Activation of inflammatory pathways in the kidney following prenatal exposure to a low protein diet

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ABSTRACT

The Developmental Origins of Health and Disease hypothesis proposes that events that occur *in utero* programme susceptibility to chronic adult diseases. Evidence demonstrates that certain organs, such as the kidney, are particularly vulnerable to structural and functional changes in response to adverse nutritional conditions during fetal life, and that the mechanisms may be sex-specific. At present many of the mechanisms of fetal programming that ultimately lead to inflammation and renal damage are not yet understood, and this project hypothesised that IκB-α and Monocyte Chemoattractant Protein-1 (MCP-1) may play a role. IκB-α is an inhibitor of the transcription factor nuclear factor-κB, which activates MCP-1, a protein with an active role in inflammation and renal damage. Levels of IκB-α and MCP-1 in the kidneys of offspring exposed to a low protein diet prenatally were measured and compared to a control group. Furthermore, the effects of gender were considered. Wistar rats were fed a control diet or a maternal low protein diet during pregnancy. After delivery all offspring were fed a standard laboratory chow diet, until they were culled at 8 weeks old. Renal IκB-α protein expression was assessed by Western blot, and renal MCP-1 mRNA expression by real-time PCR. Results were analysed using SPSS by two-way ANOVA or Pearson Correlation. There were no significant differences in IκB-α and MCP-1 levels between MLP or control rats, nor were there any significant effects of gender. There was a significant positive correlation between IκB-α and MCP-1 (R^2= 0.21, P< 0.01), but this correlation was weak. Hypothesis 1 – prenatal exposure to a low protein diet leads to decreased expression of IκB-α – was rejected. Hypothesis 2 - prenatal exposure to a low protein diet leads to increased expression of MCP-1 – was rejected. Hypothesis 3 - IkB-α and MCP-1 are negatively correlated – was rejected as the correlation was positive. However the validity of the IκB-α results is questionable due to inconsistencies in the Western blot gel transfer. The data for MCP-1 suggests it does not increase in response to prenatal protein restriction.

INTRODUCTION

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that events that occur *in utero* programme susceptibility to chronic adult diseases, such as type 2 diabetes, coronary heart disease (CHD), and hypertension.[1, 2] Early in the development of the fetal origins hypothesis it was suggested that limiting nutrient supply to the fetus was an important programming factor.[3] The term "fetal programming" refers to the concept that an insult or stimulus at a critical point during fetal development leads to a long-term effect.[4] This 'plasticity' of fetal development means that developing tissues can adapt to their current circumstances by altering gene expression in response to the prenatal environment. If this occurs during critical developmental periods, the availability of nutrients could therefore have permanent effects on tissue structure and function.[4]

Epidemiological evidence supports the theory that nutritional factors during fetal development can have long-term implications for the health of the offspring. If an insult takes place during a critical period of development for the fetus then growth may be restrained, and the resulting low birth weight may be a predictor

of disease later in life.[5] Low birth weight and/or disproportion at birth have been linked to CHD,[6] type 2 diabetes,[7] and hypertension.[8] Birth weight is, however, only a proxy measure of events during intrauterine development, and is not necessarily part of the causal pathway between maternal diet and offspring health and disease. In addition to this, epidemiological studies have been criticised on a number of other fronts, including weaknesses in terms of cohort selection and failure to correct for all confounding factors.[2, 4] Another limitation of epidemiological studies in the area is that many are historical; the cohorts are not contemporary, and may not reflect current lifestyles and diet. These limitations mean that animal studies have been critical to demonstrating the biological plausibility of the programming effects of prenatal nutrition, and are now a valuable tool for investigating the mechanisms involved. Many different dietary interventions have been studied in animal models, and they tend to have similar programming effects, which suggests there may be common mechanisms involved. Hypertension has been reported in rats exposed prenatally to high fat,[9] low iron,[10] and low protein diets.[11] Using a maternal low protein diet is considered to be a mild nutritional restriction and this model has been used in many studies.[4] Worldwide many women have protein-restricted diets during pregnancy, so the use of the MLP model is particularly appropriate and relevant.

