

## IV. STUDIES ON MOLECULAR BIOLOGY

### 1. ROLE OF DIETARY N-3 PUFA OR TRANS FATTY ACIDS IN FOETAL PROGRAMMING OF INSULIN RESISTANCE IN RATS: BIOCHEMICAL AND MOLECULAR MECHANISMS

During development of fetus there are critical and restricted periods which are often coincident with periods of rapid cell division during which individual tissues/organs differentiate and mature. According to theory of 'fetal origins' of chronic adult disease, nutritional deprivations/imbalance *in utero* alters physiology and metabolism of developing tissues/organs postnatally and increases the risk of chronic adult diseases. Long chain polyunsaturated fatty acids (LC PUFA) are integral components of cell membrane and are important determinants of fetal growth and development. Docosahexaenoic acid is one of the abundant fatty acid present in brain, other neural tissues and the retina.

In India, low birth weight has been a major problem for quite a long period and in recent years there has been a steep increase in insulin resistance and its sequelae leading to diet-related chronic diseases. The major target tissues of insulin action are skeletal muscle and adipose tissue. Recent evidence indicates that although adipocyte glucose uptake is small, it plays a significant role in insulin resistance. Adipocytes release free fatty acids, leptin and adipocytokines. LCPUFA content of skeletal muscle and adipocyte plasma membrane have been correlated with insulin sensitivity. Further, PUFA regulate the expression of genes involved in carbohydrate and lipid metabolism by binding to nuclear receptors known as PPARs. n-6 and n-3 PUFA have distinct biological effects and therefore both absolute levels and n-6/n-3 ratio are important for various physiological functions. Our recent studies have shown that keeping total PUFA in the diet constant and increasing  $\alpha$ -linolenic acid (18:3n-3, vegetable oil) or LCn-3 PUFA (fish oil) increased insulin sensitivity in target tissues in sucrose induced insulin resistant rats. On the other hand Trans fatty acids (TFA) from Indian vanaspati increased insulin resistance. The present study is designed to evaluate whether dietary fatty acids (TFA or 18:3n-3 or LCn-3 PUFA) affect foetal programming of insulin resistance.

#### Hypothesis

Low maternal n-3 PUFA nutritional status or high TFA intake may predispose the pups to insulin resistance in adult life.

#### Aims and Objectives

To investigate the effects of increasing dietary n-3 PUFA (decreasing n-6/n-3 ratio) or TFA before conception, during pregnancy and lactation predisposes to insulin resistance in adult rats.

#### Methodology

Study design: WNIN female weanling rats (n=32) were divided into 4 groups and fed cereal pulse based diets containing 10% fat. The total dietary PUFA was ~ 10en% but the n-3 PUFA levels varied and were as follows:

Group I	: ~ 0.2en% 18:3n-3 (n-6/n-3 ratio ~30)
Group II	: 2.5 en% 18:3 n-3 (n-6/n-3 ratio 2)
Group III	: 0.2 en% 18:3n-3 + 0.5 en% LC n-3 PUFA (n-6/n-3 ratio 10)
Group IV	: Vanaspathi, which furnished 1en% TFA and 10en% total PUFA

WNIN male weanling rats (n=16) were fed stock colony diet.

The above diets were fed for 90 days. After 90 days of feeding, blood was collected from rat in-groups I to IV. After 5 days of bleeding (95 days feeding) rats in groups I to IV were mated with males fed stock colony diet (2 females + 1 male). The pregnant rats were continued on the respective diets throughout the period of pregnancy and lactation. The pups were weaned at 21 days. Half of the pups in each group were continued on the respective diets for 90 days whereas the other half were switched to diets providing n-6/n-3 ratio = 30. At the end of 45 and 90 days, blood was collected after 18hr fasting. Plasma glucose, insulin, total and HDL cholesterol and triglycerides were estimated. At the end of 90 days following blood collection, animals were sacrificed; liver, pancreas, epididymal and retroperitoneal fat pads and skeletal muscle (diaphragm) were dissected out. Adipocytes were isolated from epididymal fat pads and the following parameters were estimated.

1. Plasma free fatty acids
2. Adipocyte glucose transport
3. Adipocyte lipolysis and antilipolytic effect of insulin
4. Liver TBARS
5. Liver antioxidant enzyme activities (Catalase, GSH-px and SOD)

## Results

1. The fasting plasma free fatty acid levels were comparable between the groups.
2. Adipocyte lipolysis, insulin mediated antilipolysis and glucose transport were similar among different groups.
3. Compared with offspring on the control diet (n-6/n-3 ratio of 30), offspring on n-3 PUFA diet which provides n-6/n-3 ratio of 2 (vegetable oil) or 10 (fish oil) had significantly higher activities of liver catalase and glutathione peroxidase. There were no significant differences in liver TBARS among the groups.

