Chapter 3

Mechanism of Action of TNFα in Gluconeogenesis Mediated via Foxa2
3.1. INTRODUCTION

Type 2 diabetes, which accounts for almost 90% of the total diabetic population, stems from the decreased responsiveness of the body to insulin (insulin resistance), accompanied by the failure of pancreatic β-cells to secrete insulin to counteract this insulin-resistant state. Obesity is invariably associated with diabetes and a parallel increase in the occurrence of both is evident across all populations (Mokdad et al., 2003, Zimmet et al., 2001). Obesity-induced insulin resistance is thereby characterized by a loss of insulin sensitivity mediated by factors released from adipocytes, mainly free fatty acids and proteins, termed adipocytokines, which act to control various metabolic functions (Pittas et al., 2004) with well-described physiological effects (Boden and Shulman, 2002). One such adipocytokine is tumour necrosis factor-α (TNFα), which has been identified as a significant contributor to insulin resistance, and its levels have been reported to be increased significantly in obese diabetic individuals and in several animal models of obesity (Hamann et al., 1995; Hotamisligil et al., 1993; Hotamisligil et al., 1995; Katsuki et al., 1998; Kern et al., 1995).

The liver is a major insulin target tissue and plays a significant role in glucose homeostasis, as it can alternate between cycles of glucose output and its inhibition to maintain normal circulating glucose levels (Granner et al., 1983); it is this precisely regulated cycle that is disturbed under conditions of insulin resistance and type 2 diabetes. Nuclear transcription factors that are crucial in governing this metabolic switch are regulated by circulating levels of insulin and glucagon (Spiegelman and Heinrich, 2004). Insulin triggers the activation of a series of phosphorylation cascades that are lost in insulin-resistant states, thereby preventing insulin from correctly regulating glucose and fat metabolism (Saltiel and Kahn, 2001).

The hepatocyte nuclear factor 3 (Hnf-3) forkhead family of nuclear transcription factors, which includes three members designated as Foxa-1 (Hnf-3α), Foxa-2 (Hnf-3β) and Foxa-3 (Hnf-3γ) (Kaestner et al., 1994; Wolfrum et al., 2003; Brennan, 1993), play an important regulatory role in the maintenance of normal glucose homeostasis; they do so by regulating the gene expression of rate-limiting enzymes of gluconeogenesis and glycogenolysis, including phosphoenolpyruvate carboxykinase.
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(PEPCK) and glucose-6-phosphatase (G6Pase), and by regulating glucagon and Pdx-1 gene expression in the pancreas (Wolfrum et al., 2003; Gerrish et al., 2000; O'Brien et al., 1995; Shih et al., 1999; Wang et al., 2000). In addition, although some reports have shown the regulation of gluconeogenic enzymes by another forkhead transcription factor, Foxo1 (Brunet et al., 1999; Zhang et al., 2006), others have reported that the over expression of Foxo1 carries the message to G6Pase only and that PEPCK levels remain unaffected (Nakae et al., 2001; Barthel et al., 2001). Thus, the mechanisms involved in the regulation of gluconeogenic enzymes are very controversial, and it is thereby hypothesized that both of these factors contribute to insulin action on glucose production by regulating the expression of different gluconeogenic enzymes (Wolfrum et al., 2003), and/or synchronize with other transcription factors to regulate the same. Therefore in this chapter, the role of Foxa2 (HNF-3β), if any, in the regulation of gluconeogenesis in HepG2 cells, and the effects of TNFα pretreatment on this phenomenon was studied with an objective to decode its regulation in obesity and insulin resistance.

3.2. MATERIALS AND METHODS

3.2.1. Cell Culture

All experiments were performed in HepG2 (human hepatocellular carcinoma) cells obtained from the National Centre for Cell Science, Pune, India. Cells were maintained in DMEM supplemented with 10% fetal calf serum and 1% antibiotic–antimycotic (100units/mL penicillin, 0.1mg/mL streptomycin and 0.25µg/mL amphotericin B) at 37°C in a humidified atmosphere of 5% CO2. All incubations were carried out after overnight serum starvation.