The processes of proliferation and differentiation within a developing fetus require oxygen and nutrients, and it has been shown that restricting nutrient intake may lead to fewer cells within a particular tissue, or changes in the balance between cell types[12] This may lead to disruption of, or changes to, organ size and function[4], and metabolic and/or endocrine control.[5] Figure 1 shows some potential ways in which under-nutrition may affect the function of organs and systems. Under-nutrition may also lead to permanent changes in gene expression via epigenetic mechanisms, which may programme altered endocrine or physiological function in later life.[4, 5]

There is currently very strong evidence that certain organs, such as the kidney, are particularly vulnerable to structural changes in response to adverse conditions *in utero.* Low protein diets have been demonstrated to result in smaller kidneys with fewer nephrons,[13] misshapen kidneys,[14] and fewer glomeruli.[15] This is associated with altered renal function. MLP rats have been found to have lower creatinine clearance, and higher blood urea and urinary albumin excretion,[14] reduced glomerular filtration rate,[16] and reported increased urinary excretion of vasoactive prostaglandins.[17]

The renin-angiotensin system (RAS) regulates long-term blood pressure and the volume of extracellular fluid, through the actions of renin, angiotensin I and II, and aldosterone.[18] This system has been studied extensively as playing a potential role in the mechanisms underlying the programming of hypertension in animal models. Angiotensin II is considered to be the main effector hormone of the RAS, promoting vascular smooth muscle growth and inflammation, vasoconstriction, and sodium reabsorption.[19] Angiotensin II works through cell surface receptors called AT_1 and AT_2, and current evidence suggests an alteration in the balance between these receptors may be involved in the programming effects of maternal diet on postnatal renal function and blood pressure control.[20]

Evidence relating suggests that the mechanisms and progression of the programmed phenotype may be sex-specific. Glucocorticoids are involved in the regulation of gene expression and the renin-angiotensin system, and are thought to mediate some of the effects of maternal diet on the developing organs of the fetus.[21] One study[20] observed that offspring of MLP rats had higher systolic blood

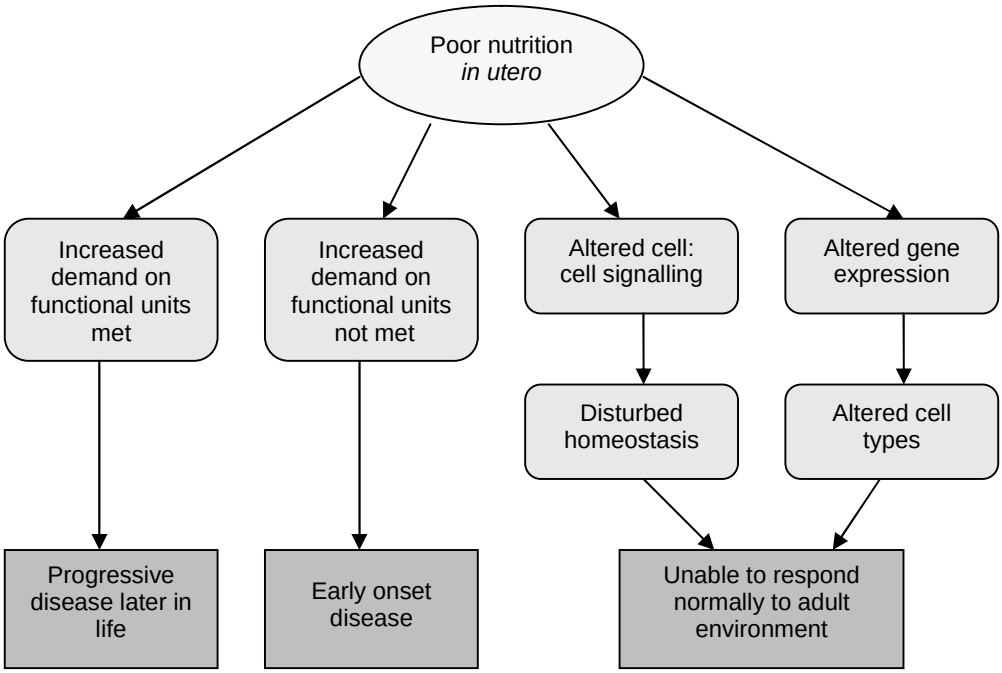

Figure 1: Potential ways in which tissue remodelling may occur.

