TITLE: A key role for interferon regulatory factors in mediating early-life metabolic defects in male offspring of maternal protein restricted rats

Running Title: IRFs and Programmed Obesity

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Abstract

An adverse intra-uterine environment, induced by maternal consumption of diets high in saturated fat or low in protein have been implicated as a potential trigger for development of metabolic disease in later life. However, the underlying mechanisms responsible for this programming of obesity have yet to be described. Recent studies have demonstrated that interferon regulatory factors 3 (IRF3) and 4 (IRF4) function to repress adipogenesis. We investigated whether impaired IRF3 and IRF4 function may predispose to development of metabolic disease in a model of programmed obesity. Changes in IRF3 and IRF4 levels, adipogenic gene expression and adiponectin signalling were measured in white adipose tissue from programmed male offspring of rat dams fed a low-protein diet (MLP), which are predisposed to obesity. 3T3L1 adipocytes were used to determine novel regulatory mechanisms governing IRF expression.

IRF3 and IRF4 levels were suppressed in MLP rats, together with raised lipogenic and adipogenic gene expression. Adiponectin and adiponectin receptor 1 and 2 mRNA levels were reduced in MLP rats, along with levels of PPAR and activity of AMPK, two downstream targets of adiponectin. Further studies determined that both IRF3 and IRF4 are induced by adiponectin, with adiponectin-AMPK and adiponectin-PPAR signalling regulating IRF3 and IRF4, respectively.

We demonstrate that impaired ability to repress adipogenesis and lipogenesis, through dysregulated adiponectin-PPAR -AMPK-IRF signalling, may play a causal role in predisposing MLP offspring to development of obesity and metabolic disease in later life.

Introduction

An adverse intra-uterine environment has also been implicated as a potential trigger for development of obesity in later life (1-3). Examples of poor maternal nutrition include maternal consumption of diets high in saturated fat or a diet low in protein diet (1, 4). Maternal consumption of a low protein (Maternal Low Protein; MLP) diet results in offspring with a low birth weight in
humans and animal models (4). Moreover, there exists a strong positive correlation between low birth-weight and the development in later life of obesity, insulin resistance and type 2 diabetes (5). Epidemiological and experimental evidence suggests that an imbalance between prenatal (e.g. maternal protein restriction) and post-natal (e.g. excess dietary carbohydrate) nutrition can predispose to development of obesity (1). In agreement, children exposed to growth-restricted conditions during development exhibit increased adiposity in childhood and later life (6), whilst the offspring of rats fed a low-protein diet display a greater predisposition to develop obesity and related metabolic disorders in response to high-fat feeding compared to controls (1, 5). This phenomenon, termed the ‘thrifty phenotype’, suggests that fetal malnutrition may lead to physiological changes that programme the fetus for survival in what it predicts to be a poor nutritional environment. This evolutionary programming is less advantageous when food is abundant, potentially predisposing to obesity (5). However, the underlying mechanisms responsible for this programming of obesity have yet to be described.

Recent studies have identified the immunoregulatory proteins, interferon regulatory factors 3 and 4 (IRF3 and IRF4) as key regulators of adipocyte function (7, 8). IRF4 increases during adipocyte differentiation and functions to repress adipogenesis and lipogenesis and induce lipolysis in white adipose tissue. IRF4 expression is repressed by insulin, and consequently IRF4 levels are reduced in insulin resistant mouse models, as well as being elevated during fasting and reduced during refeeding. Adipocytes lacking IRF4 display increased expression of lipogenic genes including fatty acid synthase (FAS; gene code: Fasn) and sterol regulatory-element binding-protein 1 (SREBP1). Moreover, IRF4 knockout mice have larger adipocytes, are obese and develop T2DM. Less is known about the regulation of IRF3 (7, 8).

To further examine the early life mechanistic changes following exposure to an adverse intra-uterine environment responsible for onset of obesity and metabolic syndrome in later-life, we examined the role of IRF3 and IRF4 in the MLP model of fetal programming. Further to this, we investigated novel mechanisms involved in regulation of IRF3 and IRF4 in adipocytes and white adipose tissue.