## 2. IS RESISTIN A PRO-INFLAMMATORY MOLECULE ?

Resistin, an adipocytokine, is found to be elevated in genetic and diet-induced mouse models of obesity. This protein is expressed exclusively in adipocytes in rodents. However, in humans it is secreted mainly by macrophages. Reduced insulin-stimulated glucose uptake in mice that were administered recombinant resistin and reversal of the same by anti-resistin IgG indicates a role for this molecule in the development of insulin resistance. Plasma resistin levels are elevated in individuals with type 2 diabetes mellitus. Diabetics with insulin resistance and reduced glucose uptake suffer from cytokine-induced acute-phase inflammation.

Inflammation in relation to obesity and insulin resistance has often been correlated with the over-production of the pro-inflammatory cytokine TNF- $\alpha$ . TNF- $\alpha$  is one of the major inflammatory mediators secreted by macrophages upon stimulation with pro-inflammatory molecules. TNF- $\alpha$  is expressed

constitutively at a low level in monocytic cells. This basal level expression has been shown to be altered by the inflammatory milieu leading to either its upregulation or downregulation. In monocytes, the nuclear factor NF- $\kappa$ B has been established as an important transcription factor in the expression of cytokine genes including TNF- $\alpha$ . It is interesting to elucidate the immunomodulatory functions of human resistin. The effect of resistin on the production of pro-inflammatory cytokines in macrophages was demonstrated. The results showed that human resistin acts as a pro-inflammatory molecule, stimulating the synthesis and secretion of TNF- $\alpha$  and IL-12 and this involves the activation of NF- $\kappa$ B transcription factor. Given a direct positive correlation between TNF- $\alpha$  (and/or) inflammation and resistin expression we investigated whether resistin itself directly activates macrophages for the production of TNF- $\alpha$ . These studies were done in *in vitro* maintained U937 cell line as a source of human monocyte/macrophage lineage. Cells were seeded in 35 mm dish and were differentiated in the presence of 25 nM PMA for 24 h. These differentiated macrophages were then stimulated by the addition of hResistin (30 g/ml) for 48 h. As a positive control, macrophages were also stimulated with bacterial LPS. TNF- $\alpha$  level was assayed by EIA in these cells. Incubation of cells with hResistin protein results in induction of TNF- $\alpha$  (Figure 13A & 13B). As compared to unstimulated U937 macrophages, the stimulation of TNF- $\alpha$  by hResistin was about two fold. Similar experiments were performed using mouse RAW 264.7 macrophages. These results, therefore, conclusively demonstrate that addition of hResistin to macrophages induces TNF- $\alpha$  secretion in both human as well as in mouse macrophages.

It is known that macrophage activation during inflammation also leads to increased production of other cytokines such as IL-12. The level of IL-12 in the culture supernatant was measured by EIA at 48 h post-stimulation (Figure 14A & 14B). These results categorically demonstrate the unique property of hResistin to act as an inducer of pro-inflammatory cytokines in both murine and human macrophages. The stimulation of TNF- $\alpha$  was also analysed at the transcriptional level. RAW 264.7 cells were stimulated with human resistin and the mRNA levels of TNF- $\alpha$  were measured by quantitative RT-PCR. Stimulation of mouse macrophages with recombinant resistin lead to the increased transcription of TNF- $\alpha$  gene. These results demonstrate that treatment of RAW 264.7 cells by hResistin also leads to an increase in *de novo* synthesis of TNF- $\alpha$  mRNA. Since human resistin is a secretory protein, there is always a possibility that recombinant resistin purified from a bacterial expression system under denaturing conditions might not have refolded into its native form. To further address the issue of protein folding, we cloned human resistin gene into mammalian expression vector pCDNA 3.1. The recombinant plasmid pCDNA-AShRes carrying resistin gene under the transcriptional control of CMV promoter was used for transient transfection (Figure 15A). The expression of human resistin in transfected RAW 264.7 cells was confirmed by RT-PCR. TNF- $\alpha$  mRNA expression was analysed as a function of time in the transiently transfected cells by RT-PCR (Figure 15B).