pressure at 4 weeks of age than the control group. However, this appeared to be dependent on glucocorticoids in males but not females. AT_1 receptor mRNA expression was not significantly different between the MLP and control groups, whereas AT_2 receptor mRNA expression was lower in MLP-exposed females only. Other studies reported MLP male offspring had lower renin and angiotensin II levels compared to the control group, but these effects were not seen in females[16] unless protein was more severely restricted.[22] Together this work suggests that sex-specific mechanisms may be involved in the programming of hypertension or that females may be protected by a factor presumed to interact with sex steroids.

Whilst angiotensin II and AT_1/AT_2 receptors are well characterised regulators of blood pressure, emerging evidence suggests their involvement in the inflammatory process too. One common feature of renal disease is monocyte infiltration.[19] Angiotensin II has a direct role in the inflammatory response by activation of Nuclear Factor Kappa B (NF-κB) in the kidney via the AT_1 and AT_2 receptors, and increases degradation of IκB-α, which is an inhibitor of NF-κB.[19] AT_1 also regulates monocyte chemoattractant protein-1 (MCP-1) via the NF-κB pathway.[23]

Some studies have demonstrated interactions between NF-κB, IκB-α and MCP-1. Angiotensin II activates NFκB directly via AT_1 and AT_2 receptors and indirectly via its metabolite angiotensin IV, which resulted in increased MCP-1.[24] Vascular injury in mice resulted in increased NF-κB and MCP-1 and a decrease in IκB-α,[25] and in humans with diabetic nephropathy there was a strong positive correlation between NFκ-B and MCP-1 levels.[26]

At present many of the exact mechanisms of fetal programming that ultimately lead to inflammation and renal damage are not yet understood. Previous research has shown a link between renal damage and NF-κB, IκB-α, and MCP-1, and has considered how these proteins interact with one another. It is possible that these inflammatory pathways could be involved in the progression of programmed hypertension via the altered balance of angiotensin receptors which has been

observed in animal models. This study investigated this theory by testing three hypotheses:

1. Prenatal exposure to a low protein diet leads to decreased expression of IκB-α in the kidney of the offspring postnatally,
2. Prenatal exposure to a low protein diet leads to increased expression of MCP-1 in the kidney of the offspring postnatally, and
3. IkB-α and MCP-1 are negatively correlated: a decrease in IkB-α leads to an increase in MCP-1.

MATERIALS AND METHODS

Animals

Female Wistar rats were mated, and once pregnancy was confirmed were allocated to either the control diet (180 g casein/kg) or a low protein diet (90 g casein/kg). The rats were fed these diets until they gave birth at 22 days gestation, and then both groups of mothers were fed a standard laboratory chow diet while suckling the pups. The offspring were weaned onto a chow diet at 4 weeks of age, and then were killed at 8 weeks of age, and their kidneys were removed. The kidneys were frozen in liquid nitrogen and then stored at -80°C prior to use. There were 19 offspring in both the control and maternal low protein groups; ten male and nine female in each group.