Materials and Methods
Experimental Animals

Studies were conducted in adherence to the regulations of the U.K. Animal Scientific Procedures Act (1986), under project license PPL 70/7276. All animals were maintained on a 12 h light/12 h dark cycle (light from 07:00). All experiments were conducted in the fed state, unless stated otherwise. Animals were sacrificed under CO$_2$ asphyxiation.

Maternal Low Protein Model

Pregnant female Wistar rats (250 – 300 g; Charles River, Kent, UK) were randomly assigned to either control (CON; 20% protein) or an isocaloric maternal low protein (MLP; 8%) diets (Hope Farms BV, Woerden, Netherlands) and maintained on the respective diet throughout pregnancy and lactation (12 rats/group). Pregnant dams fed the low-protein diet did not exhibit any differences in caloric intake compared to controls. Despite this, MLP offspring weighed significantly less at 3 days of age than CON offspring (28%; P<0.05). Male offspring were weaned at 24 days and maintained on standard rodent diet thereafter. CON and MLP offspring were killed at 8 weeks and visceral WAT depots were snap-frozen for analysis, whilst blood was collected for measurement of glucose and insulin.

PPARα$^{-/-}$ Mice

Male PPARα$^{-/-}$ mice, bred onto a SV/129 genetic background, were provided by Drs. J. Peters and F.J. Gonzalez (National Institutes of Health, Bethesda, MD). Wild-type SV/129 mice (from Charles River, UK) were used as controls.

Metformin treatment

Eight-week old male C57Bl/6 mice (Charles River, Kent, UK) maintained on standard rodent diet were administered metformin (250 mg/kg/day) or an equal volume of water daily for seven days by oral gavage.

3T3L1 Culture and Differentiation
3T3-L1 preadipocytes were cultured (37°C; 5% CO₂) in Dulbecco’s Modified Eagle Media (DMEM; 25 mM glucose) supplemented with 10% bovine calf serum (BCS), (both Sigma Aldrich, Poole, UK) until confluent. Two days post-confluence (Differentiation day 0), to induce differentiation media was changed and replaced with DMEM/10% foetal calf serum (FCS) supplemented with insulin (1 µg/ml), dexamethasone (0.25 mM) and isobutyl-1-methylxanthine IBMX (0.5 mM). After 2 days (differentiation day 2), media was replaced with DMEM/10% FCS supplemented with insulin (1 µg/ml). After 4 days (differentiation day 4) media was replaced with DMEM/10% FCS. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin (both Sigma Aldrich, Poole, UK). Differentiation of pre-adipocytes to adipocytes was confirmed by measurement of PPARγ gene expression. Treatments, carried out on differentiation day 8, were as follows; adiponectin (30 µg/ml), WY-14643 (10 µM) for 24 h. All treatments were carried out in DMEM containing 10% FCS.

Immunoblotting

Solubilized protein samples (10 µg; measured and equalized in each fraction using an RC-DC BioRad system (BioRad, UK)) were separated by SDS-PAGE and transferred onto PDVF membrane (GE Healthcare, Amersham, UK). Blots were blocked with 5% (w/v) bovine serum albumin (BSA) or 5% milk in Tris-buffered saline/0.1% Tween-20 (TBS/T) solution and then incubated overnight in primary antibody. Antibodies used in this study are anti-phospho(Thr¹⁷²)AMPK, anti-AMPK, anti-phospho(Ser¹⁷¹)ACC and anti-ACC (Cell Signaling Technologies, MA, USA). Detection of bands was achieved by using the chemiluminescence substrate SuperSignal West Pico (Pierce, Rockford, IL, USA). Reference protein measurements were made with mouse monoclonal anti-β-actin (clone AC-15; Sigma, Poole, UK) primary antibody in a 3% (w/v) milk-TBS/T solution, at 4°C.

Quantitative RT-PCR

Gene expression was measured by qRT-PCR, using Taqman® or Sybr green® methodology. Gene expression was determined by ΔΔCt methodology, normalised against 18S ribosomal RNA (Applied
Biosystems, UK). Changes in gene expression are represented as fold change relative to one, where control equals one. All primer and probes are from Eurogentec, UK. A list of primers can be found in supplemental table 1 (supporting data).