3.2.2. Western Blotting

HepG2 cells were plated in six-well plates and incubated with TNFα at 1nM for 24 h (As stated above in Chapter 2, TNFα depicted similar pattern of inhibition in insulin stimulated Akt activation at 0.5 and 1nM at 12 and 24 h respectively) or insulin at 50nM for 15 min, or preincubated with TNFα followed by insulin treatment. On termination of incubation, cells were lysed in ice-cold lysis buffer 10mM Tris, 50mM
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NaCl, 1% Triton X-100, 5mM EDTA, 20mM sodium pyrophosphate, 50mM NaF, 100µM Na3VO4, 5µg/mL each of leupeptin, aprotinin and pepstatin, and 1mM phenylmethylsulphonyl fluoride (pH 7.4)]. Lysates were centrifuged at 10000 g for 10 min at 4°C, and the supernatant was used as the cytosolic fraction. To the pellet, 50µL of 10mM Tris (pH 7.5) containing 10% v/v glycerol, 0.1M KCl, 0.2mM EDTA, 20mM sodium pyrophosphate, 50mM NaF, 100µM Na3VO4, 5µg/mL each of leupeptin, aprotinin and pepstatin, and 1mM phenylmethylsulphonyl fluoride was added and stirred at 4°C for 30 min. These nuclear extracts were centrifuged at 15000 g for 20 min at 4°C, and the supernatant was used as the nuclear fraction. Equal amounts of nuclear and cytosolic proteins were resolved by SDS-PAGE, transferred to poly (vinylidene difluoride) membranes and probed with p-Akt, Akt, Foxa2 and Foxo1 antibodies. Blots were probed identically for β-actin or TBP as the loading controls, and also to assess the purity of nuclear and cytosolic preparations. Bands were analysed densitometrically as described below.

3.2.3. Immunofluorescence Microscopy

HepG2 cells were treated as described above with TNFα at 1nM and/or insulin at 50nM, or in the absence of any of these (control). On termination of incubation, cells were fixed for 15 min at room temperature with 3.5% paraformaldehyde. The cells were then permeabilized with 0.5% Triton X-100 and incubated with anti-Foxa2 IgG (1:50) for 2 h at room temperature. After washing, the cells were treated with anti-goat secondary IgG linked to fluorescein isothiocyanate (1:100) for 2 h at room temperature. The cells were then washed thoroughly, 4’, 6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 1 µg/mL and the cells were visualized in a fluorescent microscope at 20x magnification (Carl Zeiss Inc., New York, NY, USA).

3.2.4. Chromatin Immunoprecipitation Assay

Cells were treated with either TNFα at 1nM for 24 h or insulin at 50nM for 15 min alone, or pretreated with TNFα followed by insulin incubation. On termination of incubation, chromatin was isolated according to the method of Buser et al. (Buser et al., 2007). Twenty per cent of the chromatin preparation was reserved as the total
input control and the remainder was incubated overnight at 4°C in the presence of either normal IgG or anti-Foxa2 IgG (5µg). Immune complexes were reverse crosslinked and the Foxa2 enrichment of the target DNA fragments in the immunoprecipitated DNA was checked by PCR and quantified by real-time PCR. In both cases, the sequences of sense and antisense primers used were 5'-GCCTGTGTGTCCCTCAAAAACC-3' and 5'-GCAACTGTCCCTTGTCAAAA-3', respectively, which were specific to the Foxa2 binding site within the human PEPCK promoter. PCRs were performed in the presence of 0.25mM dNTPs, 1.5mM MgCl2, 10pmol of each primer and 0.5U Taq polymerase, and consisted of 35 cycles of denaturation at 94°C for 45s, annealing at 58°C for 30s and extension at 72°C for 30s (10 min last cycle; GeneAmp PCR System 9700, Applied Biosystems). PCR products were separated on a 1.0% agarose gel and photographed with the Alpha Innotech gel documentation system and the intensity of each band was analysed densitometrically and plotted after normalization to that of the input DNA. For real-time PCR, reaction components were put together using the SYBR Green PCR Master Mix (Applied Biosystems), and the reactions were performed according to the manufacturer’s instructions (ABI 7500, Applied Biosystems). Reactions were performed in triplicate and the relative quantity was determined by the relative standard curve method. Values were normalized to those of input DNA and the control was arbitrarily assigned a value of unity.

