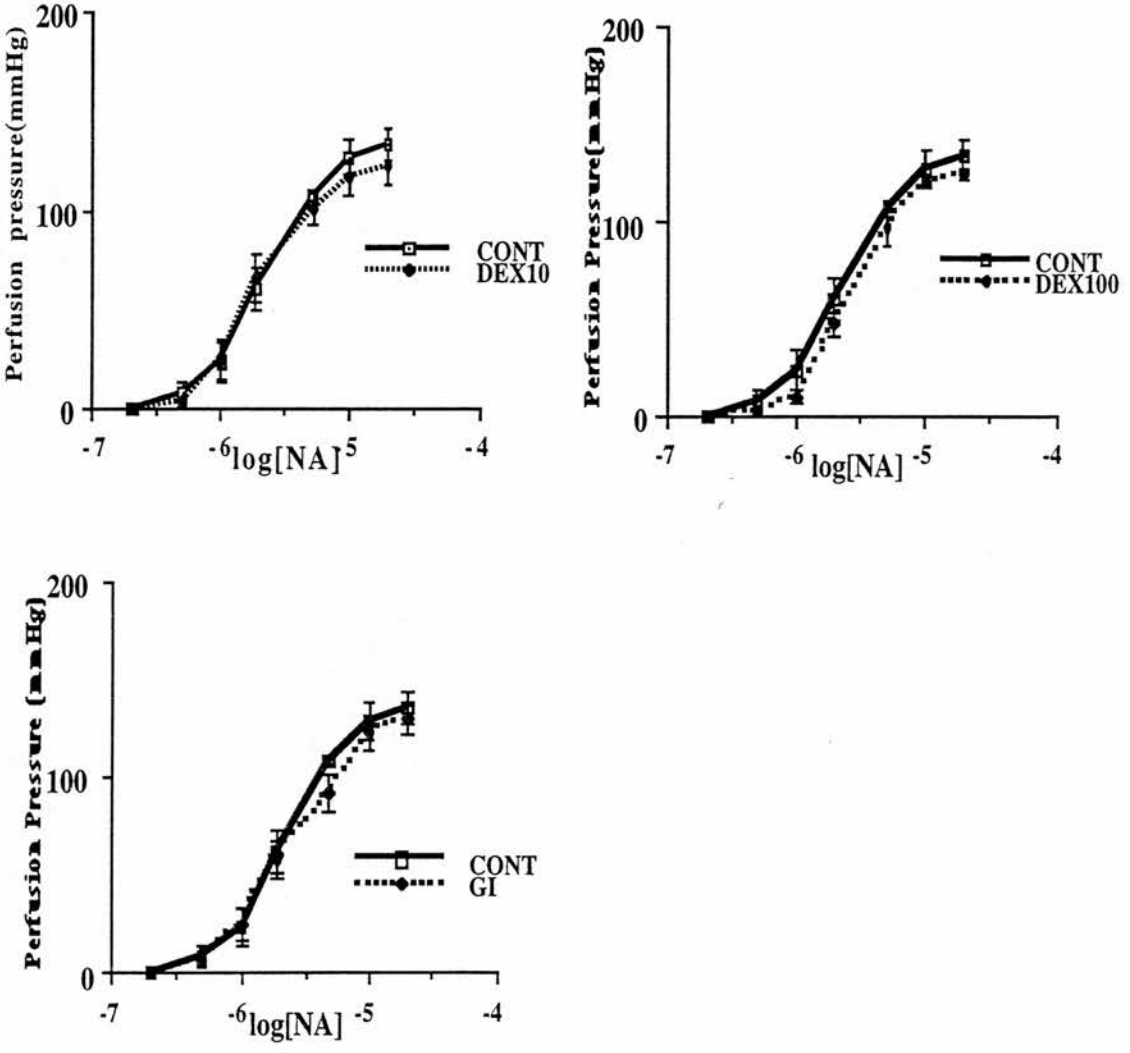


Figure 11: Dose response of mesenteric artery perfusion pressure to stepped exposure to noradrenaline. CONT n=3; DEX 10, n=4; DEX 100, n=6; GI, n=5. Data are expressed as mean ± SEM, no significant differences were observed.

Figure 12

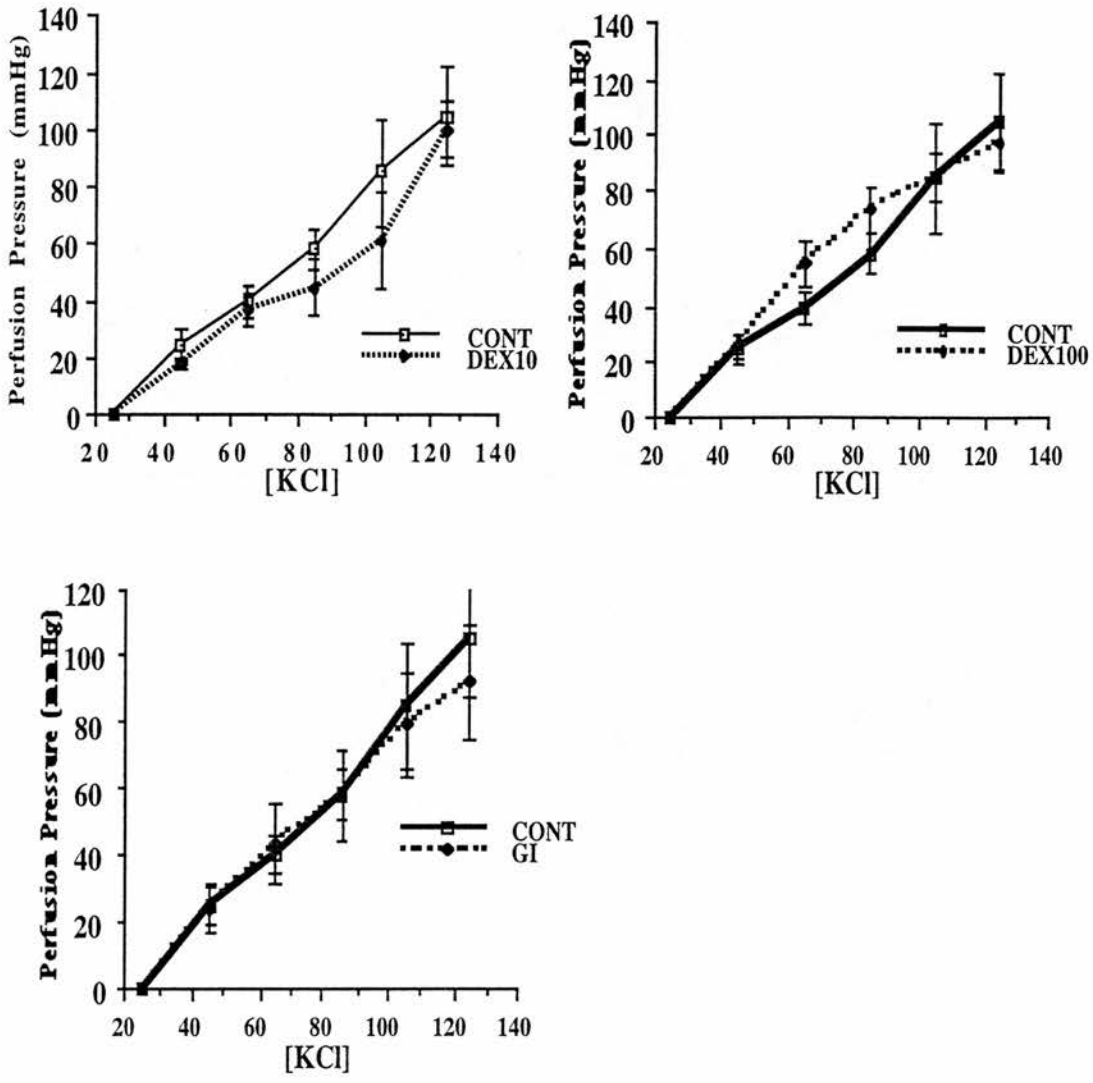


Figure 12: Dose response of mesenteric artery perfusion pressure to stepped exposure to potassium chloride CONT n=3; DEX 10, n=4; DEX 100, n=6; GI, n=5. Data expressed as mean ±SEM. No significant differences were observed.

Figure 13

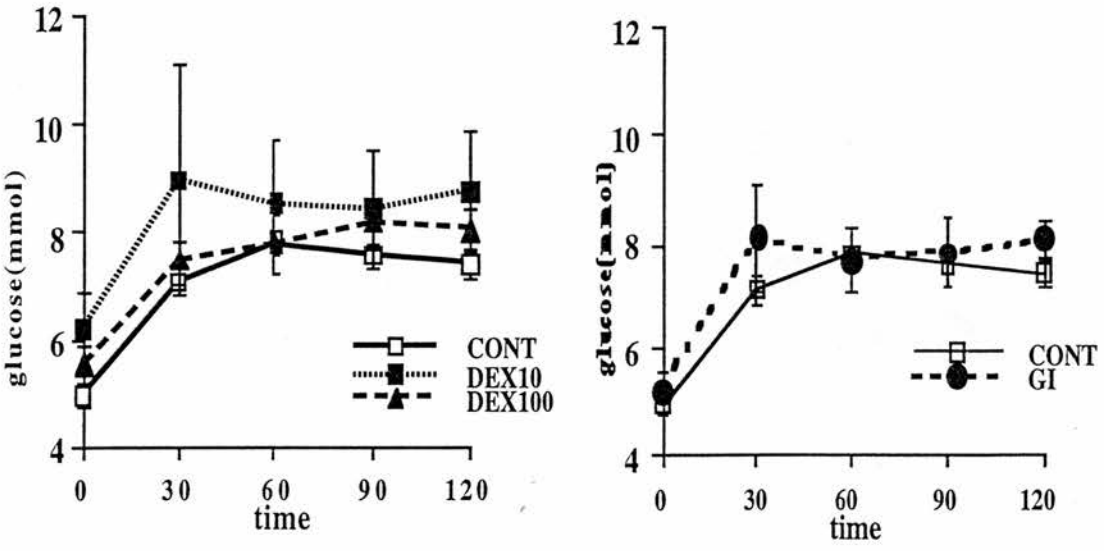


Figure 13: Effect of treatment on glucose tolerance at 17 months. Plasma glucose after OGTT in the male offspring of dams treated with dexamethasone or glycyrrhizic acid. Data expressed as mean ±SEM. No significant differences between groups CONT, n=6, DEX 10, n=4, DEX 100, n=6, GI, n=5.

Figure 14

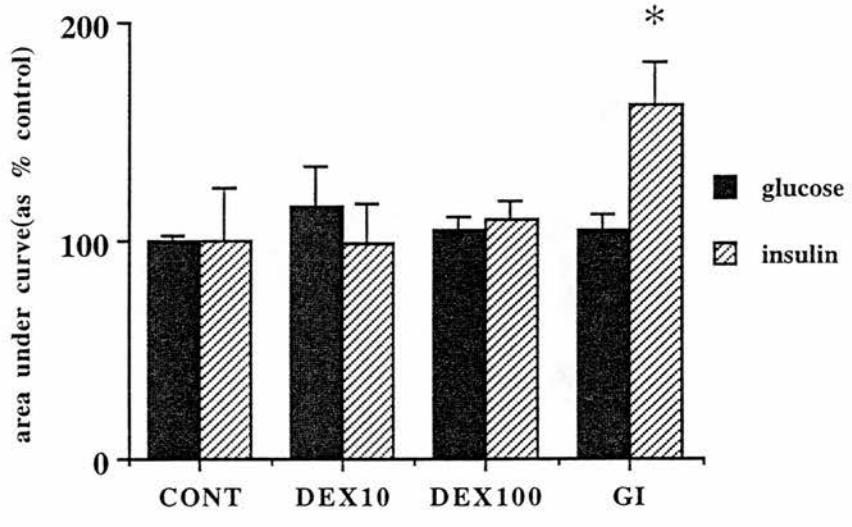


Figure 14: Area under glucose and insulin curves after oral glucose tolerance test. The area under the insulin curve for GI (*) is significantly higher compared to control (ANOVA, F=3.4, P=0.05; Newman Keuls' test)

Discussion

I have again demonstrated a modest increase in blood pressure in animals treated in utero with dexamethasone. The perfused mesenteric bed has been extensively used to model the responses of the peripheral vasculature in the whole animal. In the Spontaneously Hypertensive Rat (SHR) increased responses to a variety of stimuli have been observed including noradrenaline[381], endothelin-1[381], potassium[388] and angiotensin II[388], the abnormal response becoming more marked in older age groups[388]. Such effects may arise because of increased sensitivity to specific stimuli or non-specifically secondary to the changes in the structure of small vessels proposed in hypertension[376]. The lack of difference in dexamethasone treated animals renders unlikely a long lived effect on noradrenaline sensitivity and would also argue against structural changes in the vasculature, at least as assessed by this technique.