**Serum Insulin and Glucose**

Insulin was analysed by ELISA (Mercodia, Uppsala, Sweden) using rat insulin as standard. Plasma glucose was measured by a glucose oxidase/peroxidase method (Roche Diagnostics, Lewes, U.K.).

**Statistical analysis**

Results are expressed as mean ± SEM. Statistical comparisons were obtained using GraphPad (GraphPad Software, CA, USA). Statistical differences were calculated using an unpaired t-test.

**Results**

**Metabolic data for MLP offspring**

Pregnant dams fed a low-protein diet did not exhibit any differences in food intake compared to controls (data not shown). Despite this MLP offspring weighed significantly less at 3 days of age than control litter mates (28%; \( p<0.05 \)). After 8-weeks consumption of standard rodent diet, MLP offspring displayed modest but insignificant increases in fasting insulin (16.0 ± 3.2 µU/ml) compared to controls (14.4 ± 1.3 µU/ml) \( (p=0.65) \). Additionally, fasting plasma glucose levels were mildly but not significantly increased in MLP offspring (4.36 ± 0.13 mmol/l) compared to control (4.11 ± 0.21 mmol/l) \( (p=0.33) \), whilst glucose area under the curve (AUC) analysis for glucose tolerance tests resulted in a modest increased AUC values for MLP (9.76 ± 1.29 mmol/L/min) compared to control (7.45 ± 0.79 mmol/L/min) \( (p=0.16) \) (Table 1).

**MLP offspring display altered adipogenic and lipogenic gene expression**
Whilst no significant changes in metabolic data were observed at this early stage in the MLP rats, it is possible that mechanistic changes may occur during this early-life period which would predispose to later development of metabolic disease. Therefore, by focussing on altered lipogenesis and adipogenesis in white adipose tissue, we next attempted to identify early-life metabolic changes which would likely lead to onset of the established later-life metabolic defects induced by MLP. IRF3 and IRF4 are recently identified repressors of adipogenesis and lipogenesis (7, 8). Gene expression of IRF3 and IRF4 was markedly reduced in white adipose tissue of MLP rats compared to controls (Figure 1a and 1b). These changes occurred in parallel with increased mRNA expression of SREBP1c, elevated mRNA and protein levels of FASN as well as increased ACC protein (Figure 1c – e), consistent with a role for IRF4 in repressing transcription of these genes. This data implies that early life changes in IRF3 and IRF4 occur in MLP rats and may contribute to increased risk of development of obesity and metabolic syndrome in later life.

IRF3 and IRF4 suppression is associated with impaired adiponectin signalling.

Previous studies have demonstrated that insulin exerts regulatory control of IRF4, whilst less is known about IRF3 regulation in the context of obesity and metabolic syndrome. Insulin was reported to repress IRF4 expression in vitro in adipocytes and in vivo under fed conditions, and in obese, hyperinsulinaemic rodent models (7). However, despite marked reductions in IRF3 and IRF4 expression, we did not observe significant changes in serum insulin levels in our models (Table 1). This suggested the existence of an alternative regulatory mechanism controlling IRF4, and potentially IRF3, in white adipose tissue. Adipokines, such as adiponectin, are bioactive peptides secreted from adipose tissue which exert metabolic control over a number of insulin sensitive tissue, including, in an autocrine manner, adipose tissue. We next investigated potential alterations in adiponectin signalling in MLP rats, with aim of determining alternative mechanisms of IRF3 and IRF4 regulation. Remarkably, we found that mRNA levels of adiponectin (ADIPOq; Figure 2a) and adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2) were dramatically suppressed in MLP rats by ≈90%
relative to control (Figure 2b – c), suggesting that impaired adiponectin signalling may lead to decreased IRF3 and IRF4 expression. To investigate this further, we used the 3T3L1 adipocyte cell line. Consistent with impaired adiponectin signalling and reduced IRF expression in the white adipose tissue of MLP rats, incubation of 3T3L1 adipocytes with adiponectin led to increased mRNA levels of IRF3 and IRF4 (Figure 2d – e). This suggests that, in addition to insulin, adiponectin provides an additional level of regulatory control over IRF3 and IRF4 in white adipose tissue.