3.2.5. RNA Isolation, RT-PCR and Quantitative Real-Time PCR

The subsequent effects of TNFα incubation prior to insulin treatment, or insulin or TNFα treatments alone, on the transcript levels of gluconeogenic genes were examined as described by Gabbay et al. (Gabbay et al., 1996). Cells were incubated either in the presence of TNFα (1nM) for 24 h, followed by insulin (50nM) for 4 h, or with insulin or TNFα alone, or in the absence of any of these agents. Total RNA was extracted using the RNeasy kit (Qiagen), reverse transcribed and amplified (GeneAmp PCR System 9700, Applied Biosystems) with gene-specific primers (PEPCK: sense, 5'-GGTTCCCAGGGTGCGATGAAA-3'; antisense, 5'-CAGCTAGGGTGAAATCCGTCAG-3'; G6Pase: sense, 5'-ATGAGTCTGGTTACTACAGGCA-3'; antisense, 5'-
AAGACAGGGCCGTCATTATGG-3’). Real-time PCR for quantification was performed as described in Chapter 2. To further validate the role of Foxa2, HepG2 cells were transfected with 100nM of either control or Foxa2 siRNA (Santa Cruz Biotechnology Inc.), according to the manufacturer’s instructions. After allowing the cells to grow in fresh DMEM for 48 h, they were incubated with insulin or TNFα, or pretreated with TNFα prior to insulin, as mentioned above. Cells incubated in the absence of any of these were taken as the control. On termination of incubation, RNA was isolated and the status of PEPCK was determined by real-time PCR, as described previously.

3.2.6. Glucose Production Assay

Glucose production was carried out essentially as described by Choi et al. (Choi et al., 2005) with slight modifications. Briefly, after an overnight serum starvation, HepG2 cells were incubated with TNFα at 1nM for 24 h or insulin at 50nM for 24 h, or pretreated with TNFα prior to insulin incubation. Glucose released into the medium was assayed by subsequent incubation in glucose production medium [glucose- and phenol red-free DMEM containing the gluconeogenic substrates, sodium lactate (20mM) and sodium pyruvate (2mM)] and measurement of the glucose concentration using the glucose assay kit (Merckotest Glucose kit, Merck). This was normalized with total cellular protein measured using the protein assay kit (Biorad Laboratories).

3.2.7. Densitometric Analysis

Each band, when mentioned, was analysed by alpha digidoc 1201 software (Alpha Innotech Corporation, San Leandro, CA, USA). The same sized rectangular box was drawn surrounding each band and the intensity of each was analysed by the program after subtraction of the background intensity.

3.2.8. Statistical Analysis

All experiments were performed in triplicate and the data are presented as the mean ± standard error of the mean (SEM). Student’s t-test was used for statistical analysis and P < 0.05 was taken to be statistically significant.
3.3. RESULTS

3.3.1. Incubation of HepG2 Cells with TNFα Attenuates Insulin-stimulated Akt Phosphorylation and Nuclear Exclusion of Foxa2

Initially, we determined whether the cells were insulin unresponsive in our study at the dose and period of TNFα preincubation prior to insulin treatment used. Akt is one of the most important insulin signalling intermediates, and is well known to be activated by insulin, an effect that is equally well known to be prevented in cells preincubated with TNFα prior to insulin incubation. Earlier reports (Gupta et al., 2007, Gupta and Khandelwal, 2004) have demonstrated that insulin-stimulated Akt phosphorylation is significantly prevented in HepG2 cells preincubated with TNFα prior to insulin incubation.

![Figure 3.1: Effect of TNFα on insulin-stimulated Akt activation in HepG2 cells.](image)

Figure 3.1: Effect of TNFα on insulin-stimulated Akt activation in HepG2 cells. Serum-starved HepG2 cells were incubated in the absence or presence of TNFα (1 nM, 24 h) and then with or without insulin (50 nM, 15 min). Cellular lysate (50 μg) from each group was resolved by SDS-PAGE, transferred to poly(vinylidene difluoride) membranes and probed by western blotting with p-Akt and Akt (total) antibodies. Each band is a representative of three independent blots (A). Signals were scanned, analysed densitometrically and intensities are expressed as arbitrary units (B). Values are the means ± SEM of three experiments. *P < 0.001 when compared with control; **P < 0.01 when compared with incubation with insulin alone.
Interestingly, HepG2 cells overexpressing a constitutively active form of Akt demonstrate restoration of this preventative effect of TNFα on insulin action (Gupta and Khandelwal, 2004). As our study was directed towards the underlying mechanisms of insulin and TNFα pretreatment on gluconeogenesis within the hepatocyte, we started by studying the status of Akt under these conditions. Insulin significantly stimulated Akt phosphorylation relative to the control (P < 0.001), and this effect was decreased significantly on TNFα pretreatment (P < 0.01) (Figure 3.1A, B).