We had observed no significant changes in the oral glucose tolerance of the dexamethasone treated animals. There was however a trend to higher fasting glucose levels and with small numbers it is possible that we were missing an effect due to a type 2 error. The higher insulin response of the GI treated animals was also intriguing, raising the possibility of the development of insulin resistance in these animals. This possibility is pursued more completely in the offspring of the carbenoxolone treated dams (Section 5).

Section 3: Effect of Dexamethasone in utero on vascular structure and the PRA axis

Introduction

The renin angiotensin system is an important regulator of blood pressure and fluid and electrolyte homeostasis in fetal, newborn and adult life, in addition angiotensin II influences cellular growth and differentiation in the fetus. Inhibition of the renin-angiotensin system by angiotensin converting enzyme inhibitors in early postnatal life acts to exert life long influences on blood pressure in animal models, even after withdrawal of the drug[389, 390]. Thus, early modulation of the renin-angiotensin system may exert the kind programming effects on blood pressure suggested by epidemiological studies in humans.

We have previously observed that dexamethasone exposure in utero leads to life long increases in blood pressure in the rat. Glucocorticoids may influence several aspects of the renin-angiotensin system; angiotensinogen is induced by glucocorticoids in vivo, the rat angiotensin gene containing a glucocorticoid response element[391] and prenatal treatment with dexamethasone leads to increases in hepatic angiotensinogen levels in both fetus and dam[371]; glucocorticoids do not appear to exert direct effects on renin and prorenin expression[392] but renin expression is induced by renal sympathetic innervation in early life[393] and renal sympathetic influences are in turn enhanced in the long term by dexamethasone treatment in utero[340]. Effects of glucocorticoids in utero have been extensively studied in the sheep where cortisol infusion is active to increase fetal blood pressure[369, 337] and to alter AT1 receptor RNA expression in a tissue specific manner[372]. Higher plasma angiotensinogen levels have been observed in relation to lower fetal weight at 18 weeks supporting a potential role for glucocorticoid exposure in the lower birth weight infants[394].

A further potential mechanism for the effect of dexamethasone lies in the alteration of renal function. Reductions in glomerular filtration area arising from fetal life have been postulated as of importance in the connection between low birth weight and later

high blood pressure[373], the hypothesis being that the total glomerular number is largely fixed in early life and this will in turn influence the propensity to renal disease and to high blood pressure. Part of this hypothesis rests upon the observation that there is a wide variation in weight, cortical volume and nephron number in the normal population[395]. That this biological variation is of potential importance to high blood pressure is supported by both animal models and human studies[396] where lower glomerular numbers have been related to higher blood pressure and, in particular, salt sensitive hypertension in adult life. Further, nephrotoxic insults in fetal life have been shown to reduce glomerular number in adult animals[397] but blood pressure has not yet been examined in these studies.

Glucocorticoids have been shown to hasten the earlier maturation of renal function[335-337] and angiotensin II to promote renal growth and differentiation via the renal AT1 receptor[398] in turn increased by glucocorticoid treatment in utero[372]. Intra uterine exposure to glucocorticoid might therefore act to increase blood pressure promoting premature maturation of the kidney and perhaps, by reducing glomerular number.

We therefore decided to examine the PRA axis in animals treated with dexamethasone *in utero* measuring the response of blood pressure and aldosterone to infusion of angiotensin II as well as examining vascular structure in detail in the mesenteric bed and glomerular number in the kidney.

Methods

Treatment of Dams

Female Wistar rats (200-250 g) were maintained under conditions of controlled lighting (lights on 07.00-19.00 h) and temperature (22°C) and allowed free access to food (standard rat chow; 56.3% carbohydrate, 18.3% protein, NaCl 0.7%; B.S.&S Scotland Ltd. Edinburgh) and tap water. The rats were time-mated and then given either dexamethasone (DEX: 100μg/kg/day in 4% ethanol-saline, 0.1 ml, s.c.) or

vehicle alone (CON) throughout pregnancy. No further treatment was given (to mothers or pups) after delivery and all offspring were weighed at three days of age.

Chronic effects of Angiotensin II

At twelve weeks, female offspring were habituated to measurement of blood pressure by application of a tail cuff (see Methods). The technique is non-invasive and used in conscious animals. Animals had blood pressure measured on a twice weekly basis and each measure was taken as the mean of five readings.

After three baseline measurements animals were subjected to a short halothane anaesthaesia allowing insertion of two mini pumps (Alzet model 2002, Palo Alto, California, US): the first mini-osmotic pump containing 5-bromo-2'-deoxyuridine (BrdUrd; Sigma Chemical Company, Poole, Dorset, UK) dissolved in a 1:1(v/v) mixture of dimethyl sulfoxide and O.154 M NaCl, and the second containing angiotensin II (Hypertensin; Ciba Geigy, Basle, Switzerland, 6 mg/ml delivered at 200 $ng.kg.min^{-1}$) or vehicle.

Animals were killed by decapitation and trunk blood collected for measurement of renin, corticosterone and aldosterone. Tissues were fixed by infusion of formalin into the inferior vena cava and tissues then dissected and stored in formalin.

Mesenteric tissues were prepared as in the Methods section, in brief cut into 3μm transverse sections serially incubated with a primary anti-BrdUrd monoclonal antibody and a secondary rabbit anti-mouse antibody conjugated to alkaline phosphatase and subsequently visualised using alkaline phosphatase substrate.

After staining vascular structure was assessed by measurement of medial area, number of nuclei, and number of BrdUrd positive nuclei using an image analysis system, in coded sections with the observer blinded to treatments. Between 42 and 68 vessels were measured in each treatment group ranging in size of medial area from 60 to 20,000 μ^2. The BrdUrd index was defined as the number of BrdUrd positive nuclei in relation to the total number of nuclei.

Adrenal slices were prepared identically and cellular area and BrdUrd index identified under image analysis.

Acute effects of Angiotensin II

Animals were subjected to a short halothane anaesthaesia and the left carotid and right jugular vein cannulated using a teflon cannulae. 48 hours after cannulation blood pressure was measured directly as described in the Methods. Baseline blood pressure was measured in conscious unrestrained animals prior to the infusion of ANG II via the venous cannula at a continuous rate of 75ng.kg.$^{-1}$min.$^{-1}$ in 0.9% saline. Blood pressure was recorded throughout and mean blood pressure before and after infusion taken as the mean of 8-10 readings throughout the record.

Statistics

Data was analysed either by unpaired t-tests (for two groups) or ANOVA (for four groups) followed by Newman-Keuls' post-hoc multiple comparisons test, as appropriate. The response of blood pressure to acute and chronic ANG II was analysed by repeated measures ANOVA. All data are expressed as mean ± SEM.

Results

Effect of Dexamethasone treatment on pregnancy and offspring weight

Dams administered dexamethasone gained weight through pregnancy to a lesser extent than controls (CON 116± 5.9g, n=5; DEX 71± 6.0g, n=4; P<0.01). Treatment did not affect litter size(CON 9.8± 0.12g, n=5; DEX 11.8± 0.6g, n=4) or the length of gestation(CON 22.2± 0.5 days, n=5; DEX 21.8± 0.6 days, n=4), but did result in an 18% reduction in weight in offspring at 3 days (CON 9.06± 0.19g, n=49; DEX 7.44± 0.10g, n=47; P<0.01).

Effect of dexamethasone treatment on blood pressure and the PRA axis

At the onset of blood pressure measurement there was no significant difference in body weight (CON 249± 2.2g, n=16, DEX 246± 3.2g, n=16). Both dexamethasone treatment in utero and ANG II treatment in adulthood led to higher blood pressures (Figure 15) with data analysed either with repeated measures ANOVA across all readings (effect of dexamethasone in utero; $F=4.8$, $P<0.05$: and effect of ANG II in adulthood;$F=18.0$, $P<0.01$), or when a mean of the last three blood pressure readings were taken from the end of the study period (CON 120±3.5 mmHg, n=8; DEX 126±2.2 mmHg; CON/ ANG II 134±4.2 mmHg, n=8; DEX/ ANG II 145±3.6 mmHg, n=8: effect of dexamethasone in utero; $F=5.9$, $P<0.05$: and effect of ANG II in adulthood;$F=23.1$, $P<0.01$).

Hormonal measurements at sacrifice revealed an expected fall in plasma renin activity in ANG II treated animals (plasma renin activity: CON 0.54± 0.06; CON/ ANG II 0.24± 0.07; DEX 0.64± 0.07; DEX/ ANG II 0.12± 0.6: two way ANOVA effect of ANG II $F=10$, $P<0.01$) while aldosterone levels were higher in the animals treated with dexamethasone in utero, but not those treated with ANG II in adult life (aldosterone levels: CON 990± 127; CON/ ANG II 800± 101; DEX 1171± 82; DEX/ ANG II 1348± 252: two way ANOVA effect of dexamethasone in utero $F=5$, $P<0.05$). Corticosterone levels were not different in any group at sacrifice (CON 590± 77; CON/ ANG II 496± 102; DEX 660± 71; DEX/ ANG II 624± 56).

Effect of dexamethasone treatment in utero on adrenals and vasculature

Neither dexamethasone treatment in utero nor ANG II treatment in adult life led to any change in organ weights (adrenal, kidney, liver, heart, thymus; data not shown). Analysis of mesenteric vessels stained for 5-bromo-2'deoxyuridine displayed evidence of vascular hyperplasia in response to ANG II (BrdUrd Index CON 1.1±1.4%; CON/ ANG II 9.25±1.69%; DEX 2.04±1.33%; DEX/ ANG II 7.3± 1.69%: effect of ANG II $P<0.05$) without evidence of vascular hypertrophy, as assessed by the medial area of

vessels divided by the number of nuclei(CON 240±31 μ^2; CON/ ANG II 223±30 μ^2; DEX 206±15 μ^2; DEX/ ANG II 215± 29 μ^2). There was no additional effect of dexamethasone treatment in utero on any measure in these vessels. Adrenal slices appeared morphologically identical. Measurement of cells in the zona glomerulosa identified no evidence of either cellular hypertrophy(CON 60±3.1 μ^2; CON/ ANG II 61.4±1.3 μ^2; DEX 61.8±3.0 μ^2; DEX/ ANG II 67.2± 3.6 μ^2) or hyperplasia as assessed by the BrdUrd index(BrdUrd Index CON 0.05±0.02%; CON/ ANG II 0.03±0.01%; DEX 0.06±0.03%; DEX/ ANG II 0.03± 0.01%).

Effect of acute angiotensin treatment on blood pressure

Blood pressure was measured in animals with indwelling carotid cannulae before and after infusion of ANG II. ANG II infusion resulted in an acute rise in blood pressure evident a few seconds after the onset of the infusion (Figure 16: repeated measures ANOVA $F=23.9$, $P<0.01$), while there was a trend to higher basal blood pressure in the animals treated with dexamethasone in utero there was no difference in the incremental rise in blood pressure (incremental rise CON 23.7± 8.7 mmHg, DEX 17.9± 2.5 mmHg).

Figure 15

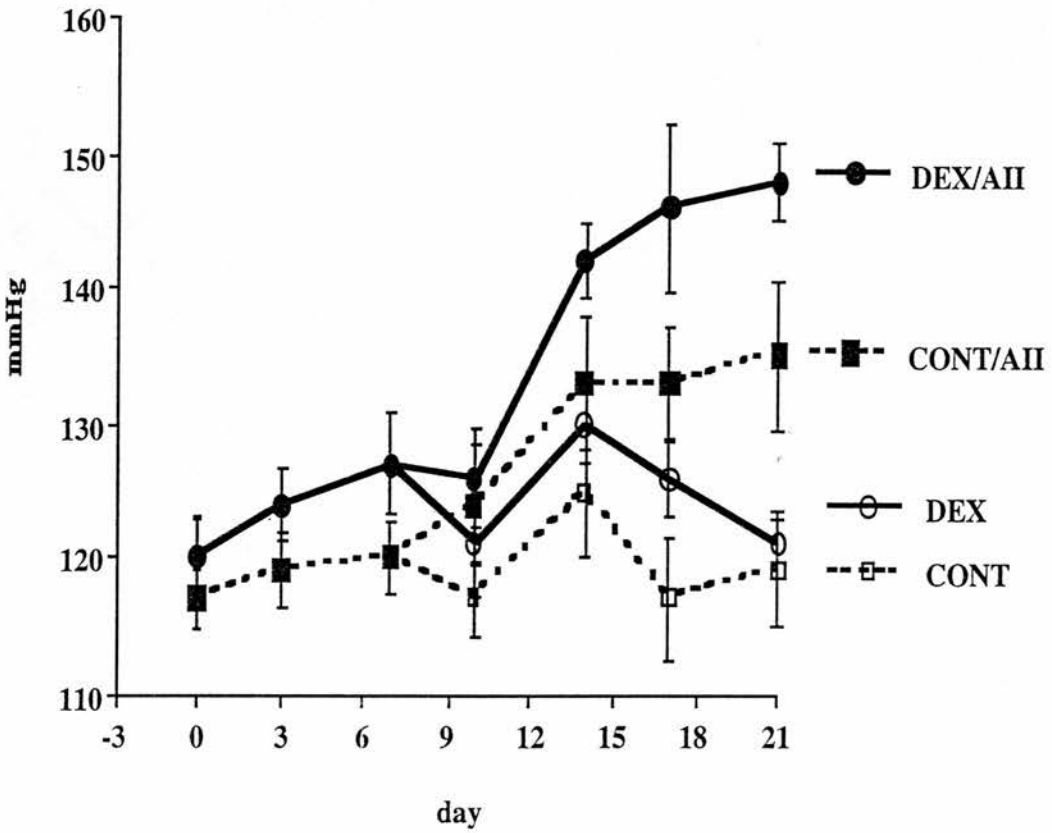


Figure 15: Serial blood pressures before and after treatment with ANG II in adulthood in animals treated with dexamethasone in utero. Repeated measures ANOVA displays a significantly higher blood pressure in animals treated with dexamethasone in utero ($F=4.8$, $P<0.05$).