In order to evaluate the mechanism of action of resistin in eliciting a pro-inflammatory response in macrophages, the role of NF- $\kappa$ B family of transcription factors, which are known to be involved in regulating inflammatory responses, was investigated. This was carried out initially by assaying for nuclear translocation of NF- $\kappa$ B using electrophoretic mobility shift assay in macrophages exposed to resistin. U937 cells were stimulated with increasing concentrations of purified recombinant human resistin for four hours. It can be clearly seen that nuclear extract derived from U937 cells treated with hResistin shows increased DNA-Protein complex corresponding to the p50 and p65 heterodimer (NF- $\kappa$ B) (Figure 16). Furthermore, this complex is generated in a dose dependent manner. These results provide the first line of evidence that exogenous resistin stimulates the p50/p65 heterocomplex formation and its localization into the nucleus. The induction of pro-inflammatory cytokines in macrophages by the addition of resistin therefore appears to be mediated through the NF- $\kappa$ B transcription factor. The NF- $\kappa$ B activity in macrophages was blocked by using a dominant negative I $\kappa$ B $\alpha$  plasmid. U937 cells were transfected with dominant negative I $\kappa$ B $\alpha$  plasmid lacking Ser<sup>32/36</sup> residue. In the absence of the serine residue, phosphorylation of I $\kappa$ B $\alpha$  is inhibited and consequently, the translocation of NF- $\kappa$ B into

the nucleus is blocked. U937 cells were then allowed to stabilize for 12 hours followed by stimulation with purified human resistin. TNF- $\alpha$  levels were checked by ELISA after 24 hours and compared with macrophages transfected with control vector (pBSK from Stratagene). Cells transfected with dominant negative I $\kappa$ B $\alpha$  plasmid secrete significantly lower levels of TNF- $\alpha$  in response to human resistin as compared to cells transfected with pBSK plasmid alone (Figure 17A). This therefore clearly suggests that in the absence of functional NF- $\kappa$ B the pro-inflammatory action of resistin is greatly reduced. To provide additional evidence on the role of NF- $\kappa$ B, PDTC, an inhibitor of I $\kappa$ B $\alpha$ , was used. PDTC blocks the dissociation of NF- $\kappa$ B/ I $\kappa$ B $\alpha$  complex thereby inhibiting the translocation of NF- $\kappa$ B (Figure 17B). Although, resistin is believed to be a link between type 2 diabetes and obesity, it is tempting to suggest that the overproduction of resistin during obesity actually influences type 2 diabetes through activation of TNF- $\alpha$ . TNF- $\alpha$  is not only a major candidate for induction of inflammation, as observed frequently in diabetic patients, it has also been associated with increased insulin resistance in obesity.

