RESEARCH PAPER

Pioglitazone attenuates prostatic enlargement in diet-induced insulin-resistant rats by altering lipid distribution and hyperinsulinaemia

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BACKGROUND AND PURPOSE

Increased incidence of benign prostatic hyperplasia among insulin-resistant individuals suggests a role for hyperinsulinaemia in prostatic enlargement. We have already reported increased cell proliferation and enlargement of prostate gland in insulin-resistant rats. The present study aimed to elucidate the molecular mechanisms underlying the reversal of prostatic enlargement in insulin-resistant rats by the peroxisome proliferator-activated receptor γ agonist pioglitazone.

EXPERIMENTAL APPROACH

Sprague–Dawley rats were fed a normal pellet or a high-fat diet for 12 weeks with or without pioglitazone (20 mg·kg^{-1}). Subgroups of animals fed different diets were castrated. Effects of dietary manipulation and pioglitazone were measured on insulin sensitivity, lipid distribution, cell proliferation and apoptosis.

KEY RESULTS

A high-fat diet led to the accumulation of fat in non-adipose tissues, insulin resistance, compensatory hyperinsulinaemia and prostatic enlargement in rats. Pioglitazone treatment altered fat distribution, improved insulin sensitivity and normalized lipid and insulin level in rats on the high-fat diet. The improved metabolic parameters led to decreased cellular proliferation and increased apoptosis in the prostate gland. High-fat diet feeding and pioglitazone treatment did not change plasma testosterone levels. However, significant prostatic atrophy was observed in castrated rats irrespective of dietary intervention.

CONCLUSIONS AND IMPLICATIONS

Our results show a previously unexplored therapeutic potential of pioglitazone for prostatic enlargement under insulin-resistant condition and further suggest that targeting distribution of lipid from non-adipose tissue to adipose tissue and insulin signalling could be new strategies for the treatment of benign prostatic hyperplasia.

Abbreviations

BAT, brown adipose tissue; BPH, benign prostatic hyperplasia; ERK-1/2, extracellular signal-regulated kinase; GTT, glucose tolerance test; HFD, high-fat diet; IGF, insulin-like growth factor; IR, insulin receptor; IRS, insulin receptor substrate; ITT, insulin tolerance test; MEK, mitogen-activated protein kinase kinase; NPD, normal pellet diet; PCNA, proliferating cell nuclear antigen; PI3-kinase, phosphatidyl inositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; WAT, white adipose tissue

Introduction

Benign prostatic hyperplasia (BPH) is a highly prevalent condition of prostate in older men, characterized by non-malignant enlargement of the gland (Alonso-Magdalena *et al.*, 2009). More than 60% of men aged over 50 years have histological evidence of BPH and, after the age of 70, the proportion increases to 80% (Berry *et al.*, 1984). Despite the prevalence of BPH, the pathogenesis of the disease is far from completely known. Several epidemiological studies indicate obesity, dyslipidemia and hyperinsulinaemia as the risk factors for BPH, together with type 2 diabetes, and further corroborate the association between these two conditions (Hannah and Howard, 1994; Nandeesha *et al.*, 2006; Ozden *et al.*, 2007; Vikram *et al.*, 2010b). Our previous report has emphasized that hyperinsulinaemia during insulin resistance is primarily responsible for the increased cell proliferation and enlargement of the prostate gland and has provided mechanistic explanation for the link between insulin resistance and BPH (Vikram *et al.*, 2010a). Supporting evidence from experimental (Cai *et al.*, 2001; Rahman *et al.*, 2007; Vikram *et al.*, 2008; 2010a; Escobar *et al.*, 2009) and clinical (Hammarsten and Hogstedt, 1999; Nandeesha *et al.*, 2006; Parsons *et al.*, 2006) studies strengthens the hypothesis that hyperinsulinaemia plays an important role in the pathogenesis of BPH. Compensatory hyperinsulinaemia affects androgen as well as insulin-like growth factor-1 (IGF-1) signalling and both are known to contribute, individually, to prostatic growth. Steroidal hormones have been largely implicated in the normal as well as pathological growth of the prostate gland (Imamov *et al.*, 2004; Wu *et al.*, 2007). Insulin and IGF-1 signalling are reported to activate androgen signalling through direct interaction of Foxo 1 with the androgen receptor (Fan *et al.*, 2007). However, the role of androgen signalling under hyperinsulinaemic condition remains to be explored.

Recently Escobar *et al.* (2009) reported the growth-promoting effects of saturated dietary fatty acids in the prostate of rat and indicated the peroxisome proliferator-activated receptor γ (PPARγ; receptor nomenclature follows Alexander *et al.*, 2009) as a possible link between diet and augmented prostatic growth. Excessive intake of dietary fat results in its impaired distribution and insulin resistance, key features of the metabolic syndrome, a condition which has recently been acknowledged as an important risk factor for BPH (Kasturi *et al.*, 2006). Based on published data and our own experience, we postulated that pharmacological intervention with a PPARγ agonist might improve the lipid distribution as well as insulin sensitivity and would eventually result in the resetting of the cellular equilibrium and reversal of the prostatic enlargement. In the present study, we have made an attempt to investigate the possible molecular mechanisms responsible for the reversal of prostatic enlargement in insulin-resistant rats treated with the PPARγ agonist pioglitazone. Our results suggest that pioglitazone improves lipid distribution, glucose intolerance, insulin sensitivity and hyperinsulinaemia, and attenuates enhanced cell proliferation and enlargement of the prostate gland in diet-induced insulin-resistant rats.