Figure 16

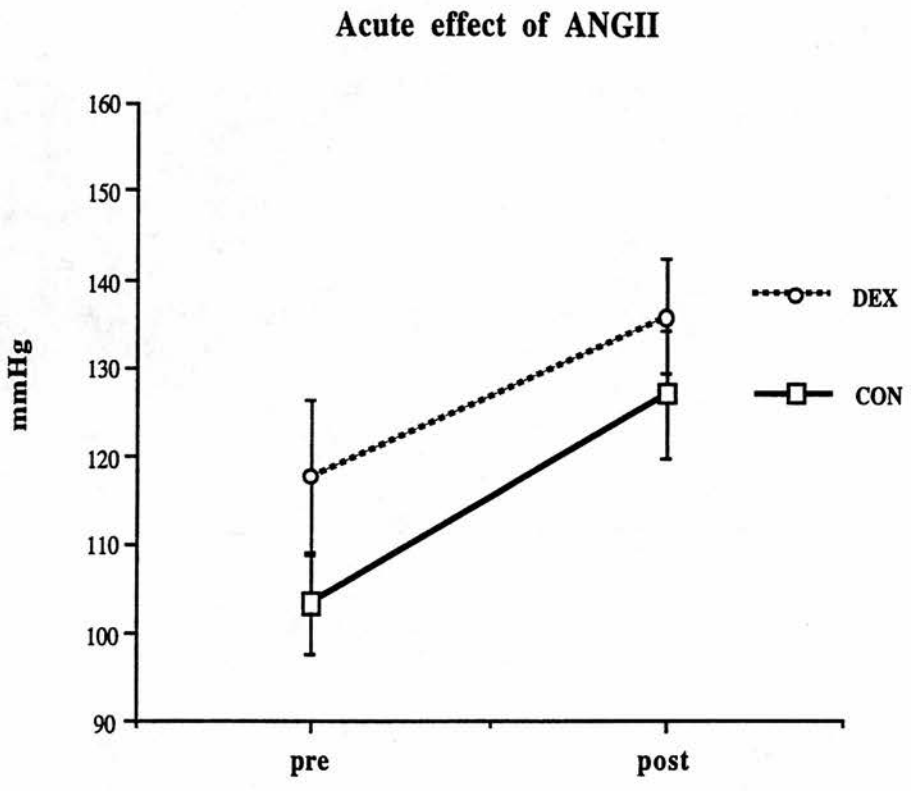


Figure 16: Acute effects of ANG II. Rise in blood pressure in the offspring of dams treated either with dexamethasone or vehicle in utero and a ten minutes after infusion of ANG II in adulthood. No significant differences were observed.

Discussion

In this study we have confirmed that dexamethasone in a low pharmacological dose, which does not influence fetal viability, leads to permanent increases in blood pressure. We have not demonstrated structural alterations in arterioles, at least in the mesenteric bed, which might account for this change in blood pressure, and the gross weight of organs including the kidney were not altered. Blood pressure was increased by ANG II infusion both acutely and chronically but, while starting from a higher baseline, the effect of ANG II did not seem to be potentiated by dexamethasone treatment in utero. Aldosterone levels do however appear to be higher in the dexamethasone treated animals and to be more reactive to ANG II. Plasma renin activity was not reciprocally suppressed as might be expected with short term excess of mineralocorticoid. While chronic exogenous administration of large amounts of exogenous aldosterone in animal models and endogenous mineralocorticoid in human disease is associated with suppression of plasma renin activity, more modest doses, still associated with hypertension may be associated with low normal plasma renin concentrations. As such, the lack of reciprocal suppression of plasma renin activity in this experiment does not preclude chronically raised aldosterone levels from being the mechanism of the long term programming of blood pressure in these animals. Dexamethasone in early life can alter expression of aldosterone synthase[399] and further studies will examine whether this enzyme has been permanently programmed by dexamethasone in utero.

Section 4: Prenatal dexamethasone treatment in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in adult offspring

Introduction

Low birth weight is associated with a some of abnormalities comprising Syndrome X. Abnormalities of glucocorticoid metabolism have been associated with both android obesity[400] and hypertension[401], although the clinical evidence of this is, as yet, often conflicting. Such abnormalities could potentially act either in a causative role in the metabolic syndrome, or as an additional cardiovascular risk factor[402, 403]. Two studies have now suggested that lower birth weight may also be associated with alterations in the HPA axis[404, 405]. Phillips et al have found that morning cortisol levels are increased in adults of lower birth weight[405], while Clark et al have detected an inverse correlation between birth weight and urinary excretion of both adrenal androgens and glucocorticoids in children aged 9[404]. These studies suggest that those of lower birth weight might have a higher adrenal secretion of both adrenal androgens and glucocorticoids. These data support a possible link both between the metabolic syndrome and abnormal glucocorticoid secretion or metabolism, and between lower birth weight and alteration in the HPA axis. Could changes in the central control of glucocorticoid secretion underlie the changes in blood pressure observed in dexamethasone treated fetuses?

There are established paradigms of programming by steroids in early life, and these favour central mechanisms in such imprinting effects. The two best described examples are of imprinting by androgen exposure and of alteration in the hypothalamic-pituitary adrenal axis by handling in early life.

It is observed that exposure of newborn rats in the first ten days of life permanently alter the pulsatility of hypothalamus to male or female patterns[358, 359]. This effect is functionally important as the changes engendered by testosterone exposure in early life in female animals may lead to permanent alterations in fertility[358]. It is

proposed that such phenomena arise from organisational effects on neural pathways of estrogens formed by aromatisation of injected or endogenous testosterone and that such sensitive time windows extend into late intrauterine life in the rat and may also exist in mid gestation in the human (reviewed in [361]).

The stress axis is also be subject to programming effects. It is known that exposure of neonatal rats to handling early in life leads to permanent alterations in the pattern of response of the hypothalamic-pituitary -adrenal(HPA) axis[366]. The mechanism is uncertain but shows features in keeping with programming: if exerted at the key time in early life, handling appears to alter the "hard-wiring" of the developing HPA axis with measurable differences both in the peripheral corticosterone response to stress and in the central expression of glucocorticoid receptors[367]. An increase in the expression of hippocampal mineralocorticoid receptor (MR or type 1 corticosteroid receptor) is observed which is believed to be of importance in sensitivity to glucocorticoid feedback leading to a reduced duration of corticosterone pulse after stress in the adult animals. Again such effects have functional correlations with such animals displaying a reduced propensity to subsequent neurodegenerative disease, itself potentiated by long term glucocorticoid exposure[368].

In such experiments the environmental change occurs in early life. Can similar effects be exerted in utero? In animal studies maternal exposure to stress has been associated with a number of sequelae such as reduced birth weight, increased perinatal mortality [295], and changes in behaviour and motor development in offspring [406, 407]. Furthermore, alterations, albeit inconsistent, have been reported in the HPA axis in newborn and adult offspring. These include: normal basal corticosterone concentrations and prolonged elevation of corticosterone after exposure to novelty stress[408], and elevated mean weekly corticosterone concentrations, but diminished corticosterone responses to short, but not long-term foot shock stress[295]. The mechanism whereby these changes occur is unclear. Maternal glucocorticoids secreted during stress could play a decisive role in this phenomenon. Generally the fetus is protected from exposure

to maternal glucocorticoids by placental 11ß-OHSD and, as previously discussed, at least theoretically the extent of available enzyme activity should be sufficient to convert most maternal cortisol to cortisone[268]. The biological variability of enzyme activity is unknown in normal populations, however, and passage of maternal glucocorticoids is observed in some circumstances in animal models [409].

The altered HPA axis response in the offspring of rats exposed to stress during gestation, was associated with a reduction in hippocampal glucocorticoid and mineralocorticoid receptor numbers[408]. Further, there are considerable data supporting a role of corticosteroid receptors in the central nervous system in the development of mineralocorticoid induced hypertension and in the regulation of blood pressure [410-412].

The developmental window for the action of dexamethasone on blood pressure and any association of prenatal exposure to dexamethasone with later HPA axis responsiveness, is also unknown. The aim of this study was to investigate the effect of late prenatal exposure to dexamethasone on corticosterone responses to restraint stress and glucocorticoid and mineralocorticoid receptor gene expression in areas of the brain postulated to mediate the central effects of corticosteroids on HPA axis suppression and blood pressure.

Methods

Female Wistar rats (200 - 250 gms) were maintained under conditions of controlled lighting (lights on 7 a.m. to 7 p.m.) and temperature (22°C) and allowed free access to food (standard rat chart); 56.3% carbohydrate, 18.3% protein, 0.7% NaCl; BS&S Scotland Ltd, Edinburgh) into tap water. The rats were time mated and given either dexamethasone (100 µg/kg/day) or vehicle subcutaneously on days 15 to 20 of gestation. The offspring were studied at 16 weeks of age as follows:-

Blood pressure measurement.

Blood pressure was measured directly after carotid cannulation in conscious

unrestrained animals (DEX n=7, CONT n=7) using a pressure transducer for 10 minutes on three consecutive days and recorded as the mean of the three readings as previously described.

Hypothalamic-pituitary-adrenal axis

Plasma corticosteroid concentrations were measured basally and in response to 30 minutes of restraint stress.

Corticosteroid receptor mRNA expression.

Glucocorticoid and mineralocorticoid receptor mRNA expression was examined by in situ hybridisation. Freshly dissected brains were frozen on dry ice and the following regions were sectioned: hippocampus, area postrema, organosum vascularum laminae terminalis, subcommisural organ and nucleus tractus solitarius. The detailed methods for in situ hybridisation are described in the methods section.

Plasma corticosterone concentrations were measured by radio immunoassay using previously described methods (the intra assay coefficient of variation was 3.8%).

Statistics

All results are expressed as mean ± SEM. ANOVA was used to evaluate the significance of the differences which were accepted at a $P < 0.05$ level.

Results

Pregnancy

Treatment with 100 µg/kg.day-1 dexamethasone during the last week of rat pregnancy did not alter gestation length (CONT 21.7± 0.3 days: DEX 22.3± 0.3 days) or offspring number (CONT 9.7± 0.9: DEX 10±2) or viability. Maternal weight gain was similar in the two groups for the first 15 days of gestation (before treatment CONT

gained 38± 4g: DEX 41± 4g) but thereafter the dexamethasone treated dams gained less weight (days 15-21 weight gain CONT 55±7g: DEX 21±5g; P< 0.05).

Blood pressure and Hypothalamic-Pituitary adrenal axis in adult animals

At 16 weeks of age there were no significant differences in offspring weight (CONT 410± 10.7g, DEX 432± 9.2g). The mean systolic and diastolic blood pressures (Figure 17) were significantly higher in the offspring of the dexamethasone treated rats compared to the offspring of the control rats by 11 mmHg for systolic and 13 mmHg for diastolic blood pressures, respectively.

Mean basal plasma corticosterone concentration was 154.5 ± 28.6 nmol/L in the offspring of the dexamethasone treated rats and 79.3 ± 14.8 nmol/L in the offspring of the control rats (P = 0.03). The responses to restraint stress were similar in the two groups (Table 6).

In Situ Hybridization

In the offspring of control pregnancies, GR mRNA expression was higher in the dentate gyrus and CA1 regions of the hippocampus compared with the CA2 and CA3 regions. MR mRNA showed significantly higher expression in CA2 than other subfields. In the offspring of the dexamethasone treated rats there was a significant reduction in the GR mRNA gene expression in the dentate gyrus (21% fall) and CA1 region (15% fall: both P < 0.01) compared to the offspring of the control rats. MR mRNA expression was lower in the CA1 (24% less, P<0.05) and CA2 (25% less, P<0.05) areas of the hippocampus in the dexamethasone treated rats compared to the control rats.

There was low but detectable expression of MR mRNA in the sub-commisural organ, area postrema, nucleus tractus solitarius and OVLT and much higher expression in the motor IX nucleus. No differences in MR gene expression were noted in these

regions between rats exposed to dexamethasone and controls. GR mRNA was clearly expressed in the subcommisural organ, area postrema, OVLT and nucleus tractus solitarius, albeit at lower levels than in the hippocampus. There were also no differences in GR mRNA expression in these areas between the two groups.