Adiponectin regulates IRF3 and IRF4 through AMPK and PPARα

Adiponectin, via signalling through adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2) has been reported to exert metabolic effects through activation of the energy sensing enzyme AMP-activated protein kinase (AMPK) and the nuclear receptor peroxisome-proliferator activated receptor α (PPARα) (9). Both AMPK and PPARα play important roles in regulating lipogenesis and fatty acid oxidation (9-11). Consistent with observed reductions in adiponectin and AdipoR1 expression, MLP rats also displayed decreased protein levels of phospho(Thr^{172})-AMPK (Figure 3a). Since phosphorylation at Thr^{172} leads to AMPK activation, this observation suggests reduced AMPK activity in MLP rats. In addition, protein levels of phospho(Ser^{79})-ACC, a downstream target of AMPK, were also reduced in MLP offspring compared to control (Figure 3a). Total protein levels of AMPK were unchanged, demonstrating that decreased AMPK activation did not occur because of MLP-induced reductions in total protein. In contrast, total-ACC protein levels were elevated in MLP mice, reflecting increased lipogenesis, changes that are consistent with increased lipogenic gene expression (Figure 1e). Furthermore, reflecting decreased AdipoR2 expression, we also observed decreased mRNA levels of PPARα in MLP rats, compared to control (Figure 3b). Transcriptional modulation by PPARα requires the presence of the co-activator PPARγ co-activator 1α (PGC1α). Consistent with this, PGC1α mRNA levels were also suppressed in MLP rat adipose tissue (Figure 3c).

To further analyse the potential role of adiponectin-AMPK-PPARα signalling in regulating IRF3 and IRF4 expression, we initially used 3T3L1 adipocytes. Use of 3T3L1 adipocytes allowed for specific
examination of IRF4 regulation in a model lacking macrophages, which may influence results given
the role of IRF4 in immune regulation (12). Adipocyte differentiation was confirmed by measurement
of mRNA levels of PPARγ (data not shown). Treatment of differentiated adipocytes with WY14643, a
potent PPARα agonist, had no effect on IRF3 mRNA (Figure 4a) but did induce expression of IRF4
(Figure 4b). We further analysed these putative signalling mechanism using PPARα knockout mice,
which displayed ≈90% reduction in PPARα mRNA levels in white adipose tissue, together with a
marked reduction in PGC1α mRNA levels (Figure 4c – d). Significantly, in PPARα knockout mice
the normal IRF4 responses to fasting and feeding were disrupted (Figure 4e). Consistent with previous
reports (7) IRF4 levels were induced by fasting in wild-type mice. However, this effect was blunted in
PPARα knockout mice, where IRF4 levels were reduced in fasted compared to fed mice. Similar to
MLP rats, PPARα knockout mice also displayed reduced IRF4 levels (Figure 4f), together with raised
levels of ACC and SREBP1c (Figure 4g – h), indicative of elevate lipogenesis and adipogenesis in
white adipose tissue. Taken together, these results indicate that an adiponectin-PPARα signalling
pathway induces IRF4 mRNA expression in white adipose tissue. Moreover, this pathway is
dysregulated in response MLP, and this may in part mediate the metabolic defects associated with this
MLP.

**Adiponectin-AMPK signalling may regulate IRF3.**

In contrast to IRF4, IRF3 was unaffected by PPARα agonist in vitro, indicating an alternative
regulatory mechanism for IRF3 (Figure 4a). Since adiponectin induced both IRF3 and IRF4, but
PPARα regulated IRF4 only, we reasoned that AMPK, another target of adiponectin, may exert
regulatory control over IRF3. To test this, we used metformin, as a known activator of AMPK (13)],
rather than for its actions as an anti-diabetic treatment. Administration of metformin to control mice
led to significant induction of IRF3 (Figure 5a) but had no effect on IRF4 mRNA levels (Figure 5b),
implying that adiponectin-AMPK signalling selectively induces IRF3 in white adipose tissue.