Methods

Animals and experimental design

All animal care and experimental procedures complied with the Committee for the Purpose of Control and Supervision of Experimentation on Animals guidelines and were approved by the Institutional Animal Ethics Committee. Experiments were performed on male Sprague–Dawley rats (9 weeks, 200–220 g). Animals were provided by our Institute's Central Animal Facility and were kept at controlled environmental conditions with room temperature (22 ± 2°C), humidity (50 ± 10%). A 12 h light (0600–1800 h) and dark cycle was maintained throughout the study. Rats were allowed to access to food and water *ad libitum* and acclimatized for 1 week prior to the start of experiment.

Rats were fed a normal pellet diet (NPD) or a high-fat diet (HFD; see below for composition) for 12 weeks, with or without pioglitazone (20 mg·kg⁻¹, once daily) treatment from the 9th to the 12th week. Pioglitazone was suspended in 0.5% carboxymethyl cellulose and orally administered to the animals. To investigate the role of androgens in prostatic growth, a separate study was performed with castration of the rats. Castration was carried out 1 week prior to the dietary changes and animals were kept on different diets for 12 weeks. Castration and necropsy of all the animals was carried out in a dedicated necropsy room (Thermo Electron Corporation, Waltham, MA, USA). A total of 64 rats were used in the study.

All the animals were killed at the end of the experiment by cervical dislocation. Prostate, liver, adipose tissue and skeletal muscle were carefully removed. Rodent prostate consists of bilaterally symmetrical ventral, dorsolateral and anterior prostate (Sugimura *et al.*, 1986; Marker *et al.*, 2003). In the present investigation, the ventral prostate was used for further analysis. The left lobe of the ventral prostate was preserved at –80°C and used for the molecular studies. The right lobe of the ventral prostate was divided into three parts relative to the

urethra (distal, intermediate and proximal) and used for histological examinations. The animal groups maintained on NPD or HFD were designated as NPD or HFD and interventions (if any) such as pioglitazone treatment or castration are indicated by PIO or CAS, respectively, to the name of the group.

Diet for the development of experimental insulin resistance

Insulin resistance was induced in rats by feeding HFD (5.3 kcal g^{-1}, carbohydrate 17%, protein 25%, fat 58% kcal), while the control rats were fed with NPD (3.8 kcal g^{-1}, carbohydrate 67%, protein 21%, fat 12% kcal) for a period of 12 weeks. The NPD used to feed the animal was standard rodent chow (Pranaw Agro Industries, New Delhi, India). The detailed methodology for the HFD preparation has already been described by Srinivasan *et al.* (2004). In brief, the contents of HFD include NPD powder (36.5%), lard (31%), casein (25%), vitamin–mineral mix powder (6%), cholesterol (1%), DL-methionine (0.3%), Yee-sac powder (0.1%) and sodium chloride (0.1%).

Assays of biochemical parameters

The blood samples (≈0.8 mL) were collected from the orbital plexus of rats under light ether anaesthesia in heparinized microcentrifuge tubes. The plasma was separated by centrifugation (2500× *g*, 5 min) and analysed for glucose, triglycerides, total cholesterol and high-density lipoprotein (HDL)-cholesterol using commercially available spectophotometric kits (Accurex Biomedical Pvt. Ltd., Mumbai, Maharashtra, India). Plasma insulin or testosterone was estimated by rat/mouse insulin (Linco Research, St Charles, MO, USA) or testosterone (SYNTRON, Bioresearch, Inc., Carlsbad, CA, USA) ELISA kit according to the manufacturer's instruction.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Prostatic sections originating from intermediate part of the ventral prostate (right lobe) were used for the determination of cell death using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay kit (Calbiochem, Darmstadt, Germany). The assay was performed according to the manufacturer's instruction.

Glucose and insulin tolerance tests

Animals were kept on 6 h fasting for glucose and insulin tolerance test and a basal sample was taken, followed by i.p. injection of D-glucose (1 g·kg^{-1}) or bovine insulin (0.25 IU·kg^{-1}). Blood samples were collected at 5, 15, 30, 60, 90 and 120 min and plasma glucose concentration was determined to assess impairment in the glucose tolerance and glucose disappearance with time.

Adiposity index

Adiposity index was calculated by dividing the sum of the weight of epididymal fat pad, peri-renal fat pad (white adipose tissue, WAT) and inter-scapular fat pad (brown adipose tissue, BAT) by the body weight of the animal.

Histological examinations

Rats were anaesthetized with diethyl ether and killed by cervical dislocation. Ventral prostate (distal, intermediate and proximal), liver, muscle, epididymal fat, peri-renal fat and interscapular fat were removed and stored in 10% formal saline. Paraffin blocks were prepared after completing the routine processing. Sections (3–5 μm) were prepared from paraffin blocks and stained with haematoxylin and eosin. Epithelial infoldings in the prostatic acini and morphology of luminal secretory epithelial cells were examined in the distal, intermediate and proximal parts of the ventral prostate. Histological images were captured by a charged-coupled device camera attached with the Olympus microscope (Model BX 51, Tokyo, Japan). Cellular height was measured using the photomicrograph software (OLYSIA BioReport, Cell^F, Münster, Germany). At least 100 cells from each section were measured, and a minimum of two histological sections of each part of the ventral prostate were examined and cellular height was represented as percent of control.