Figure 17

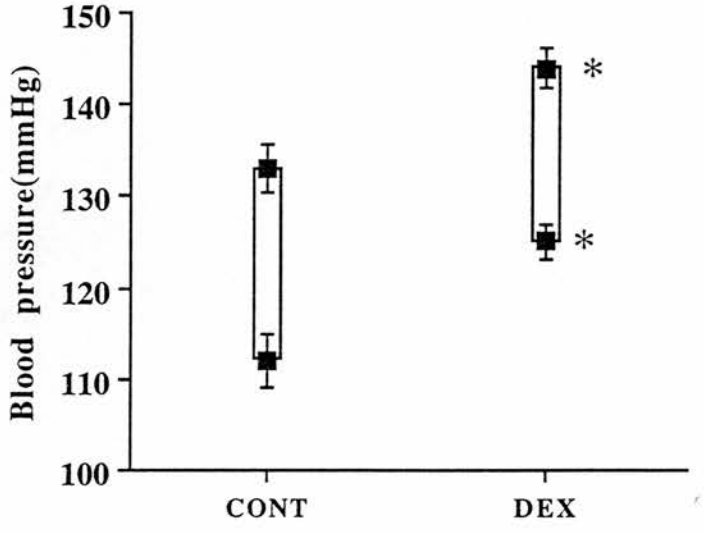


Figure 17: Blood pressure at 16 weeks in animals treated with dexamethasone in the third week in utero: CONT systolic BP 133± 2.7/ 112± 2.8 versus DEX 144± 2.1/ 125± 2.0: P<0.01 for both systolic and diastolic blood pressures).

Table 6

	CONT	DEX
Basal	79.3 ± 14.8 nmol/l	154.5 ± 28.6 nmol/l *
30 minutes	2066± 161 nmol/l	2381± 518 nmol/l
60 minutes	1126± 264 nmol/l	1269± 397 nmol/l

Table 6: Corticosterone levels before and after restraint stress. Basal cortricosterone levels in the DEX group were significantly higher (*, P<0.05).

Discussion

Despite the administration of dexamethasone solely in the last week of pregnancy in this study, there was a similar elevation of blood pressure in the adult offspring to that observed in previous studies with administered of a similar dose throughout gestation. Thus, the developmental window for the effects of glucocorticoid exposure on the offspring includes at least the last trimester.

This study investigated the hypothesis that prenatal glucocorticoid exposure could permanently induce altered expression of corticosteroid receptors in areas of the brain postulated to play a role in the central regulation of blood pressure and in mineralocorticoid-induced hypertension and thus induce hypertension in the adult offspring. There is a large body of data indicating that mineralocorticoids act centrally as well as peripherally in inducing hypertension. Firstly, mineralocorticoid receptors are present in areas of the brain known to be important in the regulation of body fluid homeostasis and maintenance of blood pressure[413, 414]. Secondly, intracerebroventricular (ICV) infusion of a specific MR antagonist has been shown to attenuate or block the development of deoxycorticosterone acetate (DOCA) salt-hypertension or that induced by ICV administration of aldosterone [410-412]. Thirdly, lesions of the anterior part of the third ventricle (AV3V), area postrema (AP), septal area of the limbic system and paraventricular nucleus abolished or reduced the increase in blood pressure in the mineralocorticoid induced hypertension model [415].

Notwithstanding these data we found low levels of MR mRNA expression in the OVLT, AP, NTS and SCO and did not demonstrate any changes in the expression of either GR or MR nRNA to explain the elevated blood pressures. This would argue against both a specific central mineralocorticoid component and altered expression of corticosteroid receptors in the brain in the pathogenesis of the observed elevation in blood pressure.

In this study the adult offspring of the dexamethasone treated rats had elevated mean basal corticosterone concentrations, but normal corticosterone responses to

restraint stress, together with a significant reduction in GR mRNA expression in the dentate gyrus and CA1 region of the hippocampus and a reduction in MR mRNA expression in the CA1 and CA2 areas of the hippocampus. This data indicates that prenatal exposure to excessive glucocorticoids does program altered central corticosteroid receptor expression, but in a selective fashion. In a number of experimental animal models, elevated corticosterone concentrations are consistently associated with decreases in hippocampal corticosteroid receptor concentrations as well as prolonged or heightened adrenocortical responses to stress. This implies that a loss of hippocampal receptors results in reduced sensitivity to corticosterone feedback [416]. It is recognised that under basal conditions, glucocorticoids bind primarily to hippocampal MR and under circumstances of stress or at a diurnal peak hippocampal GR are largely occupied by glucocorticoids[417]. In this context it might be hypothesised that the offspring of the dexamethasone treated mothers would demonstrate prolonged corticosteroid responses to restraint stress in the presence of elevated basal corticosteroid concentrations. We have not shown this, probably reflecting that the time points chosen were too short to demonstrate this effect as it may take over 90 minutes for corticosterone levels to return to basal. Equally the expected reduced sensitivity to corticosteroid feedback may be situation or stressor-specific. In prenatally stressed adult offspring a reduction in hippocampal MR and GR receptor densities and prolonged responses to restraint stress were demonstrated, indicating that prenatal stress has long and short-term effects on HPA activity of the offspring[408]. These observations, together with those of the present study, suggest that maternal corticosterone secreted during stress and crossing the placenta could play an important role in this phenomenon [408]. A more detailed evaluation of the HPA axis, such as the diurnal rhythm and responsiveness to other forms of stress in relation to hippocampal corticosteroid receptor expression would enhance our understanding of the effects of hormone imprinting.

Section 5: Effect of inhibition of 11beta-OHSD on birth weight and blood pressure.

Introduction

Access of maternal glucocorticoids to the fetal compartment is normally limited by the placental glucocorticoid metabolising enzyme 11ß-OHSD. It was not known whether a reduction in placental 11ß-OHSD would result in changes in birth weight and further whether the effects on blood pressure we had observed with dexamethasone would also be present with enzyme inhibition. Exposure of the fetus to exogenous glucocorticoids remains an unusual occurrence in normal pregnancy and if the influences of dexamethasone on later blood pressure were to be of more general, physiological importance then it was of importance to consider whether exposure of the fetus to a greater than normal amount of endogenous, maternal glucocorticoid might exert the same effects.

Previous work had already demonstrated that there was a relationship between placental 11ß-OHSD activity and birth weight in the rat[418] and subsequently it was shown that activity of 11ß-OHSD in human placenta at term was also correlated with birth weight[419, 228], while both the syndrome of apparent mineralocorticoid excess[420] and the human mutations of 11ß-OHSD 2[234] have now also been associated with reduced birth weight.

We set out to examine the influence of inhibition of 11ß-OHSD by carbenoxolone on birth weight and later blood pressure and glucose tolerance.

Methods

Carbenoxolone treatment in adrenal intact animals

Female Wistar rats (200-250 g) were maintained under conditions of controlled lighting (lights on 07.00-19.00 h) and temperature (22°C) and allowed free access to food (standard rat chow; 56.3% carbohydrate, 18.3% protein, NaCl 0.7%; B.S.&S Scotland Ltd. Edinburgh) and tap water. The rats were time-mated and then given either

carbenoxolone (CBX; 12.5 mg/day in 4% ethanol-saline, 0.1 ml, s.c.) or vehicle alone (CON) throughout pregnancy. At birth, the offspring were weighed and then no further treatment was given (to mothers or pups). A separate cohort of pregnant rats underwent identical treatment, but were subjected to carotid cannulation under brief halothane anaesthesia on day 15 of pregnancy, to permit subsequent measurement of maternal blood pressure and blood sampling.

Carbenoxolone treatment in adrenalectomised animals

Non-pregnant female rats underwent adrenalectomy by the dorsal approach under halothane anaesthesia. Controls were sham-operated (SHAM). Blood (for plasma corticosterone estimation) was subsequently taken at 09.00 h by tail tipping to assess the completeness of adrenalectomy. Adrenalectomised rats were additionally given saline to drink. Adrenalectomised animals were mated 8-15 days after surgery and treated throughout pregnancy with carbenoxolone (ADX+ CBX: 12.5 mg/day in saline, s.c.) or saline alone(ADX) . Sham-adrenalectomised controls received saline.

Measurement of Placental 11§-OHSD activity

On days 17-21 of gestation animals (5 control, 3 treated with carbenoxolone 12.5 mg/day throughout pregnancy) a separate cohort of animals were subjected to halothane anaesthesia and the left carotid and right jugular vein cannulated to allow measurement of placental 11ß-OHSD-2 activity by infusion of ^{3}H corticosterone to achieve steady state and subsequent extraction of ^{3}H corticosterone and ^{3}H 11-dehydrocorticosterone from the blood of the dams, fetal tissues and placenta as described in the methods.

Oral Glucose Tolerance Test (OGTT) and Blood pressure measurement

At six months of age male offspring underwent an oral glucose load test as described in methods. Animals were fasted from 1600h the day before and 2 g/kg

glucose (as a 0.5g/ml solution) was given by gavage between 0800 and 0900h the next morning. Blood was taken by tail tipping at 0, 30, 60, 90 and 120 min, centrifuged immediately and the plasma stored at -70C.

After a two week gap animals were subjected to carotid cannulation to allow assessment of blood pressure. Male offspring were studied at 6 and female offspring at 8 months. A total of 60 animals (CON 13, CBX 10, SHAM 14, ADX 10, ADX+CBX 13) were initially studied, but only those surviving surgery and with patent cannulae have been included (CON 11, CBX 9, SHAM 8, ADX 10, ADX+CBX 11). The coefficient of variation for the repeated measures of blood pressure was 6.9% for mean arterial pressure.

Statistics

All data are expressed as mean ± SEM. Data were assessed for multiple comparisons by one or two way ANOVA, as detailed, with post hoc testing using the Student-Newmann Keuls' test. Students *t* -test was used for two group comparisons. Data were reported as significant when $P<0.05$.

Results

Effect of carbenoxolone on adrenal-intact pregnant rats and their litters

Carbenoxolone treatment of pregnant rats significantly reduced birth weight (CBX 4.54±0.08g, n=35; CON 5.68±0.07g, n=39; $P<0.0001$; mean 20% decrease: Figure 18). However, carbenoxolone administration did not alter litter size (CON 9.7±1.1, n=4; CBX 8.7±1.7, n=4) or the length of gestation (CON 22.2±0.2 days, n=4; CBX 22±0.1 days, n=4). Maternal weight gain through pregnancy was significantly reduced by carbenoxolone administration (CON 72±5 g, n=4; CBX 50±5 g n=4; $P<0.05$). In the separate cohort of pregnant animals with chronic carotid cannulae, treatment with carbenoxolone had no significant effect on maternal blood pressure on days 18-20 of pregnancy (CON systolic 117±2 mmHg and diastolic 86±5

mmHg, n=4; CBX systolic 119±3 mmHg and diastolic 77±4 mmHg, n=3), maternal plasma glucose (CON 5.3±0.3 mmol/l, n=4; CBX 6.4±0.3 mmol/l, n=4) or 09.00h plasma corticosterone levels (CON 692±172 nmol/l, CBX 647±101 nmol/l).

Effect of carbenoxolone in adrenalectomised pregnant rats

Plasma corticosterone levels were <60 nmol/l in adrenalectomised rats and 898±103 nmol/l in the sham-operated animals. Maternal adrenalectomy was associated with a reduction in offspring birth weight (SHAM ADX 5.34±0.15g n=36; ADX 4.86±0.06g, n=24; P<0.05), but there was no additional effect of carbenoxolone in adrenalectomised animals (ADX + CBX 5.02±0.11g, n=22). Adrenalectomy with or without carbenoxolone did not affect litter size (SHAM ADX 7.2±1.3, n=5; ADX 8±0, n=4; ADX + CBX 7.3±2.7, n=3) or the length of gestation (SHAM ADX 22.2±0.4 days; ADX 22±0 days; ADX + CBX 21.7±0.9 days). Adrenalectomised and sham-operated rats showed similar weight gains through pregnancy (SHAM ADX 84±7g; ADX 83±15g; ADX + CBX 66±18g).

Effect of carbenoxolone on placental 11§-OHSD in vivo

Maternal blood concentrations of ^{3}H corticosterone and ^{3}H 11-dehydrocorticosterone were similar in control and carbenoxolone groups at 20,40,60 and 80 min (two way ANOVA), indicative of isotopic steady state. Carbenoxolone did not alter the metabolic clearance rate for corticosterone (CON 11.8± 0.9 ml/min, n= 5; CBX 11.0± 1.3 ml/min, n=3) and infused pregnant animals had similar blood pressures (mean arterial pressure CON 77.2± 4.5 mmHg; CBX 82± 5.3). There was no difference with treatment in the percentage of total ^{3}H steroids as corticosterone in maternal blood at steady state (CON 90.8±2.1% ; CBX 89.5±3.4%).