Immunoblotting

The protein samples were heated at 90°C for 5 min, before 40 µg of each protein sample was run on a 10% SDS polyacrylamide gel as described by Laemmli.[27] The proteins were then transferred electrophoretically to a nitrocellulose membrane, as described by Towbin.[28] The Western blots were blocked with bovine serum albumin for 30 min, and then probed with a rabbit polyclonal IκB-α antibody (Santa Cruz) diluted 1:200 for 60 min. After washing, the blot was incubated with anti-rabbit IgG horseradish peroxidise conjugate diluted 1:5000 for 30 min. Protein bands were detected using enhanced chemiluminescence (ECL) and captured on ECL hyperfilm (GE Healthcare Life Sciences). A Fluor S Max multi-imager (Bio-Rad) produced digital images of the protein bands, and these were quantified using the Quantity One gel imager software (Bio-Rad).

Real-time PCR

Total RNA was isolated from frozen kidneys using the TRIzol method (Invitrogen). The RNA was treated with DNase (Promega) and subjected to phenol-choloroform extraction and ethanol precipitation. Five microgram of RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega). The amino acid sequences and accession numbers (M57441 for MCP-1 and NM_031144 for beta-actin) were entered into the software program Primer Express (Applied Biosystems), which designed the primers and probes, and these were purchased from Sigma Genosys. Real-time PCR was performed using a Roche Lightcycler 480. The PCR run included negative controls, and a relative standard curve. All MCP-1 samples were normalised to beta-actin.

Statistics

Results are shown as means ± standard error. Statistical analysis was performed using two-way ANOVA or Pearson's two-tailed test (SPSS version 15.0). Probability of <5% was considered statistically significant.

RESULTS AND DISCUSSION

Previous research has demonstrated the programming effects of a prenatal MLP diet on renal structure and function in the offspring. Langley-Evans *et al*.[13] originally demonstrated that a MLP diet impaired nephrogenesis and programmed hypertension in rats, and this finding has been reproduced in a variety of animal

models. As well as having effects on the structural development of the kidney, programming insults have been shown to affect long-term renal function and this may be secondary to the reduction in nephron number. Pressures on the filtration system due to reduced nephron number result in intra-renal compensations to try to maintain glomerular filtration rate at the expense of hypertension (both systemically, and locally in the glomeruli) which leads to progression of renal disease and increasing loss of function [29].

IκB-α

NF-κB has been shown in several studies to increase in response to renal injury and inflammation as a result of a decrease in its inhibitor IκB-α.[25, 26] Deregulation of the NF-κB pathways has therefore been proposed as a potential mechanism for the progression of inflammatory renal disease.[30] This mechanism could also underlie the programming effects of a prenatal MLP diet on long-term renal morphology and function, because it is already known that angiotensin II,[23] angiotensin III[31] and angiotensin IV,[24] all activate NF-κB via various AT receptors, which are affected by low protein diets prenatally. We therefore hypothesised that a prenatal MLP diet would lead to a decrease in IκB-α protein expression. This hypothesis was rejected, as although the means were numerically lower in the MLP groups compared to the control groups (figure 2), this was not statistically significant and so there was no evidence to suggest that IκB-α levels were lower in the rats exposed to the MLP diet prenatally. However, the validity of the data is in question due to inconsistencies in the western blot, as standards spread across the gel showed unacceptable levels of variation. Therefore the results must be viewed with some caution.