Immunoblotting and immunoprecipitation

Protein samples were resolved on 10–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidine fluoride membrane and analysed with antibodies against proliferating cell nuclear antigen (PCNA), extracellular signal-regulated kinase (ERK) and its phosphorylated form (p-ERK), mitogen-activated protein kinase kinase (MEK), p38, caspase 3 and β-actin. The antigen-primary antibody complexes were incubated with horseradish-peroxidase (HRP) conjugated secondary antibodies and visualized by Western blot luminal reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Images were captured by ImageQuant-350 (Ver. 1.0.2). The protein quantification was done with ImageQuant TL (GE Healthcare, Buckinghamshire, UK) software and intensity values were normalized to β-actin. Tissue lysate was pre-cleaned by incubating with irrelevant primary antibody and Protein A/G plus-Agarose for 90 min. The pellet obtained after centrifugation was discarded and the supernatant was used for the further processing. Pre-cleaned tissue lysates (500 μg

protein) were incubated with ERK antibody for 16 hat 4°C. Subsequently, the immune complex was precipitated with Protein A/G plus-Agarose (SantaCruz Biotechnology) for 6 h at 4°C. The immunoprecipitate was washed three times with lysis buffer. The protein sample was resolved on SDS-PAGE and immunoblotted as described above.

Immunohistochemistry

Prostatic sections originating from intermediate part of the ventral prostate (right lobe) were deparafinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM). The following rabbit polyclonal primary antibodies were used: anti-PCNA, 1:125; anti-ERK, 1:100; and anti-p-ERK, 1:50. Polyvalent biotinylated goat anti-rabbit secondary antibody and streptavidin peroxidase (STV–HRP) system was used to amplify the signals, followed by detection with diaminobenzidine as a chromogen. Slides were counterstained with haematoxylin, dehydrated with alcohols and xylene and mounted in DPX.

Statistical analysis

Statistical analysis was performed using Jandel SigmaStat statistical software (San Rafael, CA, USA). Significance of difference between two groups was evaluated using Student's t-test. For multiple comparisons, ANOVA was used and *post hoc* analysis was performed with Tukey's test. Results were considered significant if P-values were ≤ 0.05.

Materials

D-glucose and insulin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pioglitazone was a kind gift from Ranbaxy Research Laboratories, Gurgaon, Haryana, India. All the primary and secondary antibodies were obtained from SantaCruz Biotechnology, USA until unless mentioned. Cholesterol and DL-methionine was procured from HiMedia, Mumbai, India.

Results

Pioglitazone treatment restores normoglycaemia and normoinsulinaemia in insulin-resistant rats and improves insulin sensitivity

HFD feeding increased body weight and induced mild hyperglycaemia and hyperinsulinaemia in the rats. Glucose and insulin levels were decreased in the HFD-fed rats receiving pioglitazone treatment, compared to the diet-matched controls (Figure 1A–C). Further, HFD feeding led to impaired glucose tolerance and disposal following treatment with glucose or insulin in the experimental animals, compared to the NPD-fed rats. The area under curve (AUC) was increased in HFD-fed rats, compared to the NPD and NPD + PIO groups. There was partial restoration of the glucose tolerance and disposal in the HFD + PIO group, compared to the HFD-fed rats as shown by the decreased AUC (Figure 1F–I).

Pioglitazone redirects lipid to the adipose tissue and improves plasma lipid level

Plasma. The plasma triglyceride level was increased in HFD-fed rats, compared to the NPD-fed rats. Pioglitazone treatment decreased the triglyceride level in the HFD + PIO group compared to the diet-matched controls. Total cholesterol levels were also higher in HFD-fed rats than in the NPD-fed rats and pioglitazone treatment had no effect on total cholesterol. These effects of pioglitazone treatment were further analysed by determining HDL- and LDL-cholesterol separately. Although no significant difference was observed in the plasma HDL-cholesterol content between NPD, NPD + PIO, HFD and HFD + PIO groups, there was a tendency towards higher levels in the HFD + PIO group than in the diet-matched controls. However, plasma LDL-cholesterol was decreased in HFD-fed rats receiving pioglitazone treatment, compared to the diet-matched controls (Figure 2A,B).

Liver. Dietary manipulation increased liver weight. Triglyceride and cholesterol levels were also significantly increased in the liver of HFD-fed rats, compared to the NPD-fed rats. Liver triglyceride and total cholesterol content were significantly improved by pioglitazone in the HFD-fed rats (Figure 2C–E). Further, histological assessment revealed excessive deposition of fat in the hepatocytes of HFD-fed rats and its reversal with subsequent pioglitazone treatment (Figure 2G).

Muscle. HFD feeding increased the triglyceride content of skeletal muscle but this was not decreased after pioglitazone (Figure 2F,G).