**Discussion**
We report here that expression of IRF3 and IRF4, two key repressors of adipogenesis, are reduced in offspring of rats fed a protein-restricted diet during pregnancy and lactation, in parallel with mildly elevated fasting insulin and glucose. Human epidemiological studies have consistently reported that exposure to an adverse intrauterine environment, such as protein-restriction, leads to an increased risk of development of obesity, T2DM and cardiovascular disease in later life (3, 5, 6). Experimental model studies have reported that MLP offspring gain more weight, have increased susceptibility to development on metabolic syndrome and display impaired adipocyte function when placed a high fat diet (1, 14, 15). However the underlying regulatory mechanisms driving these processes are yet to be fully elucidated. Our data provides evidence that a loss of ability to efficiently repress adipogenesis and lipogenesis, as a result of marked early-life reductions in IRF3 and IRF4, may contribute to impaired adipocyte function and increased risk of obesity in later life. Whilst metabolic changes observed in this model are mild, the early life mechanistic changes reported here would likely make a key contribution to the expected, and previously reported, metabolic deterioration likely observed in these animals in later life.

Previous studies have described that IRF4 expression and function is suppressed by insulin, and that as a result, IRF4 expression is lower in white adipose tissue depots obtained from rodent models characterised by insulin resistance; HFD, ob/ob and db/db mice (7). Despite this well characterised role of insulin, our MLP model displayed a marked reduction in IRF3 and IRF4, without significant changes in serum insulin. Instead, our data point the involvement of the adiponectin-PPARα and adiponectin-AMPK signalling pathways in the regulation of IRF4 and IRF3 respectively. Adiponectin is a bioactive adipokine expressed at high levels and secreted from adipose tissue (16). Adiponectin exerts metabolic regulatory effects via autocrine, paracrine and endocrine function, through the receptors adipoR1 and adipoR2, which in turn activate AMPK and PPARα (9, 16). Conversely, PPARα agonists have been reported to upregulate adiponectin and AdipoR1 and AdipoR2, whilst adiponectin is suppressed in PPARα knockout mice (17). PPARα is a transcription factor for genes involved in lipolysis and fatty acid oxidation and PPARα null mice maintained on a high-fat diet become more obese than wild type (18). Despite suppression of insulin levels and increases in FA
supply, PPARα-deficient mice exhibit an impaired ability to adequately up-regulate hepatic FA oxidation in response to fasting [18]. Insulin resistance induced by excess of non-esterified FA and circulating TAG can be corrected by the administration of PPARα activators by actions to promote removal of intracellular lipid through tissue FA oxidation [19]. PPARα is activated under calorie restriction, increasing the expression of genes encoding mitochondrial FA oxidation enzymes, thereby increasing the capacity for FA oxidation and intracellular lipid clearance [20]. AMPK activation occurs in response to a rise in AMP associated with metabolic stresses that interfere with ATP production (e.g., hypoxia or calorie restriction/glucose deprivation) or accelerated ATP consumption (e.g., muscle contraction), so as to preserve or maintain tissue function [21]. Therefore, AMPK also plays an important role in regulating lipid homeostasis in adipose tissue, having been shown to inhibit lipogenesis and during the longer-term increase lipolysis (10). The fact that PPARα levels and other oxidation genes (PGC-1α) and proteins (AMPK) are suppressed in MLP rats, while lipogenic genes are enhanced (SREBP-1c and FAS), suggest that these mice may develop a similar phenotype to PPARα-/- mice, and that the MLP phenotype may be a direct result of reduced PPARα levels leading to increased IRF4 levels. Under both conditions, fatty acid oxidation is decreased and lipogenesis is enhanced, again potential involving an IRF-mediated mechanism. Several mechanisms for these actions of AMPK and PPARα have been described. Our data suggests a further mechanism of action, namely via induction of IRF3 and IRF4, and consequent repression of lipogenesis and induction of lipolysis in white adipose tissue. These changes were in turn regulated by adiponectin, and these signalling pathways were all impaired in MLP rats.

In addition to specific effects on lipolysis and lipogenesis, low levels of IRF4 may also reflect poorly differentiated adipocytes, since IRF4 is expressed only in fully differentiated adipocytes. Poorly differentiated adipocytes are often present in obesity and can lead to hypoxia, inflammation and an altered adipokine profile, including lower adiponectin levels (19). Dysregulation of these processes are linked to onset of T2DM. Moreover, reduced adiponectin levels occurring as a result of poor differentiation, may represent an initial cause of the impaired adiponectin signalling observed in MLP mice. Alternatively, this mechanism of impaired adiponectin signalling may induce a feed-forward
loop, with further reductions in adiponectin signalling and consequent worsening of the MLP phenotype. In addition, whilst adiponectin has been reported to induce PPARα, the reverse has also been documented, with adiponectin upregulated by PPARα agonists and suppressed in white adipose tissue of PPARα knockout mice (17). Thus, suppressed PPARα levels in MLP mice may contribute to further impairment of adiponectin signalling and consequent exacerbation of the MLP phenotype. Further studies are required to determine the exact mechanistic interactions by which PPARα regulates IRF4.