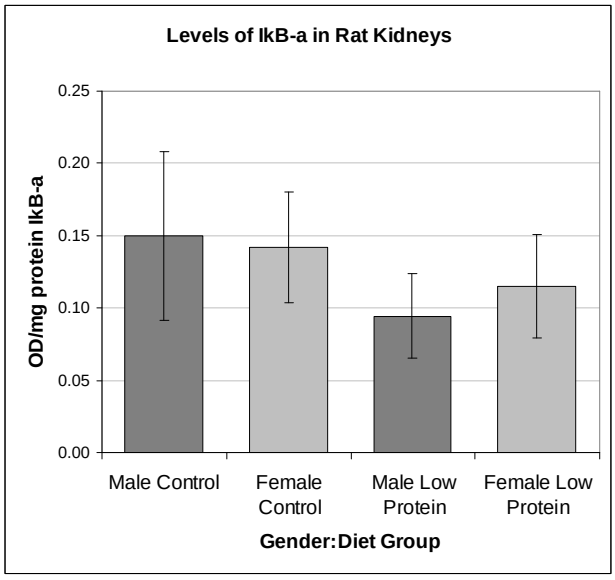

Figure 2: Levels of IκB-α protein in the kidneys of 8 week–old male and female rats who were exposed to either a standard laboratory diet (control) or a low protein diet (restricted to 50% of normal protein intake) during pregnancy. Data are shown as means ± s.e.m. n = 10 (male control and MLP groups) and 9 (female control and MLP groups). There were no significant differences. OD – optical density.

Some studies offer potential explanations as to why IκB-α may not rise in response to programming insults that alter renal function or structure. In normal healthy cells, NF-κB acts as a transcription factor for IκB-α,[32]. Therefore, if NF-κB rose initially due to an early inflammatory response then it should self-regulate itself, resulting in increased expression of IκB-α which would then act to dampen

down levels of NF-κB. It is possible that an early initial inflammatory response in MLP offspring would have been followed by a compensatory increase in IκB-α levels. The assessment of renal samples at 8 weeks of age only may have been too late to have observed the initial changes in protein expression. Alternatively, this time-point may have been too early in the advancement of renal disease to see upregulation of inflammatory pathways. Analysis of samples from multiple time-points during postnatal life would help to identify the critical ages for inflammation.

Also it could be that any programming on the NF-κB pathway is independent of IκB-α. NF-κB has several isoforms and not all are activated via the classical pathway involving inhibition by IκB-α. The alternative pathway is independent of IκB-α and involves a particular isoform of NF-κB which is not thought to be associated with IκB-α in the cytosol, but has a different inhibitory molecule.[30] Activation of this pathway could mean that levels of IκB-α remain unchanged despite the upregulation of NF-κB and subsequent activation of the inflammatory pathway. Measurement of the expression of other proteins in this pathway, including NF-κB itself, would help to resolve this issue.

MCP-1

MCP-1 is a pro-inflammatory molecule that has consistently be shown to be raised in response to inflammation,[25, 26, 31] and its transcription may be mediated by NFκB.[33] It was hypothesised that a decrease in IκB-α protein expression in response to a MLP diet would lead to activation of the NFκ-B pathway, which would in turn lead to increased transcription of MCP-1. We therefore hypothesised that a prenatal MLP diet would lead to an increase in MCP-1 mRNA expression. This hypothesis was rejected, as there were no significant differences in MCP-1 expression between the MLP and control groups, nor between males and females. A diet:sex interaction indicated a reduction in MCP-1 mRNA expression in the male MLP group only. However, this didn't quite reach statistical significance (P=0.053). Given the role of MCP-1 in the progression of renal inflammation, this trend may be worth further investigation. Variation in the data was, however, quite high (mean = 0.69; 95% confidence intervals 0.31 to 1.08) and so there was no firm evidence to suggest that MCP-1 mRNA expression was decreased in rats exposed to the MLP diet prenatally. This is perhaps not surprising, given that no change in protein expression was observed in the up-stream protein, IκB-α.

In summary, this data suggests that, although activation of the NF- κB and MCP-1 pathways are raised in response to renal inflammation[25], this particular pathway is not involved in the progression of programmed renal injury and dysfunction in this model. Instead, other inflammatory pathways may be involved. Alternatively, the stage of advancement of renal disease in these 8-week old MLP offspring may have been too early. Analysis of renal samples from older offspring may be more suited to investigating the mechanisms of progression of programmed renal dysfunction. They may not, however, be suited to investigation of the original mechanisms of programmed hypertension, which must originate early in life. Analysis of samples from multiple time-points during postnatal life is therefore recommended.

Effect of gender

In humans men are more likely to develop hypertension than women.[34] Previous rat models appear to suggest that such gender differences exist in programmed renal disease and hypertension also. This study therefore considered whether the mechanisms of renal dysfunction in MLP offspring were sex-specific.