Adipose tissue. HFD feeding increased the adiposity index in rats (Figure 1E) and histological examination of adipose tissue revealed increased adipocyte size in both BAT and WAT. Although pioglitazone treatment further increased the adiposity index, histologically, a reduction in the size of adipocytes was clearly evident in both BAT and WAT, with this effect of pioglitazone being more prominent in BAT than in WAT. In the BAT of HFD-fed rats receiving pioglitazone, several foci with small adipocytes were observed (Figure 2G).
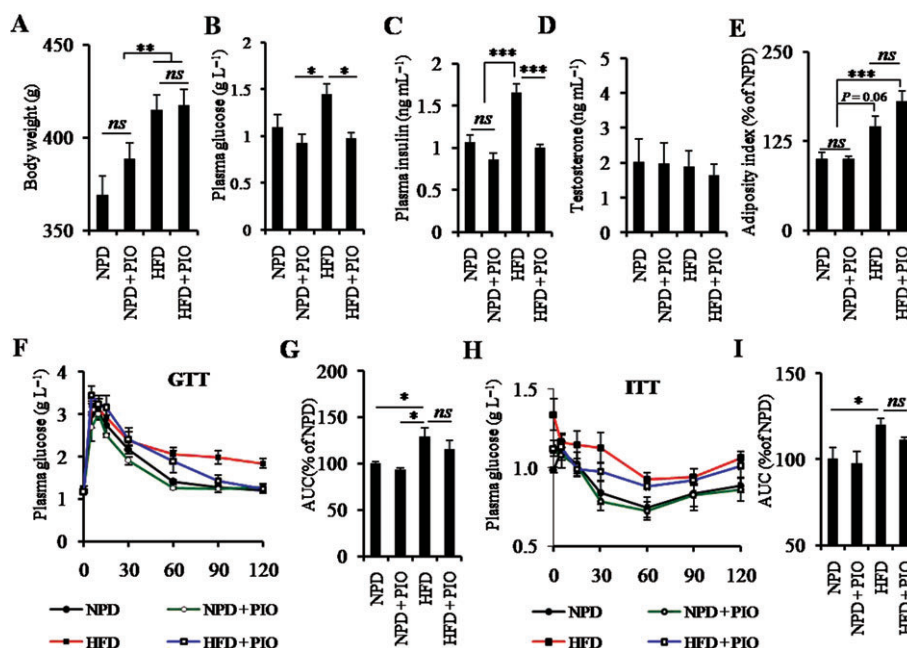
Figure 1

Pioglitazone treatment (PIO) restores normoglycaemia and normoinsulinaemia in insulin-resistant rats and improves insulin sensitivity. (A) Body weight was increased in high-fat diet (HFD)-fed rats, compared to the age-matched control ($n = 5–6$). (B,C) Pioglitazone treatment l decreased the plasma glucose (B) and insulin (C) level in the HFD-fed rats, compared to diet-matched controls ($n = 5$). (D) No change in the plasma testosterone level was observed among the different groups ($n = 4$). (E) The adiposity index was increased in HFD-fed rats and pioglitazone treatment further increased the adiposity ($n = 5$). (F–I) Pioglitazone partially improved HFD induced glucose intolerance (glucose tolerance test, GTT) and impairment in the insulin mediated glucose disposal (insulin tolerance test, ITT) as shown by changes in the area under curve (AUC) ($n = 4$). All the values are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and *ns* (not significant) $P > 0.05$. NPD, normal pellet diet.

Effect of pioglitazone treatment on the prostate gland

Both the absolute and relative weight of the ventral prostate were increased in the HFD-fed rats compared to the NPD-fed rats and both these variables were decreased in the HFD-fed rats receiving pioglitazone treatment, compared to diet-matched controls (Figure 3A,B). There was also a significant ($P < 0.001$) correlation between plasma insulins level and weight of the prostate gland (Figure 3C). Histological examination of the distal, intermediate and proximal parts of ventral prostate revealed increased infolding of epithelial layer in the prostatic acini of HFD-fed rats, compared to the NPD-fed rats. However, there was no clear effect of pioglitazone treatment on the epithelial infolding in NPD- as well as in HFD-fed rats. No differences were observed in the cellular height and morphology of luminal secretory epithelial cells among different groups (Figure 3D,E). Molecular analysis revealed significantly decreased PCNA level in the HFD-fed rats receiving pioglitazone treatment as compared to the diet-matched control. These results were confirmed by immunostaining of the prostatic sections for PCNA and quantified by counting the frequency of immunopositive cells (Figure 3H–J). HFD feeding also increased the expression of p38 in the prostate gland (Figure 3H,L).