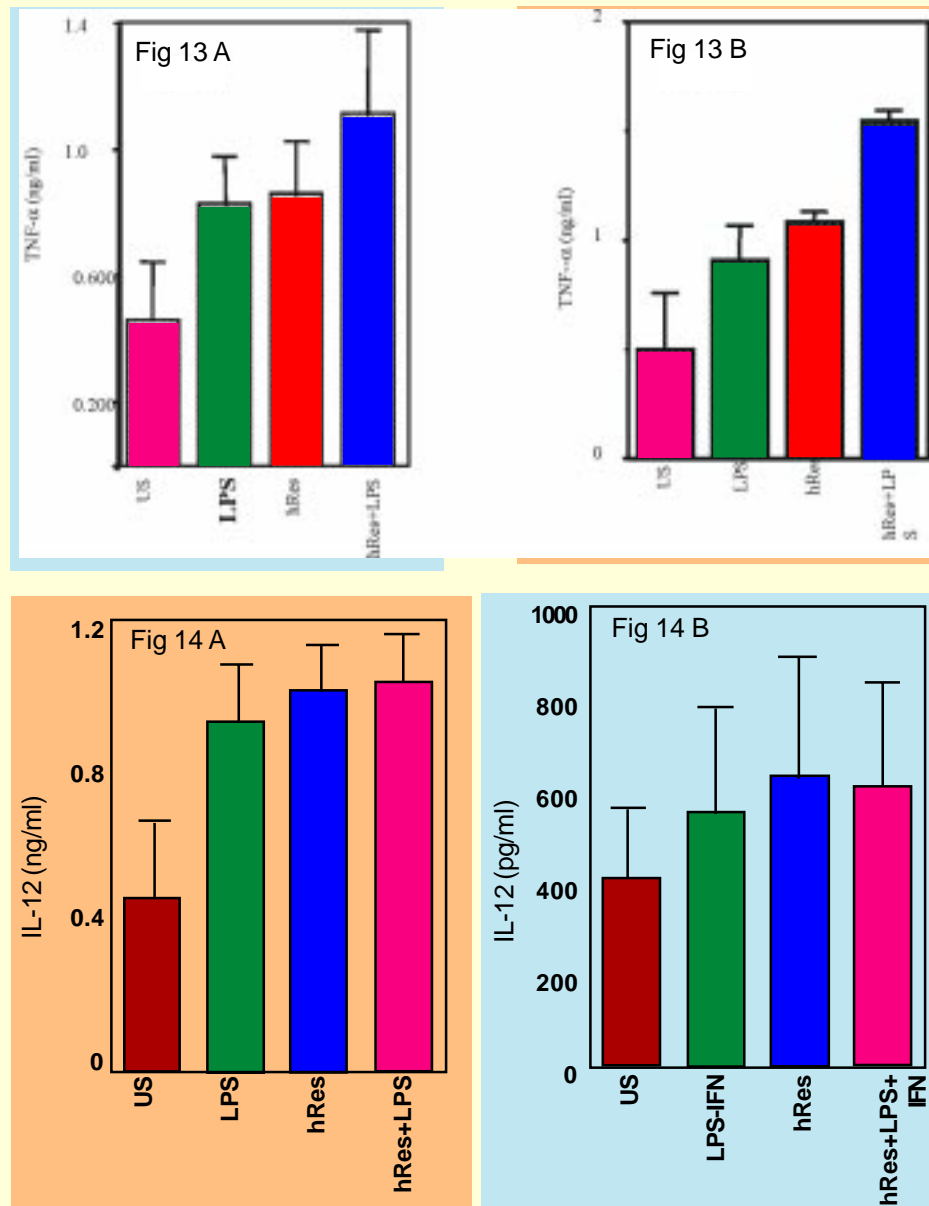


Fig. 15 A

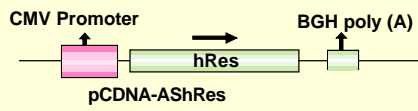


Fig. 15B

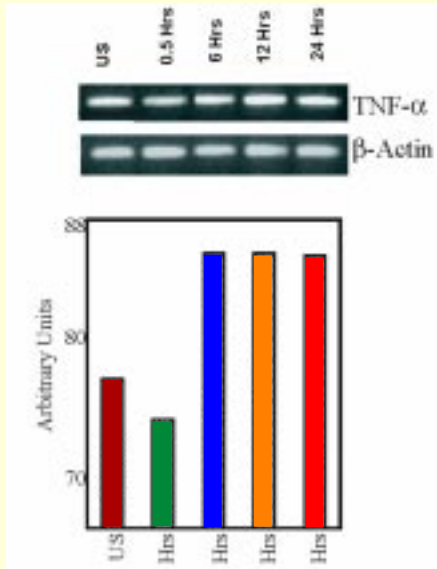


Fig. 16

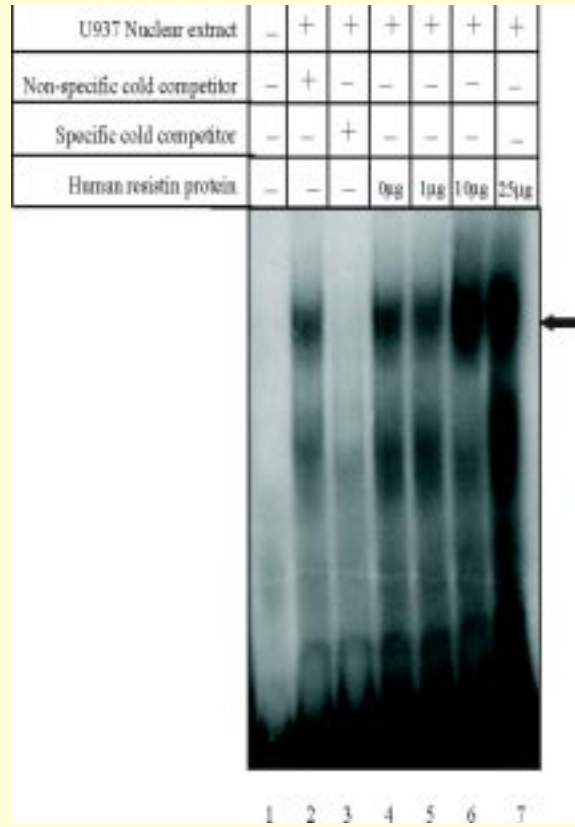


Fig. 17 A

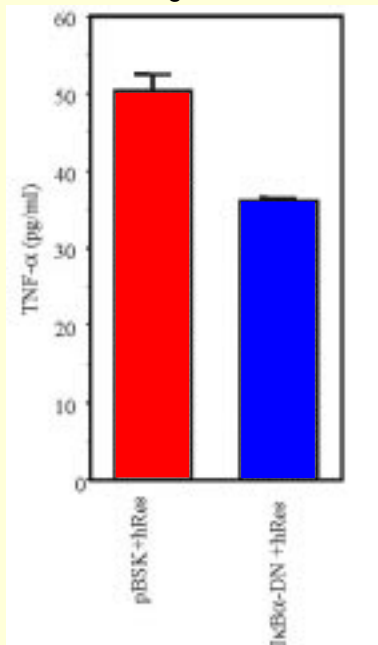


Fig. 17B