The inhibition of insulin signalling in the liver is primarily reflected by the attenuation of the insulin-mediated inhibition of gluconeogenic gene expression. As the forkhead protein, Foxa2, has been suggested to regulate, at least in part, the expression of gluconeogenic genes (Wolfrum et al., 2003; Zhang et al., 2005), we studied its status within the cell in the given experimental conditions. Foxo1, another member of the forkhead family of transcription factors, is a very well-established mediator of the effects of insulin on gluconeogenic gene expression (Zhang et al., 2006), and has also been implicated in several cellular effects of TNFα (Alikhani et al., 2005; Ito et al., 2009). Together with Foxa2, we also assessed its cellular status in the presence and absence of insulin and/or TNFα. Figure 3.2A, B shows the effects of TNFα pretreatment on insulin action on the localization of Foxa2 and Foxo1 within the cell. Incubation with 50nM insulin resulted in relative nuclear exclusion of Foxa2, with significant localization in the cytosol (P < 0.01 relative to control). An identical but more pronounced trend was observed for Foxo1, implying that it is a much stronger candidate for insulin action. Surprisingly, in cells pretreated with TNFα (1nM, 24 h), followed by insulin incubation, Foxa2 was found to be mainly localized in the nucleus (P < 0.05) and was significantly (P < 0.01) less detected in the cytosol, relative to cells incubated in the presence of insulin alone. Foxo1 also showed an almost complete nuclear localization in cells pretreated with TNFα prior to insulin incubation, whereas, in cells incubated in the presence of insulin alone, it was exclusively localized in the cytosol. As Foxo1 is already known to mediate the effects of insulin on gluconeogenic genes, we carried out further experiments to decipher the role of Foxa2 only, if any, on these series of events. There was no significant alteration in Foxa2 localization in cells treated with TNFα alone relative to cells incubated in the absence of any of these
reagents (Control) (Figure 3.2A, C). These results imply that, in the presence of TNFα, wherein cells are rendered insulin insensitive, insulin-mediated nuclear exclusion and inactivation of Foxa2 are prevented, with the result that it is primarily localized in the nucleus. Thus, although TNFα alone does not alter the status of Foxa2 within the cell, it attenuates insulin-stimulated Foxa2 nuclear exclusion, possibly by blunting insulin signalling within the cell. The subcellular distribution of Foxa2 under the conditions stated above was also checked by immunofluorescence staining with anti-Foxa2 IgG.

**Figure 3.2:** Effect of TNFα on Foxa2 and Foxo1 localization. HepG2 cells were incubated with TNFα (1nM) or insulin (50nM), or pretreated with TNFα (1nM, 24 h) followed by insulin treatment (50nM, 15 min). Cells incubated in the absence of any of these were taken as the control (Con). On termination of incubation, cells were lysed and the nuclear (50μg) and cytosolic (40μg) protein extracts were assessed for the presence of Foxa2 or Foxo1 by western blotting. Each band is a representative of three separate blots from three independent experiments. Blots were probed with TBP and β-actin antibodies and taken as nuclear and cytosolic loading controls, respectively, and also used to ascertain the purity of the nuclear and cytosolic preparations (A). Bands were scanned, quantified densitometrically and are expressed as arbitrary units (a.u.). Values depicted are the means ± SEM of three values obtained from three independent blots (B). (C) HepG2 cells were incubated as described in (A) and Foxa2 localization was detected by incubation with anti-Foxa2 IgG and fluorescein isothiocyanate-linked secondary antibody. Cells were visualized in a fluorescence microscope at a magnification of ×40. DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate. *P < 0.01 when compared with control; **P < 0.05 when compared with insulin incubation (nuclear pellet); ***P < 0.01 when compared with insulin incubation (cytosol). *P < 0.001 when compared with control; #P < 0.01 and P < 0.001 when compared with incubation with insulin alone.
In cells incubated with 50nM insulin, Foxa2 was fairly strongly detected in the cytosol, when compared with cells incubated in the absence of insulin. Although Foxa2 was not completely excluded from the nucleus by treatment with insulin, it was strongly detected in the cytosol of insulin-treated cells, but was largely absent in control cells. However, when cells were pretreated with TNFα (1nM, 24 h) prior to insulin incubation, this nuclear extrusion of Foxa2 and its localization in the cytosol were significantly attenuated, with the result that, in these TNFα-pretreated cells, Foxa2 was very weakly detected in the cytosol with the major fraction being in the nucleus (Figure 3.2C).