Effect of pioglitazone treatment on apoptosis in prostate gland

Pioglitazone treatment increased the proportion of TUNEL-positive cells in prostate tissue from the HFD-fed rats, compared to the diet-matched control. In general, when prostatic sections were examined for apoptotic cells, apart from the occasional appearance of TUNEL-positive cells, most of them were primarily located either in the central part of the epithelial layer infoldings or in the upper part of the epithelial layer. However, in the HFD + PIO group, in addition to the normal pattern of apoptotic cell distribution, a rare phenomenon was observed where groups of epithelial cells were found to be TUNEL positive (Figure 4). Further, the caspase 3/procaspase 3 level was marginally increased ($P = 0.076$) in the HFD-fed rats after pioglitazone treatment, compared to the diet-matched controls (Figure 3O).
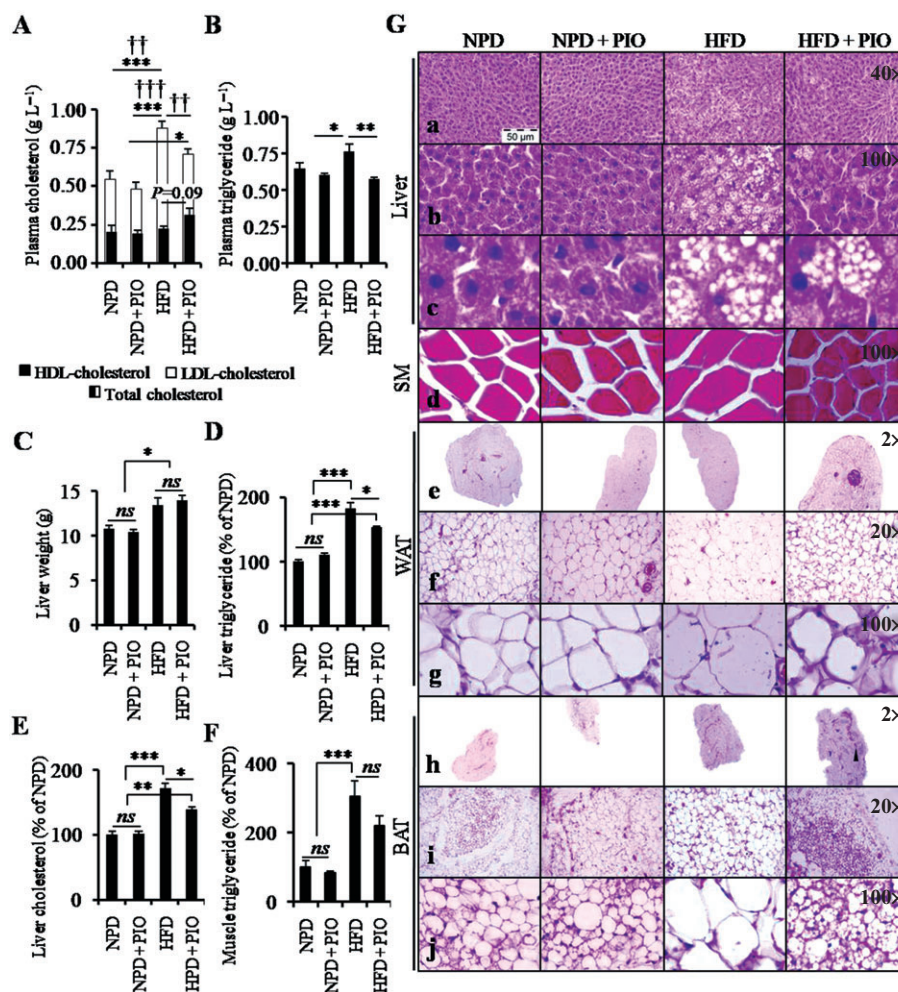
Figure 2

Pioglitazone redirects lipid to adipose tissue and improves lipid levels. (A,B) Pioglitazone treatment (PIO) decreased the plasma cholesterol (A) and triglyceride (B) levels in the high-fat diet (HFD)-fed rats compared to the diet-matched control. Although there were no significant differences between HDL-cholesterol level among all the groups, the HDL-cholesterol level tended to increase in the HFD + PIO group, compared to HFD-fed rats ($n = 5$). LDL-cholesterol was decreased in HFD + PIO group as compared to the HFD-fed rats. (C) HFD feeding significantly increased liver weight ($n = 5$). (D,E) Pioglitazone treatment decreased in the liver triglyceride (D) and cholesterol (E) level in the HFD-fed rats compared to the diet-matched control ($n = 5$). (F) Pioglitazone treatment marginally decreased the muscle triglyceride level in the HFD-fed rats compared to diet-matched controls ($n = 3$–5). (G) Representative photomicrographs showing effects of dietary manipulation and pioglitazone treatment on liver, muscle, brown adipose tissue (BAT) and white adipose tissue (WAT) at different magnifications. Panels a–c show excessive fat deposition in the hepatocytes and subsequent normalization with pioglitazone treatment. Panel d shows the histological sections of skeletal muscle (SM). Panels e–g show the enlargement of adipocytes of WAT in HFD-fed rats and normalization with pioglitazone treatment. Panels h–j shows the enlargement of adipocytes of WAT in HFD-fed rats and normalization with pioglitazone treatment. In BAT of HFD-fed rats receiving pioglitazone treatment, several foci of small adipocytes were observed and the representative photomicrographs were shown at different magnification. All the values are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns (not significant) $P > 0.05$ versus indicated group, ††$P < 0.01$, †††$P < 0.001$ versus indicated group for LDL-cholesterol. HDL, high-density lipoprotein; LDL, low-density lipoprotein; NPD, normal pellet diet.

Effect of pioglitazone treatment on the enhanced activation of ERK

In the HFD-fed rats, phosphorylation of ERK in prostatic tissue was higher than in the NPD-fed rats. Pioglitazone treatment decreased p-ERK levels in the HFD-fed rats, compared to the diet-matched controls (Figure 3F,G).