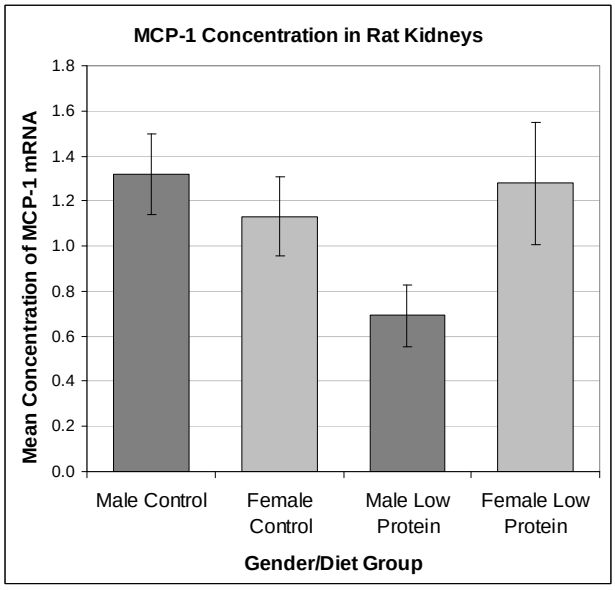

Figure 3: Levels of MCP-1 mRNA in the kidneys of 8 week-old male and female rats who were exposed to either a standard laboratory diet (control) or a low protein diet (restricted to 50% of normal protein intake) during pregnancy, Data are shown as means ± s.e.m. n = 10 (male control and MLP groups) and 9 (female control and MLP groups) There were no significant differences. MCP-1 – monocyte chemoattractant protein-1.

In this study, males tended to have slightly higher means for IκB-α and MCP-1 expression than females in the control groups but lower means after exposure to the MLP diet. However, these differences were not statistically significant and so there was no evidence to suggest that IκB-α or MCP-1 levels were different in males compared to females.

Cell types

One limitation of this experiment was that whole kidney samples were used, which doesn't allow for individual cell types to be considered. If expression was only altered in one cell type, a dilution effect may mean that the expression level in a whole kidney sample was not altered. Alternatively IκB-α or MCP-1 expression may have been differentially affected in different cell types, thus showing no change overall.

Studies have reported reduced nephron number in MLP-exposed offspring.[13, 16] If there are fewer nephrons (particularly if they are immature and therefore containing fewer functional cells) then there will be a smaller number of the cell types that occur in the nephrons compared to the kidney as a whole. One study[26] found a particularly high level of MCP-1 in tubular epithelial cells compared to other cell types. A shift in the balance of cell types could therefore alter the apparent expression of a particular gene. This could bias findings towards the null or alternative hypothesis, depending on the direction of change. Further studies using immunohistochemical methods would overcome this potential issue.

Correlation between MCP-1 and IκB-α

Previous studies[24, 25] have demonstrated that a decrease in IκB-α leads to increased translocation of NFκB to the cell nuclei, where it acts as a transcription factor for MCP-1. Conversely, increased expression of IκB-α reduces translocation NF-κB to the nucleus and thus reduces activation of MCP-1. Studies[26, 31] have also found that increased NF-κB was associated with increased MCP-1 in renal cells,

potentially via a reduced inhibitory effect of IκB-α. We therefore hypothesised that IkB-α and MCP-1 would be negatively correlated: a decrease in IkB-α leads to an increase in MCP-1.

Surprisingly, this hypothesis is rejected, as the Pearsons correlation did not show that IκB-α and MCP-1 were negatively correlated. In fact it showed that the correlation was positive (figure 4), i.e. that when IκB-α levels were lower, MCP-1 was lower too. However, although this statistically significant ($P < 0.01$) the correlation was very weak ($r^2 = 0.21$) and doubts about the validity of the IκB-α data – as previously explained – cast doubt on the reliability of the correlation.