### 3.3.2. TNFα Pretreatment Increases Foxa2 Occupancy on the PEPCK Promoter

As we observed a predominant localization of Foxa2 in the nuclei of cells pretreated with TNFα prior to insulin incubation, and considering its possible involvement in the regulation of gluconeogenic enzymes, we analysed the Foxa2 occupancy of the promoter of gluconeogenic genes, mainly PEPCK, it being the rate-limiting enzyme, to categorically determine whether Foxa2 can exert its effects on the transcriptional regulation of its targets in the absence and presence of TNFα and/or insulin. Foxa2 occupancy of the PEPCK promoter was determined by semiquantitative (Figure 3.3A, B) and quantitative RT-PCR (Figure 3.3C).

When compared with the control, insulin caused a significant (P < 0.01) decrease in Foxa2 occupancy of the PEPCK promoter. This decrease was significantly (P < 0.01) attenuated in cells preincubated in the presence of TNFα prior to insulin incubation. In cells incubated in the presence of TNFα alone, Foxa2 did not show any significant change in its occupancy on the PEPCK promoter after normalization with the input DNA and comparison with the control. All of these results indicate that preincubation with TNFα significantly abrogates the insulin-mediated decrease in Foxa2 occupancy of the PEPCK promoter, with the result being that, under these conditions, Foxa2 significantly occupies its binding element on the PEPCK promoter which, however, is not observed in cells incubated in the presence of TNFα alone.
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Figure 3.3: Effect of TNFα on PEPCK promoter occupancy by Foxa2 in HepG2 cells. Cells were pretreated with TNFα (1nM) followed by insulin incubation (50nM), or incubated with TNFα or insulin alone. On termination of incubation, nuclear chromatin was isolated and immunoprecipitated with either normal IgG or anti-Foxa2 IgG. The chromatin–antibody aggregates were pulled down with protein A-Sepharose and the occupancy of Foxa2 on the PEPCK promoter was determined by semiquantitative (A, B) and real-time quantitative (C) PCR using primers enclosing the Foxa2 binding sites on the PEPCK promoter. The relative quantity of Foxa2 occupancy was determined by the relative standard curve method. Each value presented has been normalized with that of input DNA and is the mean ± SEM of three independent values. *P < 0.01 when compared with control; **P < 0.01 and ***P < 0.05 when compared with insulin incubation.

3.3.3. Effect of TNFα Pretreatment on PEPCK and G6Pase mRNA in HepG2 Cells

Gluconeogenesis is a very significant phenomenon in the liver, and gluconeogenic enzymes, namely PEPCK, fructose-1, 6-bisphosphatase (F1, 6bpase) and G6Pase, are critical in determining the rate of gluconeogenesis and hepatic glucose production. These gluconeogenic genes are elevated under diabetic conditions. Since we had observed increased nuclear translocation of Foxa2 in cells that are insulin insensitive due to TNFα, we studied the resulting effects of TNFα pretreatment on the effect of insulin on the expression of PEPCK and another gluconeogenic enzyme, G6Pase. Compared with the control, insulin incubation caused a significant inhibition of PEPCK and G6Pase gene expression (P < 0.001, Figure 3.4A, B).
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Figure 3.4: Analysis of PEPCK and G6Pase expression following TNFα treatment. HepG2 cells were pretreated with TNFα (1 nM, 24 h), followed by insulin incubation (50 nM, 4 h), or incubated with TNFα or insulin alone for the respective indicated times. Cells in the absence of any of these were taken as the control (Con). Two micrograms of total RNA were reverse transcribed with random primers and the levels of PEPCK and G6Pase mRNA were measured by RT-PCR using gene-specific primers. 18S rRNA was taken as the internal loading control (A). Each band was analysed densitometrically and the values are depicted after normalization of PEPCK and G6Pase bands with those of 18S rRNA (B). Each point is the mean ± SEM of three sets of experiments. (C) Real-time PCR quantification of PEPCK and G6Pase mRNA in cells incubated as described in (A). Values were normalized to those of 18S rRNA and are the means ± SEM of three independent experiments. (D) Real-time PCR quantification of PEPCK in cells transfected with either control or Foxa2 siRNA prior to incubation as described in (A) above. Values are the means ± SEM of three independent experiments after normalization with 18S rRNA. *P < 0.001 when compared with control (B) and control vs. insulin and TNFα plus insulin vs. insulin alone (C); **P < 0.01 when compared with insulin alone (B) and TNFα incubation when compared with control (C); ***P < 0.05, TNFα plus insulin vs. insulin alone and TNFα alone compared with control (B). *P < 0.05 when compared with insulin alone and TNFα plus insulin, respectively, in the presence of control siRNA; †P < 0.05 when compared with insulin alone in the presence of Foxa2 siRNA (D).