Effect of castration on the prostatic enlargement in diet-induced insulin-resistant rats

Dietary manipulation (NPD/HFD) with or/without pioglitazone treatment did not lead to any change in the plasma testosterone level (Figure 1E). However, to analyse more clearly possible roles of
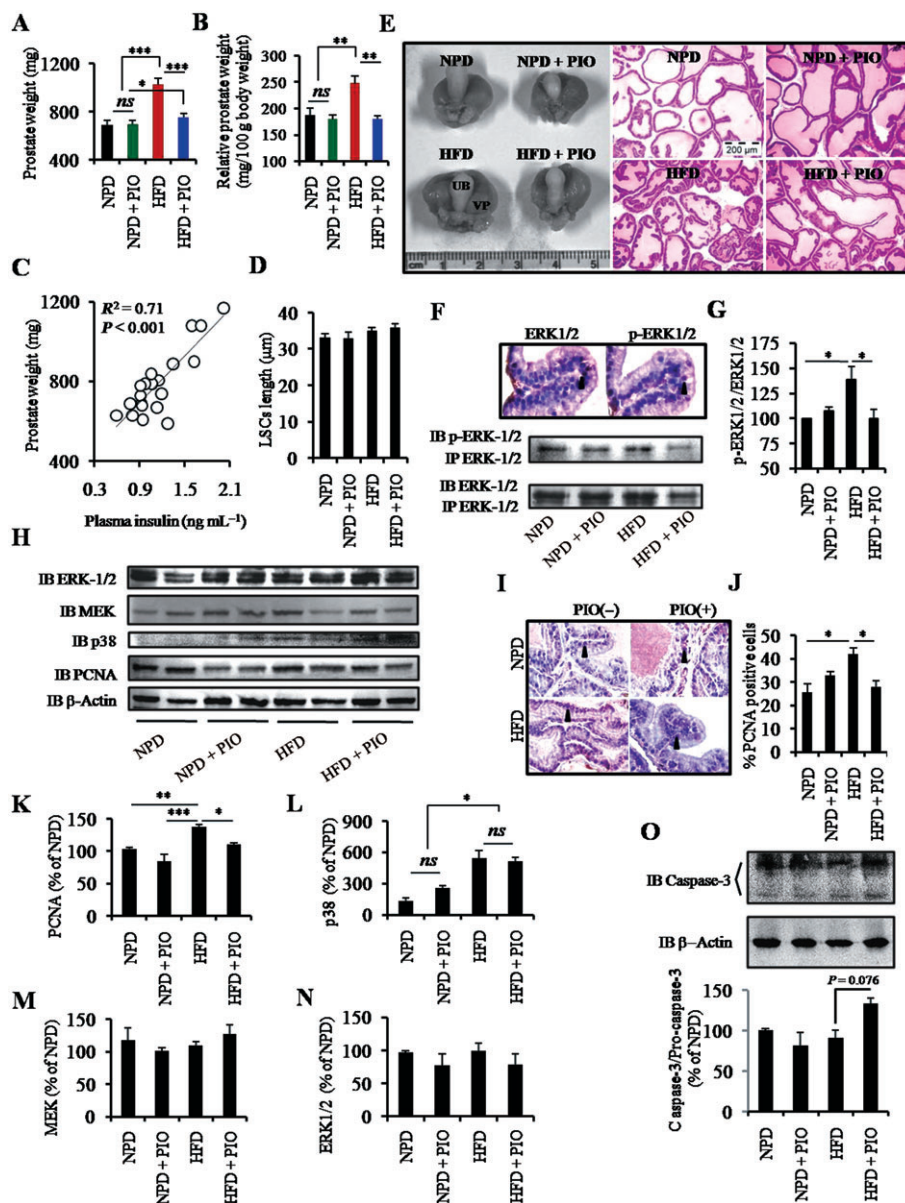
Figure 3