In addition to an established role in adipocytes, IRFs are present in macrophages and infiltrating lymphocytes. IRF4 is essential for the function and homeostasis of both mature B and mature T lymphocytes. IRF4-deficient mice exhibited a profound reduction in serum immunoglobulin concentrations and impaired T lymphocyte function [23]. The altered IRF levels could potentially represent reduced immune infiltrate into the white adipose tissue. Further research is needed to clarify this. However, given the increased inflammation and macrophage recruitment observed in response to reduced adiponectin levels in other studies (16), a reduced immune infiltrate seems unlikely, instead suggesting a specific role for adipocyte-IRF3 and IRF4 in this model.

In summary, we propose that in early life in response to protein restriction adiponectin signalling is impaired leading to reduced activity of PPARα and AMPK, which in turn lead to decreased levels of IRF3 and IRF4. This would lead to an inability to repress adipogenesis and lipogenesis, and confer increased susceptibility to development of an obese phenotype in later-life.

DISCLOSURE STATEMENT: The authors have nothing to disclose.

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References

Figure Legends

**Figure 1: Reduced IRF levels in white adipose tissue of MLP offspring.** Visceral WAT was isolated from 8-week old offspring of MLP and control Wistar rats (n = 6); Gene expression of (A) *Irf3*, (B) *Irf4*, (C) *Srebp1c* and (D) *Fasn*. (E) Protein levels of FASN and ACC. Western blots are representative. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001 vs. control.

**Figure 2: Impaired adiponectin signalling in white adipose tissue of MLP offspring leads to reduced IRF levels.** Visceral WAT and serum were isolated from 8-week old offspring of MLP and control Wistar rats (n = 6); (A) serum insulin levels; mRNA levels of (B) *AdipoQ*, (C) *AdipoR1*, and (D) *AdipoR2* in white adipose tissue. 3T3-L1 adipocytes were treated with adiponectin (30 µg/ml; 24 h) and mRNA levels of (E) *Irf3* and (F) *Irf4* were measured by qPCR. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001 vs. control.

**FIG. 3. Reduced AMPK and PPARα signalling in white adipose tissue of MLP offspring.** Visceral WAT was isolated from 8-week old offspring of MLP and control Wistar rats (n = 6); (A) protein levels of phospho(Thr172)-AMPK, total-AMPK and phospho(Ser79)-ACC; mRNA levels of (B) *Ppara* and (C) *Pgc1α*. Western blots are representative. Data are expressed as mean ± SEM. **p < 0.01, ***p < 0.001 vs. control.

**Figure 4: Adiponectin-PPARα signalling induces IRF4 expression in white adipose tissue.** 3T3-L1 adipocytes were treated with WY14643 (10 µM; 24 h) and mRNA levels of (A) *Irf3* and (B) *Irf4* were measured by qPCR. Visceral WAT and serum were isolated from 8-week old PPARα knockout and control mice (n = 6); mRNA levels of (C) *Ppara* (D) *Pgc1α*, (E – F) *Irf4*, (G) *Srebp1c* were assessed by qPCR. (H) Protein levels of ACC. Western blots are representative. Data are expressed as mean ± SEM. *p < 0.05, **p<0.01, ***p < 0.001 vs. control (FED); ###p<0.001 v. control (FAST).
Figure 5: Adiponectin-AMPK signalling may regulate IRF3 expression in white adipose tissue.

Visceral WAT was isolated from 8-week old C57Bl/6 mice administered metformin (250 mg/kg/day; 7 days) or saline equivalent (n = 6); mRNA levels of (A) Irf3 and (B) Irf4 were measured by qPCR. Data are expressed as mean ± SEM. * p < 0.05, vs. control

Table 1. Serum insulin and glucose changes between Control and MLP rats