However, TNFα pretreatment prior to insulin incubation considerably attenuated this inhibitory effect (PEPCK, P < 0.01; G6Pase, P < 0.05; when compared with insulin alone). This indicates that, in the presence of TNFα, HepG2 cells do not respond to insulin and the subsequent enhanced occupation of Foxa2 on its binding element (as observed in the case of PEPCK) leads to elevated levels of these gene transcripts.
When compared with the control, TNFα alone caused a significant \((P < 0.05)\) inhibition of PEPCK and G6Pase transcripts. However, as described in the earlier results, Foxa2 localization and occupancy on the PEPCK promoter in cells incubated in the presence of TNFα alone were not altered significantly from those of the control. These results indicate that, although PEPCK and G6Pase transcripts are decreased in cells incubated in the presence of TNFα and insulin alone, the upstream events facilitating this are possibly different, with Foxa2, at least in part, mediating the insulin effect. Real-time PCR data also depicted an identical pattern, in which PEPCK and G6Pase mRNA were significantly \((P < 0.001)\), inhibited in the presence of insulin; however, this was not observed when the cells were pretreated with TNFα prior to insulin treatment \((P < 0.001; \text{Figure 3.4C})\).

TNFα also inhibited significantly the levels of PEPCK and G6Pase gene transcripts \((P < 0.01)\). The specificity of Foxa2 was checked with the use of Foxa2 siRNA that could knock down Foxa2 protein levels by almost 70%. Incubation with Foxa2 siRNA prior to insulin treatment could only partially withdraw insulin-mediated inhibition of PEPCK gene expression \((P < 0.05, \text{Figure 3.4D})\), and a complete restoration was not observed, indicating that Foxa2 is critical, but not the sole mediator, of insulin effects. The preventative effect of TNFα on insulin-mediated inhibition of PEPCK expression was also partially reversed by Foxa2 siRNA in cells pretreated with TNFα prior to insulin incubation \((P < 0.05)\).

3.3.4. TNFα Attenuates Insulin-Induced Inhibition of Hepatic Glucose Output in HepG2 Cells

As we had observed, so far, an increase in gluconeogenic gene transcript levels in TNFα-pretreated cells as a result of a decrease in the effects of insulin, mediated in part, by the transcription factor, Foxa2, we sought to determine the effect(s) of this on glucose production from HepG2 cells, the ultimate phenotype that, together with glucose uptake, regulates the circulating glucose level within the body. The incubation of HepG2 cells with insulin inhibited glucose release by almost threefold when compared with the control \((P < 0.01)\); pretreatment with TNFα prior to insulin incubation significantly attenuated this inhibition \((P < 0.001)\), i.e. in the presence of
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TNFα, the extent of inhibition of hepatic glucose output by insulin was markedly attenuated (Figure 3.5).