It is possible, however, that there genuinely is no correlation between IκB-α and MCP-1 in these samples. IκB-α only inhibits the classical pathway of NF-κB and it is known that alternative pathways exist. NF-κB has several isoforms and some are activated via alternative pathways which do not involve inhibition by IκB-α. If MCP-1 is activated by NF-κB via the alternative pathway then a correlation with IκB-α would not be expected. It would be interesting to investigate levels of alternative inhibitory proteins to identify the inhibitory molecule in these samples. MCP-1 is also regulated by other pathways, and so a lack of correlation, with IκB-α may indicate higher activity of other upstream molecules in this tissue type.

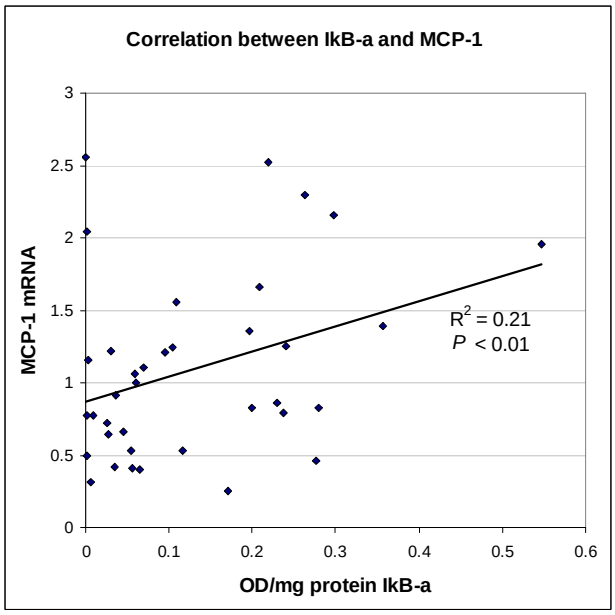

Figure 4: The Correlation between IκB-α and MCP-1. Pearson Correlation of $R^2 = 0.21$ calculated using SPSS software. Correlation is statistically significant: $P < 0.01$. N=36. OD – optical density; MCP-1 – monocyte chemoattractant protein-1.

Further research

Research into the exact mechanisms of the interaction between IκB-α, NFκ-B and MCP-1, along with the effect of prenatal diet upon the long-term levels of expression of them will lead to a greater understanding of the process of renal damage through inflammation. Ultimately this may have clinical applications in the treatment and prevention of progressive renal disease, as it may be possible to manipulate levels of IκB-α in individuals with vulnerability to renal disease in order to inhibit the transcription of inflammatory genes by NFκ-B.

It would be useful to measure NF-κB as well as IκB-α and MCP-1, and by using PCR techniques it would be possible to select primers and probes based on the regions that are unique to the different NF-κB isoforms. This would enable the effects of the classical and alternative pathways to be distinguished between. Further experiments using individual cell types would allow identification of specific cells within the kidney that are involved in the processes. Individual cell types could be studied using immunohistochemical techniques. Repeating the western blotting experiment would allow for the IκB-α data to be validated. Given the lack of evidence for involvement of NF-κB and MCP-1 in the progression of renal injury in MLP offspring, it may be more appropriate to use array technology to identify alternative target systems for analysis.

CONCLUSIONS

This study did not show IκB-α or MCP-1 to be significantly affected by exposure to a MLP diet and all three hypotheses were rejected. In addition, no significant effects of gender were seen. Although there was a statistically significant positive correlation between IκB-α and MCP-1, it was weak and this was an unreliable association as this current study has not allowed this hypothesis to be sufficiently tested due to the experimental problems with IκB-α.

Risk of developing disease is not dependent on one factor alone, but depends on a combination of genetics, programming, lifestyle and other environmental influences. Using animal models allows studies to be done that try to control for all influences apart from the dietary changes. The search for better understanding of programming mechanisms is important as in the future it may lead to methods being developed for the early detection of individuals as risk of renal disease and hypertension, or to medical interventions to help those diagnosed with them.

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Author Profile

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