Carbenoxolone treatment reduced placental 11ß-OHSD activity (activity as percentage of matched control: CON 100± 9.4% ; CBX 63.5± 8.6%; P<0.05) and led to an increase in ^{3}H corticosterone as a percentage of total ^{3}H -steroids in fetal tissues

(CON 65.6± 1.6% ; CBX 74.2±2.0%; P<0.05).

Effect of treatment on offspring in adulthood

Carbenoxolone treatment during pregnancy led to rises in offspring mean arterial pressure both in males, studied at 6 months, (CON 127± 1.4 mmHg, n=11; carbenoxolone-treated 136± 2.1 mmHg, n=9; Figure 19, Table 7) and in females, studied at 8 months (CON 113 ± 2.0, n=11; CBX 120 ±1.8 mmHg, n=8). Prenatal carbenoxolone treatment did not result in any persisting changes in body or organ weights. In particular, male offspring of saline-treated controls and carbenoxolone-treated pregnant rats were of similar weight (CON 491±6.3g, n=11; CBX 461±13g, n=9), while female offspring showed slightly higher body weight (CON 270 ±5g, n=11; CBX 298± 7g, n=7; P<0.01). There were no significant differences in organ weights (spleen, heart, kidney, data not shown) in either males or females.

The male offspring of females subjected to adrenalectomy before pregnancy were studied at 6 months. By contrast to the offspring of adrenal intact females, there was no significant change in blood pressure in the adult offspring of adrenalectomised females treated either vehicle (SHAM ADX 127± 2.3 mmHg , ADX 125± 3.1 mmHg) or with carbenoxolone (ADX + CBX 129± 3.2 mmHg, n=10; Figure 19, Table 7). The offspring of the sham and adrenalectomised mothers were lighter than the original controls but there were no differences between groups (SHAM ADX 409± 7g, n=8; ADX 415± 6.1g, n=11; ADX + CBX 419 ± 10.5g, n=10) .

Results for all 6 month male offspring were combined to consider the effects of adrenalectomy and carbenoxolone. Two way ANOVA revealed a significantly higher blood pressure in the offspring of carbenoxolone treated (F=7.6) but not adrenalectomised females. Post hoc testing revealed an effect of carbenoxolone in the offspring of adrenal intact females(CBX) both versus controls(CON) and the ADX + CBX group (Student-Newmann-Keuls', P<0.05), suggesting that carbenoxolone treatment only resulted in higher blood pressure in the offspring of adrenal intact

females.

Adult offspring response to an oral glucose load

At six months of age, the male offspring of adrenal intact rats treated with carbenoxolone during pregnancy displayed higher fasting plasma glucose levels (CONT 4.8±0.2 mmol/l; CBX 6.0± 0.3 mmol/l; P< 0.01). The plasma glucose response to an oral glucose load was also higher in the offspring of carbenoxolone-treated pregnancies (CONT vs. CBX repeated measures ANOVA, F=5.93, P=0.02, Figure 20). The area under the curve for glucose across the OGTT was significantly (10%) higher for the offspring of carbenoxolone treated pregnancies and the response of insulin in this group was also significantly (38%) greater (Figure 21). Body weights were similar at 6 months in all groups.

Maternal adrenalectomy prevented the effect of carbenoxolone on offspring glucose tolerance. There was no difference in glucose tolerance in the offspring of the adrenalectomised pregnant females with or without carbenoxolone in terms of either basal glucose or the response of plasma glucose or insulin.

Figure 18

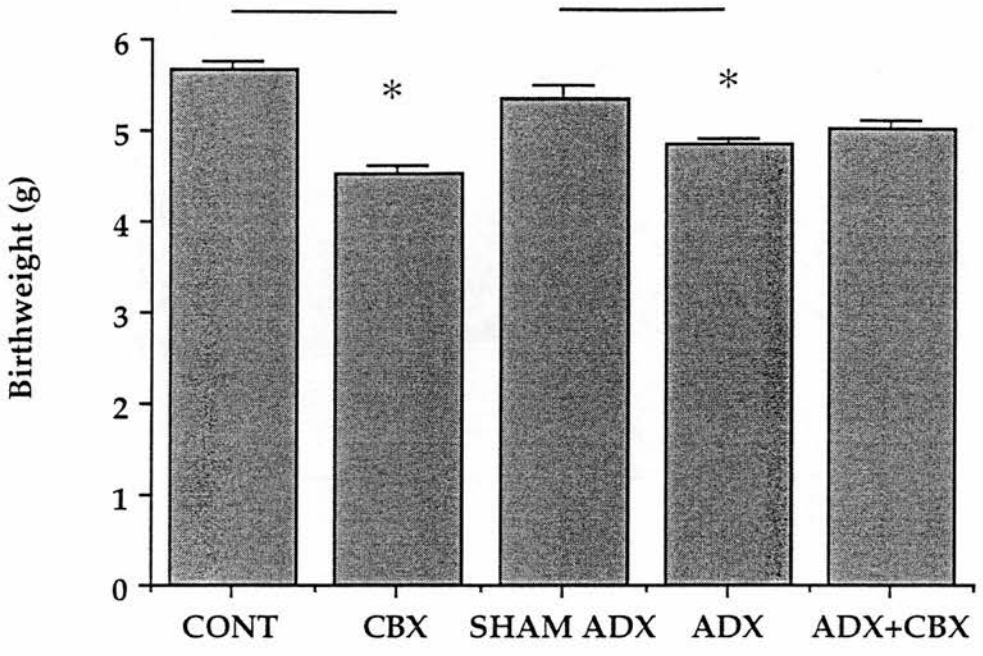


Figure 18: The effect of carbenoxolone on birth weight of litters of adrenalectomised and adrenal intact mothers. Birth weight in grams (g) of offspring of control (CONT) , carbenoxolone (CBX), sham adrenalectomy (SHAM ADX), adrenalectomised (ADX) and adrenalectomy and carbenoxolone (ADX+CBX) treated mothers. * denotes significance at 95%.

Figure 19

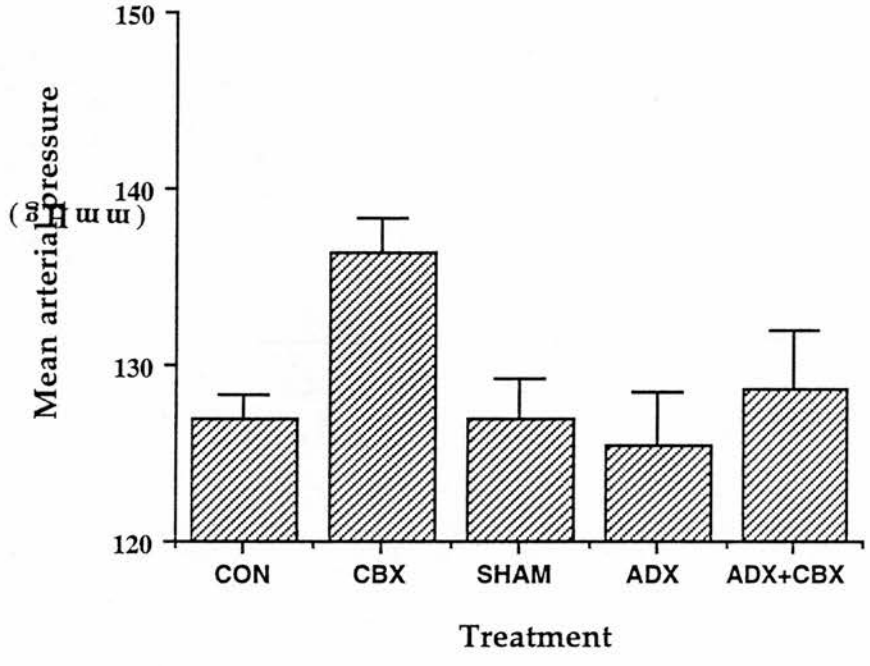


Figure 19- The effect of carbenoxolone treatment in pregnancy on male mean arterial pressure.

Mean arterial pressure in the offspring of control(n=11), carbenoxolone (CBX, n=9), sham adrenalectomised (SHAM ADX, n=8), adrenalectomised (ADX, n= 11) or adrenalectomised + carbenoxolone (ADX+ CBX, n=10) treated females. * denotes significance at $P< 0.05$ *vs* control.

Table 7

Adult offspring of adrenal intact females

		CBX	CONTROL
Male	weight(g)	461± 13	491± 6
	mean arterial pressure (mmHg)	136± 2.1 *	127±1.4
Female	weight(g)	298± 7	270± 5
	mean arterial pressure (mmHg)	120± 1.8 *	113± 2.0

Adult offspring of adrenalectomised females

		CBX+ADX	ADX	SHAM
male	weight(g)	419± 10	415± 6	409± 7
	mean arterial pressure(mm Hg)	129± 3.2	125± 3.1	127± 2.3

Table 7: Weight and blood pressure of offspring of carbenoxolone treated females. * denotes significance at $P < 0.05$.

Figure 20

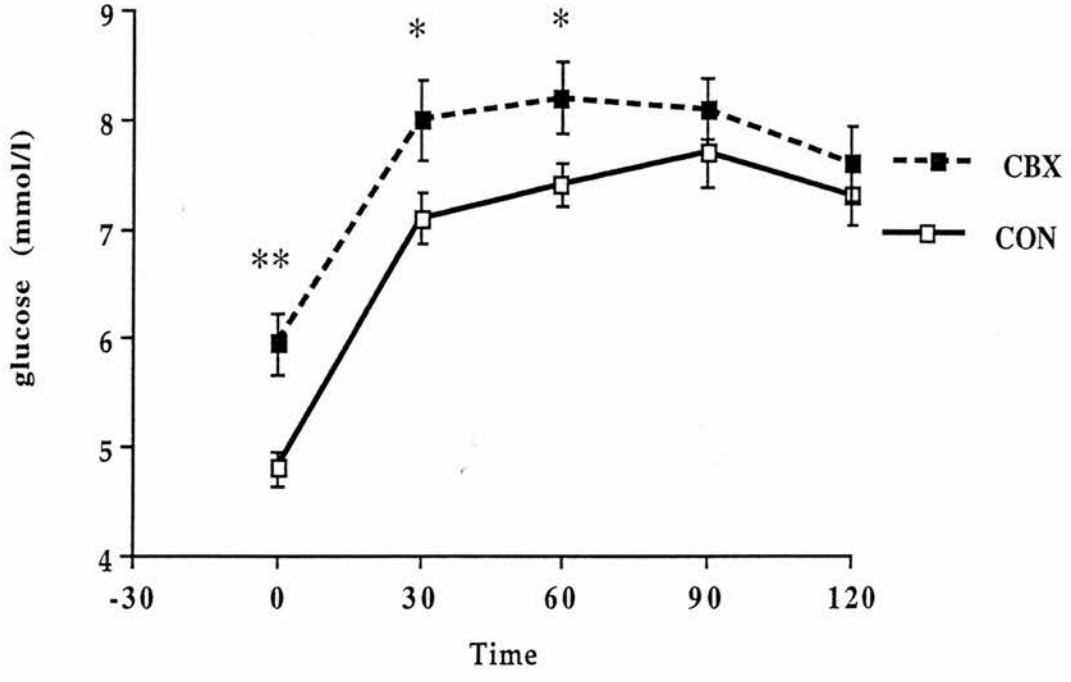


Figure 20: Plasma glucose response to an oral glucose load in offspring at 6 months. Pregnant rats received vehicle (CON, n=18) or carbenoxolone (CBX, n=14). Treatment was only during pregnancy. Repeated measures ANOVA, F=5.93, P<0.05. For individual time points ** indicates P<0.01, * P< 0.05, unpaired *t* -test vs. control.