Pioglitazone restores Hyperinsulinaemia-induced augmented cell proliferation and prostatic enlargement. (A,B) High-fat diet (HFD) feeding increased in the absolute (A) and relative (B) prostate weight and pioglitazone treatment (PIO) inhibited the diet-induced prostatic enlargement ($n = 10$). (C) Correlation between plasma insulin level and prostate weight. (D) HFD feeding and subsequent pioglitazone treatment did not affect the cellular height and morphology of the luminal secretory epithelial cells of the ventral prostate ($n = 4$). (E) Representative photomicrographs showing prostatic enlargement in the HFD-fed rats and restoration with the pioglitazone treatment. Increased infolding of the epithelial layer was observed in HFD-fed rats, compared to normal pellet diet.(NPD)-fed rats. (F,G) Effects of dietary manipulation and pioglitazone treatment on the activation of extracellular signal-regulated kinase (ERK1/2). Photomicrographs showing immunohistochemical localization of ERK1/2 and phosphorylated ERK 1/2 (p-ERK1/2) in the nucleus of prostatic luminal secretory epithelial cells and Western blot analysis of immunoprecipitated ERK for phosphorylation (E). Significantly increased p-ERK1/2 : ERK1/2 ratio was observed in the HFD-fed hyperinsulinaemic rats. Pioglitazone treatment restored the p-ERK1/2 : ERK1/2 ratio in HFD-fed rats (F) ($n = 3$). (H) Immunoblots of different proteins [β-Actin, proliferating cell nuclear antigen (PCNA), p38, mitogen-activated protein kinase kinase (MEK) and ERK1/2]. (I) Immunostaining of the prostatic sections of different groups for PCNA. (J) A decrease in cells immunopositive for PCNA was observed in the HFD-fed rats receiving pioglitazone treatment compared to the diet-matched control. (K–N) Relative expression of PCNA, p38, MEK and ERK1/2 in different treatment groups. (O) Pioglitazone treatment increased in the caspase 3/pro-caspase 3 level in the HFD-fed rats compared to the diet-matched control. All the values are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and ns $P > 0.05$. NPD, normal pellet diet; UB, urinary bladder; VP, ventral prostate; IP, immunoprecipitation; IB, immunoblotting.
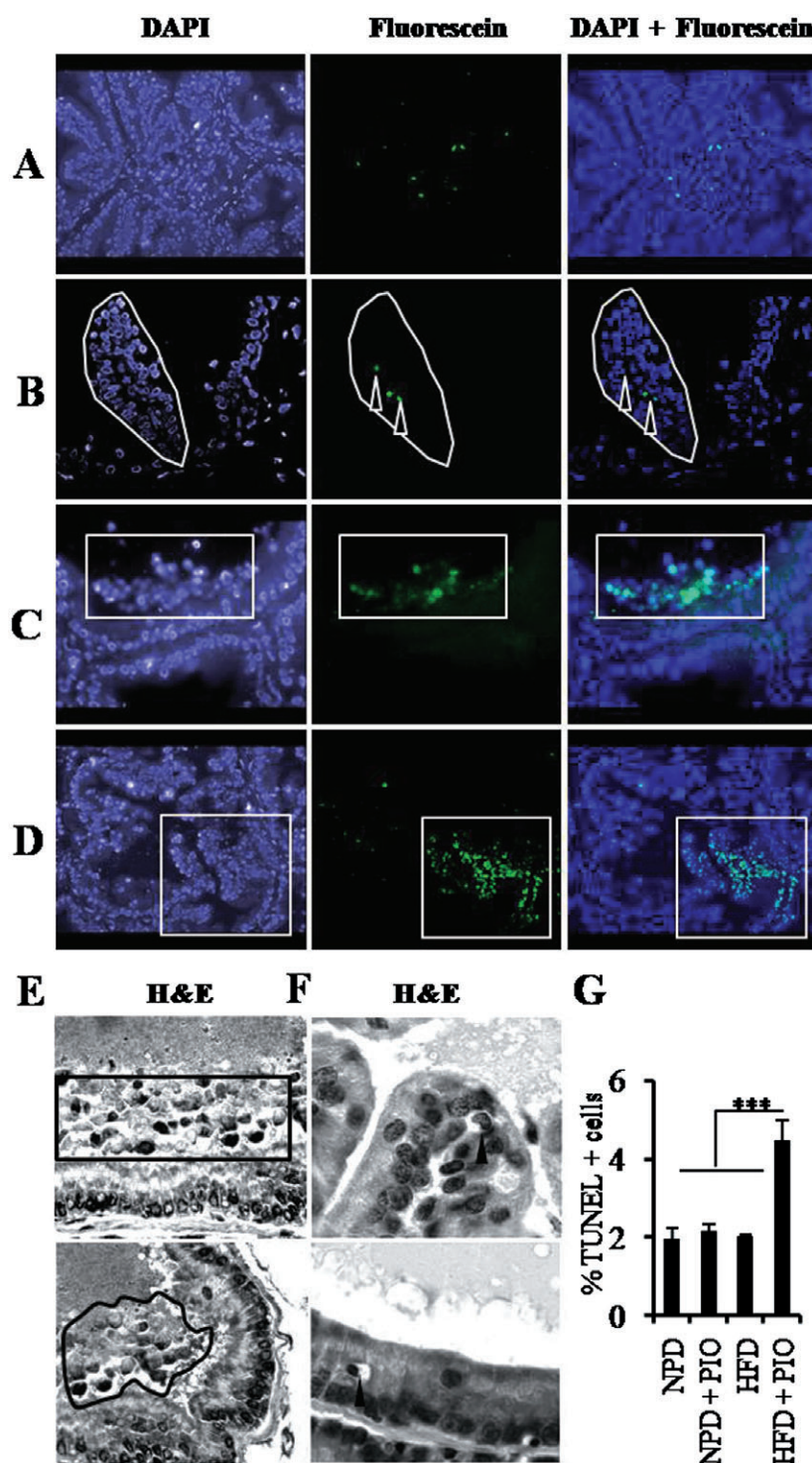
Figure 4