![Figure 3.5: TNFα attenuates insulin-induced inhibition of hepatic glucose output. HepG2 cells were serum starved overnight and incubated for 24 h in the presence of TNFα (1 nM) or insulin (50 nM) alone, or pretreated with TNFα followed by insulin for these time periods. Control cells were incubated in the absence of any of these agents. On termination of incubation, the glucose released in the medium was assayed as described in Materials and methods, and the values were normalized to the total cellular protein content. Each value is the mean ± SEM of three independent incubations. *P < 0.01 when compared with control; **P < 0.001 when compared with insulin incubation.](image)

3.4. DISCUSSION

TNFα, which is widely implicated in obesity-associated insulin resistance, impairs the insulin signalling pathway (Hotamisligil et al., 1995; Gupta et al., 2007; Rui et al., 2001; de et al., 2004); however, its role in hepatic gluconeogenesis during insulin resistance and the complex underlying mechanisms are not well understood. Impaired glucose tolerance and insulin resistance are early metabolic disturbances in the development of type 2 diabetes. Glucose homeostasis in the body is largely controlled by the liver, and hyperglycemia, as observed in type 2 diabetes, reflects increased hepatic glucose production (Meyer et al., 1998; Roden et al., 2001), as well as reduced glucose uptake (Nielsen et al., 1998). Indeed, the onset of hepatic insulin resistance typically precedes peripheral insulin resistance in humans(Clore et al.,
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1995). The stimulation of gluconeogenesis occurs invariably as a result of increased activity of PEPCK, G6Pase and F1, 6bpase, and the targeted overexpression or knockouts of these enzymes play a major regulatory role in glucose homeostasis (Niswender et al., 1997; Sun et al., 2002).

As far as the regulation of these genes is concerned, the Foxa family of transcription factors acts synergistically with other hepatocyte nuclear factors to coordinately regulate liver-specific gene expression (Hughes et al., 2003). Their transcriptional regulation particularly that of PEPCK by insulin involves the participation of several transcription factors, including Foxo1, Foxo3, PGC-1α, SREBP etc., although none can be singled out to mediate the effect of insulin. The PEPCK promoter is undoubtedly complex and possesses the binding elements of several transcription factor complexes (Figure 3.6) (Chakravarty et al., 2004).

![Figure 3.6: PEPCK gene promoter showing binding site for HNF-3β (Foxa2) and several other transcription factors](image)

The regulation by the Foxa group of transcription factors, which possess considerably identical DNA-binding domains and bind to the promoters of target genes as monomers, is even more controversial. Foxa2 plays a significant regulatory role in hepatic and/or pancreatic physiology (Brennan, 1993; Gerrish et al., 2000; Kaestner et al., 1994; Kaestner et al., 1999; O'Brien et al., 1995; Rausa et al., 2000; Shih et al., 1999; Sund et al., 2000; Vallet et al., 1995; Wang et al., 2000; Wolfrum et al., 2003). It is excluded from the nucleus as a result of its phosphorylation at Thr156 by Akt,
resulting in its inactivation and subsequent repression of the transcriptional response of key gluconeogenic enzymes (Wolfrum et al., 2003). Zhang et al. (Zhang et al., 2005) have also demonstrated that Foxa2 is required for hepatic gluconeogenesis. PEPCK activation is significantly downregulated in the absence of Foxa2, and a clear enrichment of its promoter by Foxa2 antibody has been reported (Steneberg et al., 2005; Zhang et al., 2005).

Similar results in relation to the identification of a Foxa2-binding site within the PEPCK promoter have also been reported by others (O’Brien et al., 1995; Wang et al., 1996; Wang et al., 1999; Wang et al., 2000). Wolfrum et al. (Wolfrum et al., 2003) suggested that Foxa2 may contribute to hepatic insulin resistance in Akt−/− mice as a result of an inability to phosphorylate Foxa2 and suppress the transcription of gluconeogenic enzymes. Based on their results, O’Brien et al. (O’Brien et al., 1995) reported that insulin mediates its negative effect on glucocorticoid-induced PEPCK gene transcription by inhibiting the binding of Hnf-3 proteins. However, Hall et al. (Hall et al., 2007) reported that insulin response sequences themselves are not sufficient for the complete effect of insulin on its targets. They found insulin-mediated dissociation of glucocorticoid-induced accumulation of several transcription factors, including Foxa2, from the PEPCK promoter. Taken together, several transcription factors act in tandem to regulate PEPCK gene transcription in response to insulin, and none has been definitively established as physiologically mediating the basal, as well as hormone-mediated, alterations in PEPCK gene expression.