Figure 21

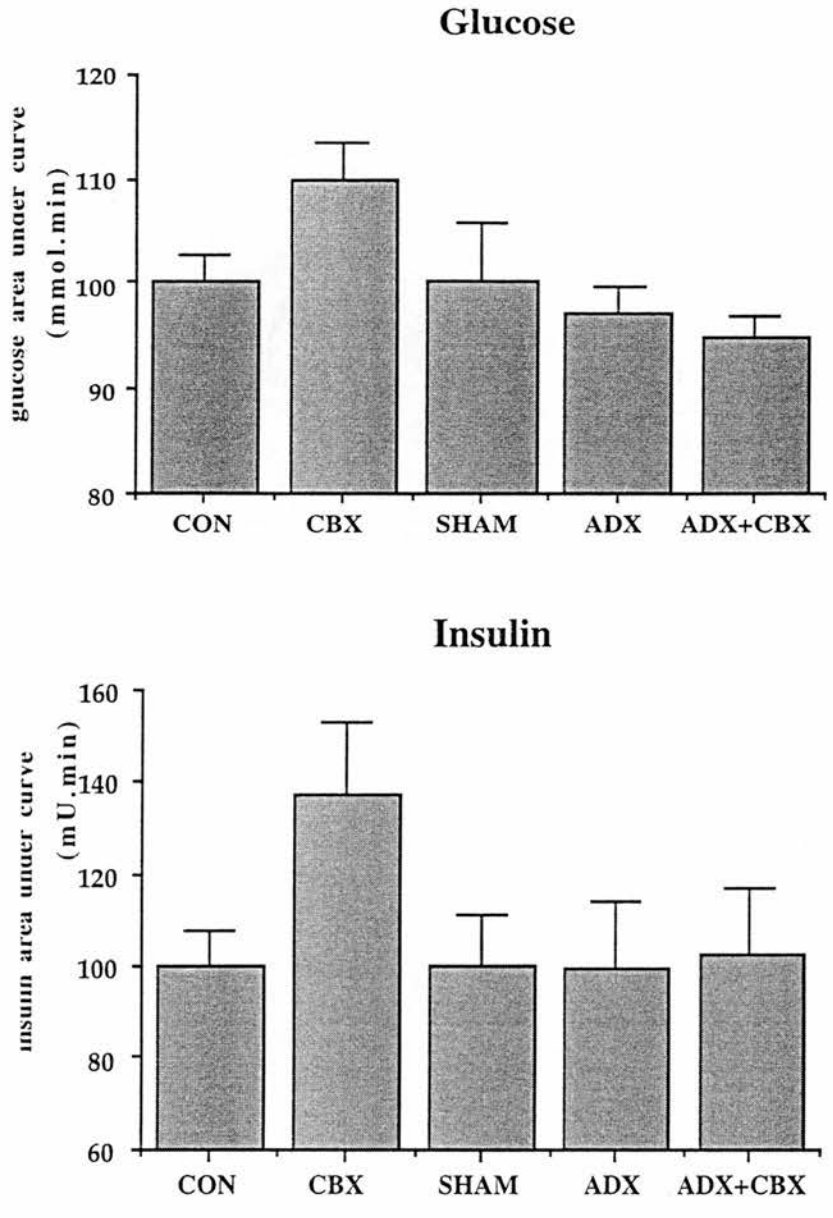


Figure 21: Glucose and insulin responses to an oral glucose load in offspring of carbenoxolone treated rats: Area under curve for glucose and insulin in 6 month old offspring of rats treated in pregnancy with vehicle control (CON, n=18), carbenoxolone (CBX, n=14), sham adrenalectomy and vehicle (SHAM, n=8), adrenalectomy and vehicle (ADX, n=8) and adrenalectomy with carbenoxolone(ADX+ CBX, n=8). * $p<0.05$ compared with control.

Discussion

Carbenoxolone acts *in vitro* as an inhibitor of placental 11ß-OHSD 2 [224]. Here we demonstrate that administration of carbenoxolone inhibits 11ß-OHSD 2 activity *in vivo* and allows increased passage of maternal corticosterone to the fetus, at least within the last week of pregnancy, in keeping with the proposed role of the placental 11ß-OHSD-2 in maintaining the lower glucocorticoid environment of the fetus [265, 268]. Carbenoxolone treatment reduced birth weight, an effect similar to that previously observed with dexamethasone and led to elevated blood pressure and raised fasting glucose in the adult offspring, supporting both a role for placental 11ß-OHSD in fetal exposure to glucocorticoids and the hypothesis [421, 422] that this exposure in excess may explain the epidemiological link between low birth weight and subsequent cardiovascular risk in humans[200].

In principle the effects of carbenoxolone on birth weight might be mediated by actions upon the mother, placenta and/or the fetus. However, some clues as to the site of action are provided by these experiments. Carbenoxolone raises blood pressure in animals models by inhibition of renal 11ß-OHSD. Elevation of maternal blood pressure might be anticipated to alter feto-placental function and perhaps growth [88]. However, no change in maternal blood pressure at the end of pregnancy was observed. This was perhaps unexpected, but might reflect the reduced efficacy of carbenoxolone in pregnant rats or a lack of hypertensive actions by a sodium and water retaining mechanism in the already volume-expanded state of pregnancy. In any event, maternal hypertensive effects *per se* appear unlikely to explain fetal growth retardation and subsequent blood pressure elevation in the offspring. The design of these studies did not allow exclusion of persisting effects of carbenoxolone treatment on maternal behaviour after birth but prior to weaning. However, since maternal treatment stopped at birth, this possibility is less likely.

Placental 11ß-OHSD is an efficient, but probably incomplete barrier to maternal glucocorticoids and a minor proportion of fetal corticosteroids normally derive from the

maternal compartment [285]. Although, within a certain tolerance, reduced fetal adrenal secretion of glucocorticoids may adjust for increased transplacental glucocorticoid passage, eventually elevated maternal glucocorticoids may overcome fetal adjustments or even flood the metabolic capacity of the enzyme, though the latter is unlikely [423]. It might be predicted that inhibition of 11ß-OHSD, which is an important pathway of glucocorticoid metabolism, would increase maternal plasma glucocorticoid levels, perhaps exceeding the limits of placental 11ß-OHSD. However, this does not appear to be the case, as maternal corticosterone levels were similar in carbenoxolone-treated and control animals. Indeed, whatever the status of glucocorticoid metabolism, corticosterone negative feedback control of hypothalamic-pituitary-adrenal axis activity will presumably ensure that plasma glucocorticoid levels are tightly controlled. However, two additional caveats concerning glucocorticoid levels and carbenoxolone bear mention. Firstly, total glucocorticoid levels do not necessarily reflect 'free' hormone concentrations, and it is conceivable that the treatments alter corticosterone-binding globulin levels, although this is perhaps unlikely to be of significance given the very similar total corticosterone values. Secondly, we have only examined glucocorticoids during the diurnal nadir. Although this pertains for the majority of the day, the diurnal peak (evening) values might be higher in carbenoxolone-treated rats. Nevertheless, there is no reason to assume this occurs and the data presented here do not support maternal glucocorticoid excess *per se* as causal of the fetal growth retardation.

With regard to the hypothesis that catch up growth *per se* would result in higher blood pressure[7] It is of note that while adrenalectomy reduced birth weight (although not as potently as carbenoxolone) it had little, if any, effect on adult blood pressure.

There are a variety of mechanisms whereby carbenoxolone might effect birth weight or fetal development apart from inhibition of 11ß-OHSD. As detailed in the introduction carbenoxolone has several cellular effects other than inhibition of 11ß-OHSD including inhibition of prostaglandin synthesis[255], alteration in hepatic

aldosterone metabolism[253], direct effects on sodium and potassium transport[256], potentiation of the effects of steroids not subject to 11ß-OHSD metabolism (aldosterone, 11-deoxycortisterone and synthetic glucocorticoid agonists)[257, 258] and direct activation of mineralocorticoid receptors[243, 244]. Carbenoxolone has been noted to enhance vasoconstrictor action in ex vivo preparations and to potentially exert damaging effects on endothelium in vivo[259]. The effects on prostaglandin synthesis might be considered of most potential importance in utero: in the sheep prostaglandin E2 levels rise towards the end of pregnancy[424] and are in turn associated with induction of ACTH in the fetal pituitary[425]. Prostaglandin synthesis, however, is only inhibited by micromolar concentrations of carbenoxolone[261] and far higher than those expected to occur *in vivo* [220, 260].

The likelihood that this effect is mediated by inhibition of 11ß-OHSD is increased by the observation that carbenoxolone was not active in the absence of maternal adrenal products. The importance of 11ß-OHSD 2 in limiting fetal exposure to maternal glucocorticoid is supported both by the effect of carbenoxolone to increase ^{3}H corticosterone access to the fetal circulation and the observation that the effects on both birth weight and offspring glucose tolerance are dependent on the presence of intact maternal adrenal glands. This suggests that carbenoxolone is not having direct effects on the dam, but acts via increased exposure of the feto-placental unit to maternal glucocorticoids.

Carbenoxolone may also have effects on the fetus itself. Fetal animals [353, 426] and humans express 11ß-OHSD [227] at some developmental stages. The function of the enzyme is unclear but it is potentially involved in protection of both mineralocorticoid and glucocorticoid receptors, as in the adult. It is possible therefore, that some of the effects we have observed may be mediated by carbenoxolone passing the placenta and inhibiting 11ß-OHSD in the fetus and this direct mechanism might be supported by the non-significant trend upward of blood pressure with carbenoxolone treatment in the absence of a maternal adrenal. Larger numbers would be neccesary to demonstrate

whether this is a real effect. Against this, given that we have demonstrated a larger effect of carbenoxolone on later offspring blood pressure in the presence of maternal adrenals and that similar effects are observed with maternally-administered dexamethasone (which is a poor substrate for the enzyme), the more likely explanation remains that the major effect is exerted by greater exposure of the fetus to maternal glucocorticoid.

Carbenoxolone could potentially alter the fetal environment in other ways. In terms of programming later glucose intolerance, the most important of these would be if maternal blood glucose had altered, given the extensive evidence for effects of maternal blood glucose on later offspring diabetes both in human populations[94,97-99] and animal models[120, 121, 126, 125]. It is important to note, therefore, that in our model there was no increase in maternal blood glucose, rendering this potential mechanism unlikely.

Glucocorticoids might act in a number of ways in the developing animal to provoke later glucose intolerance. Glucocorticoids inhibit insulin release [427] and islet beta cell replication in vitro [428, 429]. Thus increased exposure to maternal glucocorticoids may permanently reduce beta cell mass, later expressed as impaired glucose tolerance. Equally glucocorticoids may act to programme hormonal responses or metabolic pathways. Glucocorticoids exert important maturational effects on a variety of key metabolic enzymes [334, 324]. These include phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis, which is directly and potently regulated by glucocorticoids at the level of transcription [430]. Early exposure to glucocorticoids might programme these systems to alter permanently carbohydrate metabolism. Alternatively, prenatal and immediate postnatal stress (and glucocorticoids) are well-documented to programme increased hypothalamic-pituitary-adrenal axis activity producing glucocorticoid hypersecretion throughout life [366, 408]. Such an effect is indeed observed after prenatal dexamethasone exposure[431] and might of course contribute to hyperglycaemia. Finally, it is possible that glucocorticoids act indirectly by influencing fetal or placental expression of key growth factors. In this regard, glucocorticoids

regulate the synthesis of insulin-like growth factors 1 and 2, many of their binding proteins and both receptor subtypes in the fetus and placenta [432, 433].

In the next section we consider further whether such effects are mediated by insulinopenia or insulin resistance and whether dexamethasone will exert similar effects on glucose tolerance.

Section 6: Effect of carbenoxolone and dexamethasone *in utero* on insulin sensitivity

Introduction

Previous work had shown evidence of higher fasting plasma glucose and insulin resistance in the offspring of animals treated with carbenoxolone during pregnancy. This experiment was set up to assess whether treatment with dexamethasone in utero resulted in glucose tolerance and to further define whether such treatment resulted in insulin resistance or insulinopenia. Animals were assessed by oral glucose tolerance both before and after fat feeding, an intervention previously shown in vivo to be associated with the induction of insulin resistance[434].

Methods

Female Wistar rats (200-250 g) were maintained under conditions of controlled lighting (lights on 07.00-19.00 h) and temperature (22°C) and allowed free access to food (standard rat chow; 56.3% carbohydrate, 18.3% protein, NaCl 0.7%; B.S.&S Scotland Ltd. Edinburgh) and tap water as per previous protocols. The rats were time-mated and then given either carbenoxolone (CBX; 12.5 mg/day in 4% ethanol-saline, 0.1 ml, s.c.) dexamethasone in one of two doses (DEX 100: dexamethasone 100 µg/kg/day as a 0.1 ml injection in vehicle, or DEX 250: dexamethasone 250 µg/kg/day as a 0.1 ml injection in vehicle) or vehicle alone (CONT) throughout pregnancy. No further treatment was given (to mothers or pups) after birth and to minimise disturbance to the mothers offspring were weighed at three days.

At 6 months of age animals were subjected to:

i) an assessment of insulin sensitivity: animals received an intraperitoneal insulin challenge with measurement of tail tip glucose every 15 minutes for 75 minutes.

ii) After a two week gap animals were fasted from 4 pm on the night before and given a 2g/kg oral glucose tolerance test, as previously described. Following this all animals were subjected to a 30% fat diet (commercial margarine was added to our standard rat

chow resulting in a diet of 30% fat ; 39.4% carbohydrate, 12.8% protein, NaCl 0.5%) and animals weighed on a once weekly basis. Animals were housed in treatment specific groups of 8 and food intake assessed per cage per week.

Results

Effect of treatment on dams and litters

Of twenty animals timed mated 17 littered successfully (5 CONT, 4 DEX 100, 3 DEX 250, 3 CBX). In these animals there were no significant effects on litter size(CONT 9.8± 0.12; DEX 100 11.8± 0.6; DEX 250 8.7± 2.4; CBX 9± 2.1) or length of gestation (CONT 22.2± 0.5; DEX 100 21.8±0.6; DEX 250 22.7± 0.3; CBX 22.3± 0.9). There were no differences in maternal weights at baseline, but treated dams gained weight significantly less well through pregnancy in both dexamethasone treated groups (repeated measures ANOVA $P<0.05$) and showed a trend to lower weight gain with carbenoxolone treatment ($P=0.07$, Figure 22). Weight on day 3 was significantly lower in all treatment groups (DEX 100 18% lower, DEX 250 22.5% lower and CBX 18% lower) compared to control weight of 9.06 ± 0.18g (Figure 23).

Basal offspring glucose tolerance and insulin sensitivity

Insulin injection revealed no difference in the fall in glucose between groups (Figure 24). Basal glucose tolerance tests displayed higher fasting levels in the CBX animals but repeated ANOVA across the whole of the oral glucose tolerance test did not display a significant difference in either carbenoxolone (Figure 25) or dexamethasone treatment (Figure 26).

In response to fat feeding animals showed a significant increase in weight over a six week period (Figure 27). Fasting glucose showed a trend upward in all animals and a significant difference in fasting glucose in animals treated with carbenoxolone in utero (Figure 28). There was no difference from control values in the dexamethasone treated groups for fasting glucose(Figure 29) and the glucose area under the curve did not alter

for any of the groups (Figure 30) .

Insulin levels were assessed during the first oral glucose tolerance test. There were no differences in the ratio of insulin to glucose in the fasting state (CONT 1.9± 0.7, CBX 2.8± 1.0, DEX 100 1.2± 0.31, DEX 250 1.5± 0.02) or in the ratio of the rise in insulin to the rise of glucose between 0 and 30 minutes (CONT 3± 1.2, CBX 4.7± 0.9, DEX 100 4± 1.8, DEX 250 2.0± 2.6). Overall the area under the curve for glucose (CONT 889± 30, CBX 971± 28, DEX 100 904± 20, DEX 250 916± 28) and insulin ·(CONT 2162± 191, CBX 2959± 549, DEX 100 2583± 459, DEX 250 1465± 243) were not significantly different.

Figure 22

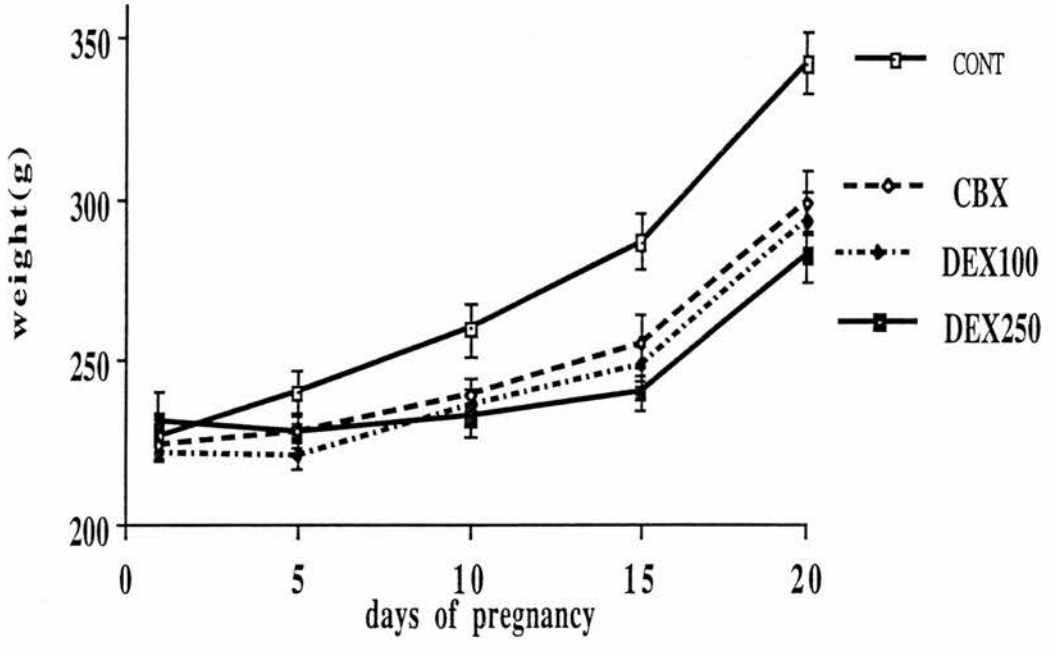


Figure 22: Dam weight gain through pregnancy in control (CONT, n=5) and dexamethasone (DEX 100, n=4, DEX 250, n=3) or carbenoxolone (CBX, n=3) treated pregnancy. Data analysed by repeated measures ANOVA with significant trend for DEX 100, DEX 250($P<0.05$).

Figure 23

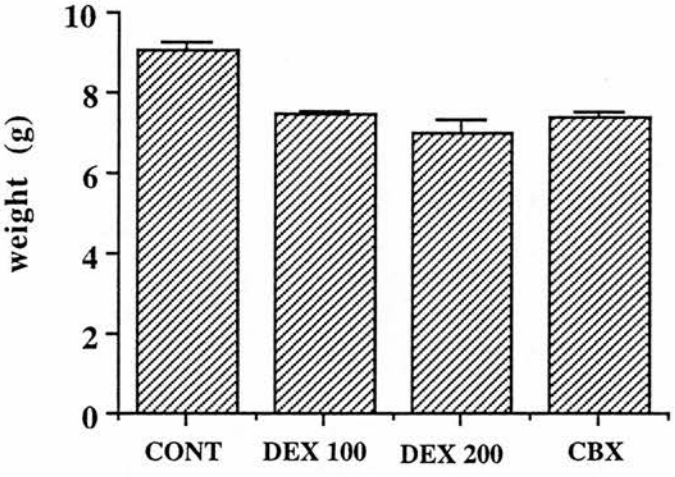


Figure 23: Offspring weight reduced by treatment. Weight at 3 days of offspring of dams treated with vehicle (n==49), dexamethasone (100µg/kg dose, n=47; 250µg/kg dose, n=27) or carbenoxoxone n=27. All treatments ANOVA F =30.8, P<0.01 with post hoc Newman Keuls' test compared to control.

Figure 24

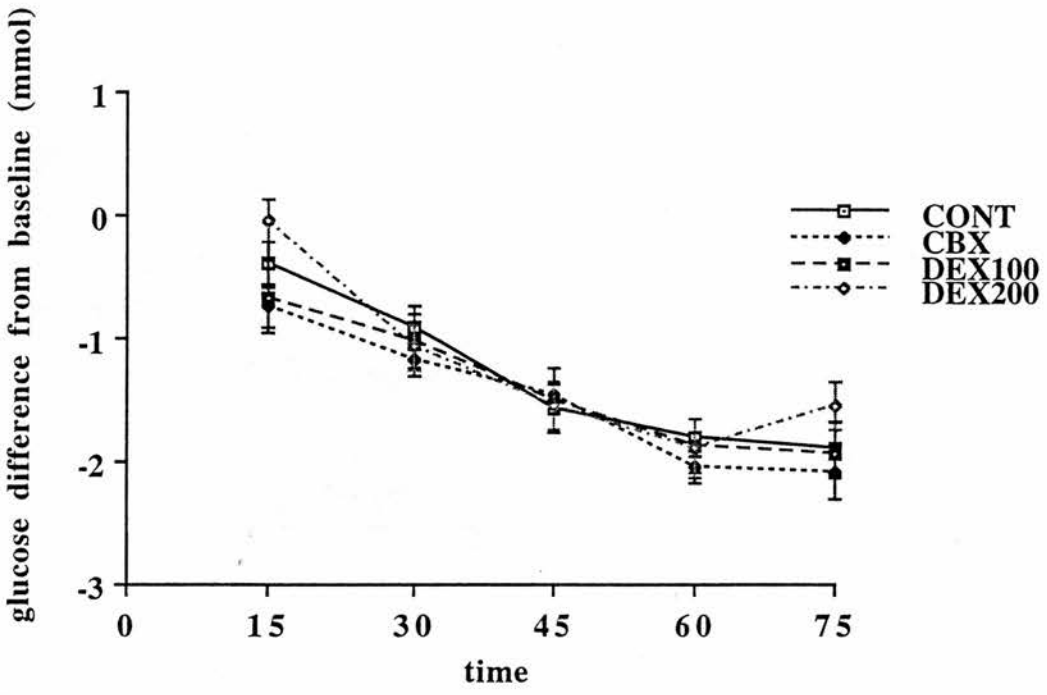


Figure 24: Effect of insulin injection on blood glucose in offspring after treatment. The offspring of dams treated with vehicle, dexamethasone or carbenoxolone during pregnancy (n=8 in each) examined with an intraperitoneal insulin injection. There were no significant differences in any group.

Figure 25

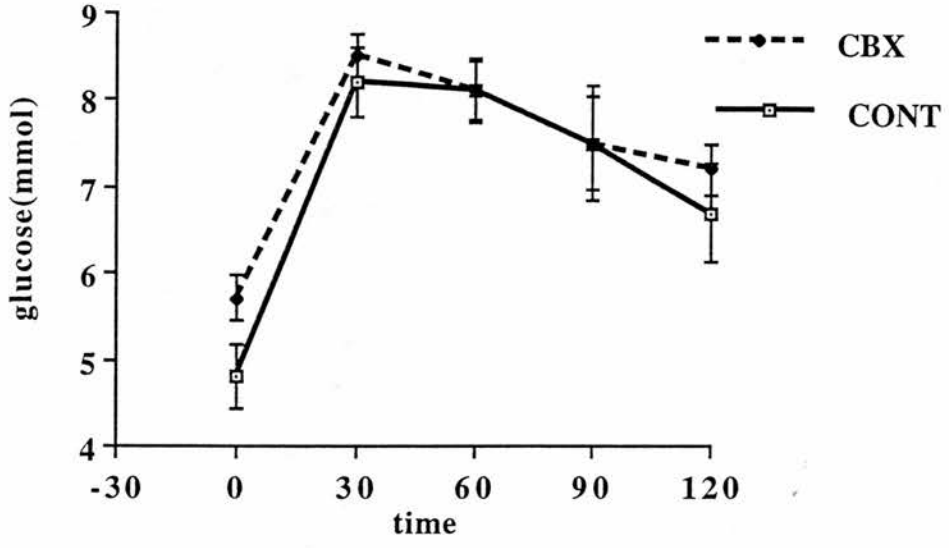


Figure 25: Basal glucose tolerance in the offspring of animals treated with carbenoxolone. Rise in glucose after an oral glucose load. There were no significant differences between groups(repeated ANOVA).

Figure 26

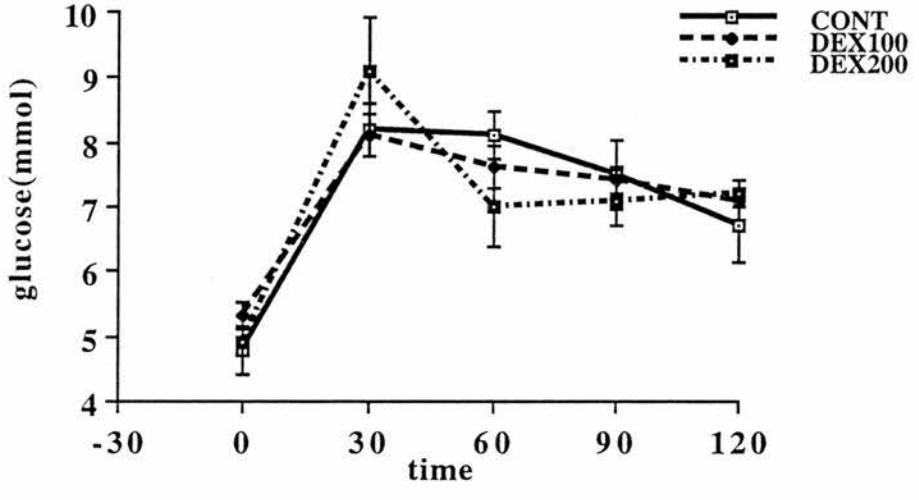


Figure 26: Basal glucose tolerance in animals treated with dexamethasone. Rise in glucose after an oral glucose load. There were no significant differences between groups(repeated ANOVA).

Figure 27

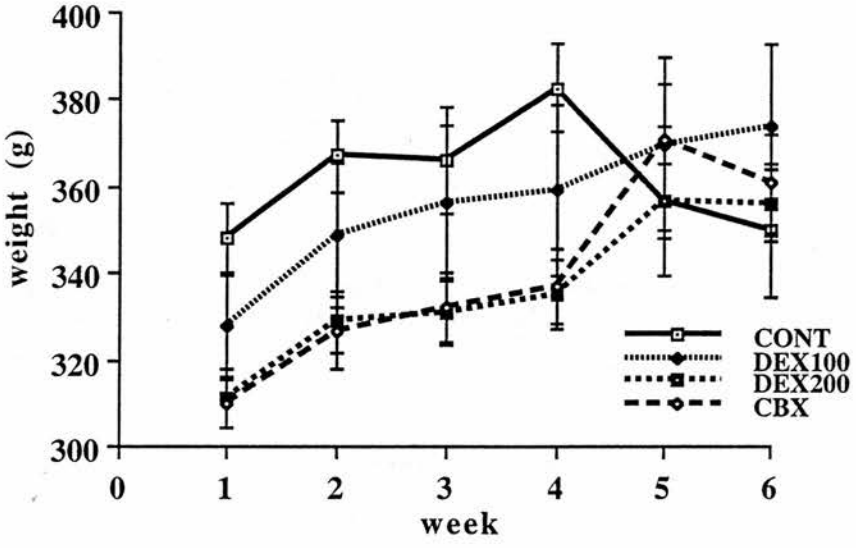


Figure 27: Weight gain with fat feeding. Rise in body weight in the offspring of dams treated in pregnancy. DEX 200 and CBX animals were lighter at baseline (ANOVA $F=4.0$, $P<0.05$). All groups showed a significant increase in weight across fat feeding(repeated measures ANOVA test for trend $P<0.01$).