In this study, we found Foxa2 to be predominantly localized in the nuclei of HepG2 cells incubated with TNFα prior to insulin incubation. As reported earlier, insulin incubation resulted in a relative increase in the nuclear exclusion of Foxa2, with it being strongly localized in the cytosol. TNFα alone, however, did not alter the status of Foxa2 localization when compared with the control. These results imply that in a TNFα mediated insulin-resistant cell, insulin-induced nuclear exclusion of Foxa2 is reasonably prevented, with the result that the majority is localized in the nucleus. Pretreatment with TNFα prior to insulin also led to enhanced binding to the PEPCK promoter by Foxa2. In our study, Foxa2 localization and its subsequent effects therefore appear to be modest, but steady, which points to the fact that other
Mechanisms and factors are also crucial in mediating the effects of insulin (Hall et al., 2007). That this is so corroborates well, considering the complexity of the PEPCK promoter, which harbours the binding elements of several transcription factors (Chakravarty et al., 2004). Another such transcription factor and a strong regulator of gluconeogenesis is the protein, Foxo1 (Zhang et al., 2006). This is a very well-studied transcription factor regulating insulin action on gluconeogenic enzymes. Our results also show an increased nuclear extrusion of Foxo1 in the presence of insulin. However, some reports have stated that insulin-mediated phosphorylation inactivates Foxo1, but, surprisingly, the message is carried only onto G6Pase and not to PEPCK, as evident from studies on epithelial kidney cells which lack Foxa2 but express Foxo1 (Nakae et al., 2001). Along similar lines, Barthel et al. (Barthel et al., 2001) reported that the overexpression of Foxo1 in rat hepatoma cells increased G6Pase transcript levels without affecting those of PEPCK. In the light of this, our results identify Foxa2 as a crucial mediator which, at least in part, plays a significant role in TNFα-mediated abrogation of insulin signaling within hepatocytes.

Consequent to the increased presence of Foxa2 in the nuclei of cells pretreated with TNFα, insulin inhibition of both PEPCK and G6Pase was significantly prevented in such cells. Experiments with Foxa2 siRNA showed that decreased levels of the Foxa2 protein marginally but significantly restored both insulin inhibition of PEPCK expression and the prevention of this by TNFα. This probably contributes towards the observed hyperglycaemic status in obese diabetics. In cells incubated in the presence of TNFα alone, although there was a significant inhibition of gluconeogenic gene transcription, we did not observe any alteration of Foxa2 localization, probably meaning that, although both insulin and TNFα alone decrease the transcription of gluconeogenic genes, Foxa2 may not be involved in the TNFα effect. This could be a possibility considering the complex promoter regulation of PEPCK (Hill & McCallum, 1992). It has been shown recently that a nuclear co repressor is required in the TNFα-mediated inhibition of PEPCK (Yan et al., 2007). Therefore, in cells preincubated with TNFα prior to insulin, insulin signalling is prevented, resulting in abrogation of this inhibitory effect on PEPCK expression. PEPCK overexpression, in turn, has been shown to attenuate insulin signalling and hepatic insulin sensitivity in
transgenic mice (Sun et al., 2002; Valera et al., 1994). Interestingly, adipose selective overexpression of PEPCK led to increased glyceroneogenesis, increased fat mass and adipose size, increased body weight and severe susceptibility to diet-induced insulin resistance (Franckhauser et al., 2002, 2006).

In this Chapter, we have demonstrated that TNFα pretreatment prevents insulin-induced inhibition of hepatic glucose output, indicating that, in such conditions, cells become insulin insensitive and therefore insulin action in the hepatocyte is attenuated; this is in agreement with studies in which the overexpression of IKKβ, a downstream mediator of TNFα signalling, leads to local and systemic insulin resistance, whereas mice lacking this enzyme in the liver retain liver insulin responsiveness (Arkan et al., 2005; Cai et al., 2005).

### 3.5. CONCLUSION

Our results have unfolded a series of events beginning with the TNFα-mediated prevention of the effect of insulin on Foxa2 localization and leading to the abrogation of insulin inhibition of gluconeogenesis and glucose output in HepG2 cells. Although TNFα-mediated inhibition of insulin signaling has been known for some time, the focus has primarily been on glucose uptake in the skeletal muscle and adipocytes. As TNFα is a major adipocytokine associated with obesity and type 2 diabetes this pathway of impairment of insulin action, as observed in HepG2 cells mediated by Foxa2, possibly explains one of the contributory mechanisms for the observed hyperglycaemia in obese diabetics.