Figure 28

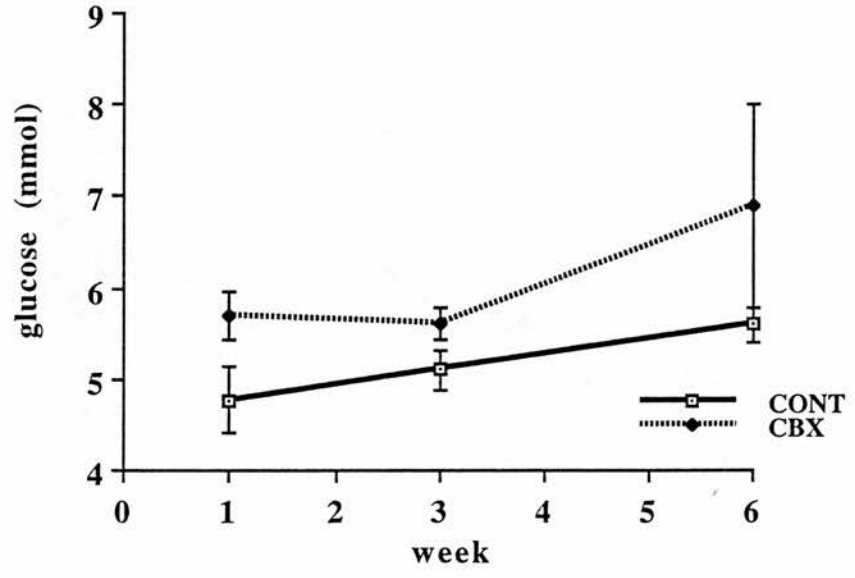


Figure 28: Change in fasting glucose after fat feeding. Offspring of dams treated eeither with vehicle or carvbenoxolone in pregnancy. Repeated measures ANOVA P<0.05 for difference between groups.

Figure 29

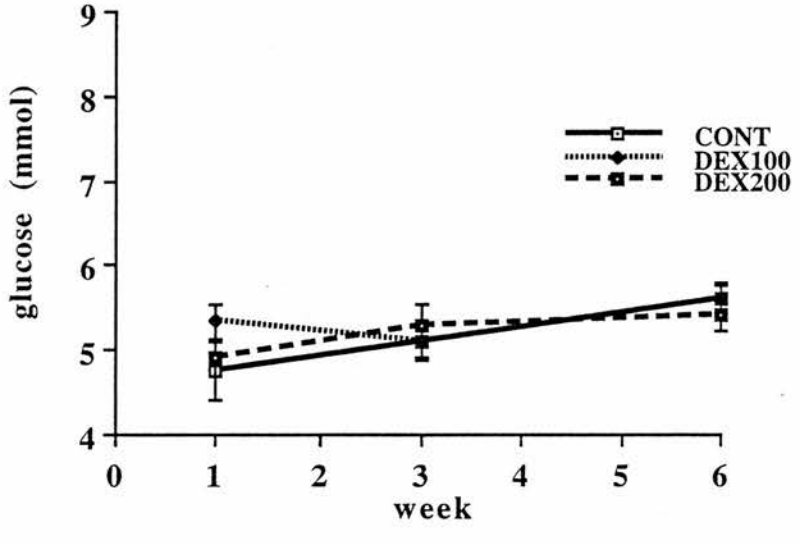


Figure 29: Fasting glucose in the offspring of animals treated with dexamethasone in pregnancy. Significant trend upward with treatment (P<0.05), no difference between the groups.

Figure 30

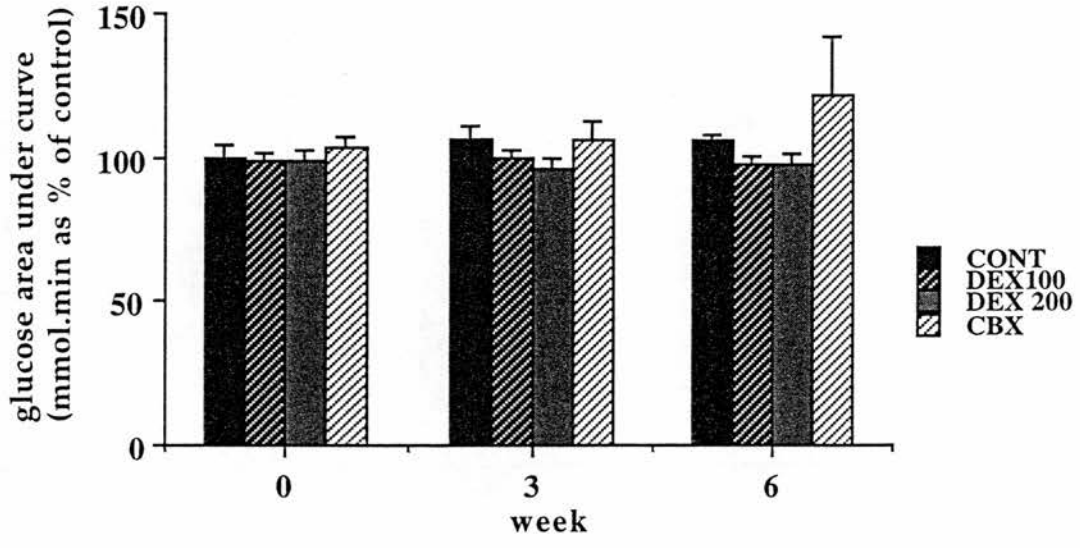


Figure 30: Effect of fat feeding on glucose area under curve. No significant differences between groups.

Discussion

We have again demonstrated the potential for carbenoxolone administered during pregnancy to reduce birth weight and raise fasting blood sugar. In non-insulin dependent diabetes there is evidence of reduced insulin secretion, peripheral insulin resistance and increased hepatic glucose output with various interrelationships between these states. Insulin resistance would be suggested by raised fasting insulin to glucose ratios and greater sensitivity to injected insulin and neither of these factors are apparent in these experiments. The oral glucose tolerance test is an imperfect way to assess insulin secretion, the closest correlations coming from the ratio of the incremental rise of insulin and glucose in the first thirty minutes after the oral glucose test. This measure is found in human populations to correlate with gold standard measures such as the intravenous glucose tolerance test. Again in this experiment there is no clear difference between groups to suggest a deficit in insulin secretion. This leaves the hypothesis that exposure to carbenoxolone might result in changes in hepatic glucose output. A variety of the key enzymes involved in glucoconeogenesis are expressed in response to glucocorticoids[430] and expression of gluconeogenic enzymes such as glucose-6-phosphatase, fructose diphosphatase, phosphoenolpyruvate carboxykinase and aspartate transaminase appear to be developmentally regulated by glucocorticoids[435, 324]. In this context we have preliminary data to suggest an increase in PEPCK activity in the offspring of carbenoxolone treated dams at least at 21 days (M.Desai personal communication).

Dexamethasone while reducing birth weight to a similar extent as previous experiments and as carbenoxolone in this experiment has resulted in no change in glucose tolerance. Clearly this raises the possibility that the effects of carbenoxolone are not glucocorticoid mediated. This would not be supported by lack of effect of carbenoxolone in the presence of maternal adrenalectomy observed in the previous section and there are other explanations. Firstly little is known of the effects of changing expression of rat placental 11ß-OHSD 2. Changes in both activity[273] and

expression[436] are recorded through pregnancy. It is possible then that the effects of carbenoxolone are different at different stages of pregnancy and that fetal glucocorticoid exposure is mainly in the third week in the presence of carbenoxolone, but throughout pregnancy in the presence of dexamethasone. Experiments assessing the effects of dexamethasone in different weeks of pregnancy might address this. It is of note that the effects of dexamethasone on sympathetic systems are mainly expressed in the last week of pregnancy.

A second area of possible difference is that the effects of carbenoxolone leading to corticosterone overexposure may be different to those of dexamethasone as corticosterone and dexamethasone may exert different cellular effects. Dexamethasone exerts cellular effects by binding to glucocorticoid receptors(GR), whereas corticosterone bind to both mineralocorticoid(MR) and glucocorticoid receptors. Since carbenoxolone might be expected to increase access of corticosterone to the fetus, cellular effects may be exerted by both MR and GR. In addition, access to MR receptors and possibly GR is, in the adult, controlled by 11ß-OHSD 2. The extent to which receptors are protected in the fetus is as yet unknown, 11ß-OHSD 2 is more extensively expressed in the fetus than the adult[436] and it is possible that the extent of inhibition by carbenoxolone may be different in different tissues. Thus carbenoxolone may lead to a subtly different pattern of glucocorticoid exposure than dexamethasone. Such effects may be difficult to model *in vivo* .

Such differential effects may be most important in the brain. Both MR and GR receptors are expressed in the brain and are present in the rat in low numbers in fetal life[437]. Expression of the MR increases to adult levels by the end of the first week of life and of the GR by the third week of life[437]. It is postulated at least in this tissue that most glucocorticoid effects will be mediated by the MR[437], making greater effects of corticosterone than dexamethasone possible.

Conclusions

In this thesis I have presented evidence that, in animal models, exposure of the fetus to glucocorticoids results in increases in blood pressure. Further, evidence is presented that at least part of the physiological control of glucocorticoid access to the fetus is dependent upon the placental glucocorticoid metabolising enzyme 11ß-hydroxysteroid dehydrogenase and that inhibition of this enzyme leads to both reduction in birth weight and later alterations in blood pressure and glucose tolerance.

I have taken as my starting point the epidemiological associations of lower birth weight to later cardiovascular risk and the overall theme of this work would be to relate placental 11ß-OHSD to cardiovascular risk in humans. The work I have presented suggests a potential for similar effects to occur in human populations, but much more work would be necessary to support such an assertion. As detailed in the introduction, it is an important feature of the work of Barker et al that the risk associated with lower birth weight extends in a graded fashion across the range of birth weight, not being restricted to the very low birth weight infant. This would indicate that whatever pathological insult or physiological process is working it must similarly act across the range of birth weight. As yet our knowledge of the influence of 11ß-OHSD on birth weight is limited. Correlations with placental 11ß-OHSD activity at term to birth weight have been shown by our group[419] and others[228] but the correlation coefficients appear modest and it would appear that at least in these studies the power of 11ß-OHSD activity to predict birth weight would be limited. The existing studies are limited however, since they take a single measure of enzyme activity at term to represent activity throughout the whole of pregnancy and by inference the glucocorticoid milieu of the fetus. This, of course may not be the case. There are obvious problems in relating a single measure of enzyme activity *in vitro* to the overall metabolising effect of that tissue[228], some of difficulties of which are answered by the approach of Benediktsson in measuring activity of the perfused whole tissue[419]. Nevertheless other variables are not accounted for including the supply of glucocorticoid to the tissue, which will be

dependent upon, among other factors, placental blood supply. In addition such experiments assume that 11ß-OHSD activity will be relatively stable throughout pregnancy so that measurement of lower activity at term infers lower activity throughout the pregnancy. The data regarding 11ß-OHSD activity through pregnancy is limited, suggesting a reduction in oxidative activity towards term[268] and does not tell us whether the level of activity is stable for an individual within a population. While it is likely that the cloning of 11ß-OHSD 2 will begin to allow these problems to be addressed our information as yet is limited. The observation that both the syndrome of apparent mineralocorticoid excess[420] and the human mutations of 11ß-OHSD 2[234] are associated with reduced birth weight are encouraging in this regard but will not explain variation within normal populations. The full blown forms of these conditions remain vanishingly rare[221] and as such even if associated with reduction in birth weight, these mutations would be expected to have little influence on birth weight in the majority.

The further study of the potential importance of 11ß-OHSD 2 to human birth weight is likely to depend on greater knowledge of 11ß-OHSD in the normal population. Firstly, whether heterozygotes for the known mutations also have lower birth weight and whether placental 11ß-OHSD activity is reduced. Secondly whether other mutations exist which might impact upon placental function but, perhaps be clinically silent in the kidney in adulthood. In addition to examine the importance of 11ß-OHSD 2 or mutations of human 11ß-OHSD-2 to human birth weight it will be necessary to gain some measure of exposure of the fetus to glucocorticoids in vivo. This is clearly difficult both technically and ethically, but without evidence in the human situation of a relationship between fetal glucocorticoid exposure and birth weight it will be difficult to establish unequivocally the physiological role of 11ß-OHSD 2 in the human situation.

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