Pioglitazone increases cell death in the prostate of HFD-fed rats. (A–D) In general, when prostatic sections were evaluated for apoptotic cells, apart from occasional appearance of apoptotic cells (A) most of them were primarily located either in the central part of the epithelial layer infoldings (B) or upper part of the epithelial layer (C). These are the three position in which all the apoptotic cells were found in normal pellet diet (NPD), NPD + pioglitazone treatment (PIO) and high-fat diet (HFD)-fed rats, while in HFD + PIO group, in addition to the normal pattern of apoptotic cell distribution a rare phenomenon was observed where groups of epithelial cells were found to be terminal deoxynucleotidyl transferase dUTP nick en labelling (TUNEL) positive (D). (E) Detached cells at the upper part of the epithelial layer, with haematoxylin and eosin (H&E) staining, resembling the pattern of apoptotic cell death in C. (F) Typical apoptotic cell under H&E staining. (G) Significant increase in the percent TUNEL-positive cells were observed in the HFD + PIO group as compared to the NPD, NPD + PIO and HFD groups. All the values are shown as mean ± SEM. ***$P < 0.001$ versus indicated group. DAPI, 4′,6-diamidino-2-phenylindole.
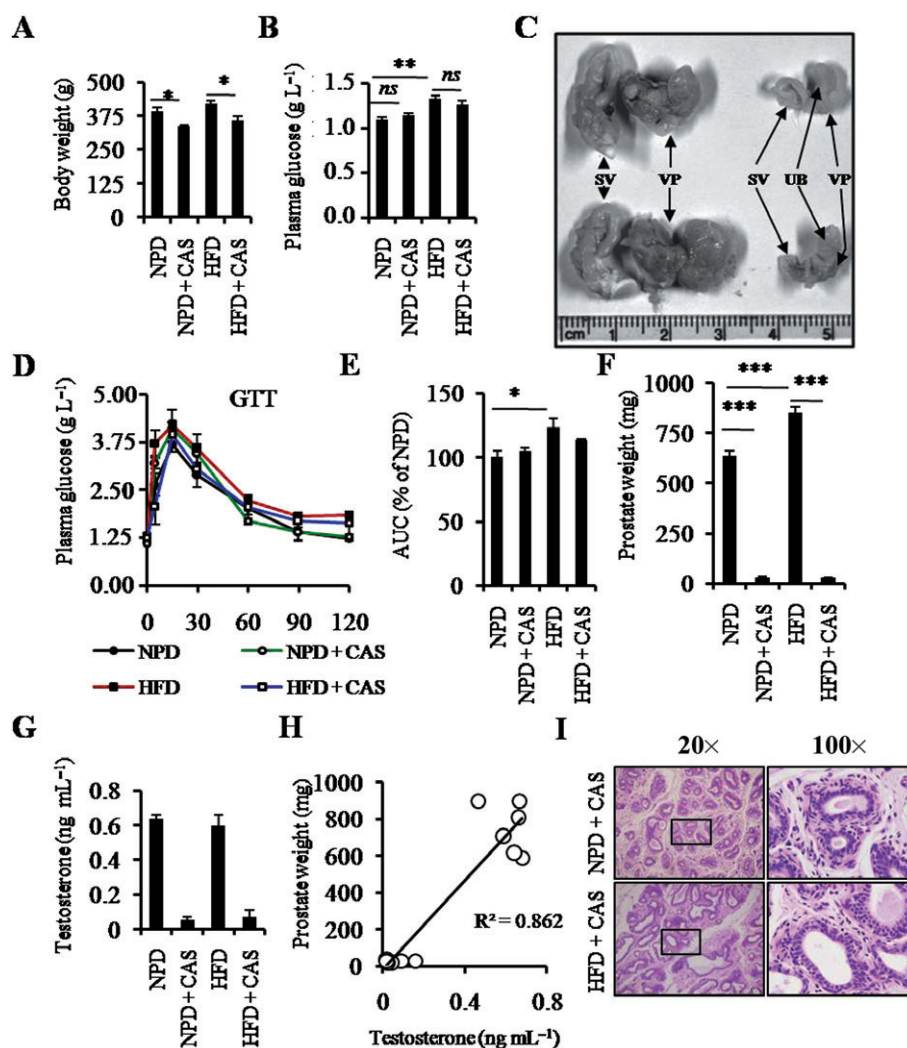
Figure 5

Effect of castration on prostatic growth. (A) Body weight was decreased in the castrated rats, compared to the diet-matched non-castrated control ($n = 4–5$). (B) High-fat diet (HFD) feeding induced mild hyperglycaemia ($n = 4–5$). (C) Representative photomicrograph showing remarkably smaller prostate in the castrated rats. (D) HFD induces glucose intolerance in Sprague–Dawley rat as determined by IPGTT ($n = 4$). (E) HFD feeding increased the area under curve (AUC) of the IPGTT in (D). (F,G) HFD feeding increased the prostate weight, compared to the age-matched control. Castration (CAS) significantly decreased the testosterone level and prostate weight in both NPD- and in HFD-fed rats ($n = 5$). (H) Scatter plot between plasma testosterone level and prostate weight indicating the crucial role of testosterone in the prostatic growth. (I) Castration reduced the height of prostatic epithelial cells in NPD/HFD-fed rats. All the values are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and *ns* (not significant) $P > 0.05$. IPGTT, intra-peritoneal glucose tolerance test; SV, seminal vesicle; UB, urinary bladder; VP, ventral prostate; GTT, glucose tolerance test.

testosterone in prostatic enlargement in our model, a subgroup of animals were castrated, kept on different diets and the consequent effects on the prostate gland assessed. Significant increases in the weight of prostate glands of HFD-fed rats were observed, compared to NPD-fed rats, confirming our earlier results. Castration led to dramatic decrease in plasma testosterone level and prostate weight in both NPD- and in HFD-fed rats. Significant decrease in the cellular height was observed in both NPD- and in HFD-fed rats (Figure 5).

Discussion and conclusions

Prostate growth and development is a complex phenomenon and is sensitive to the overall endocrine microenvironment. Previously, we have reported increased cell proliferation and contractility of the prostate gland in hyperinsulinaemic rats (Vikram *et al.*, 2008). That study provided mechanistic links between insulin resistance and BPH, and explained its association with the metabolic syndrome. In the present investigation, we report that the PPARγ

agonist pioglitazone attenuated the enhanced cell proliferation and enlargement of the prostate gland in HFD-fed insulin-resistant rats by improving glucose disposal, lipid distribution and restoring the plasma insulin level (Figure 6).
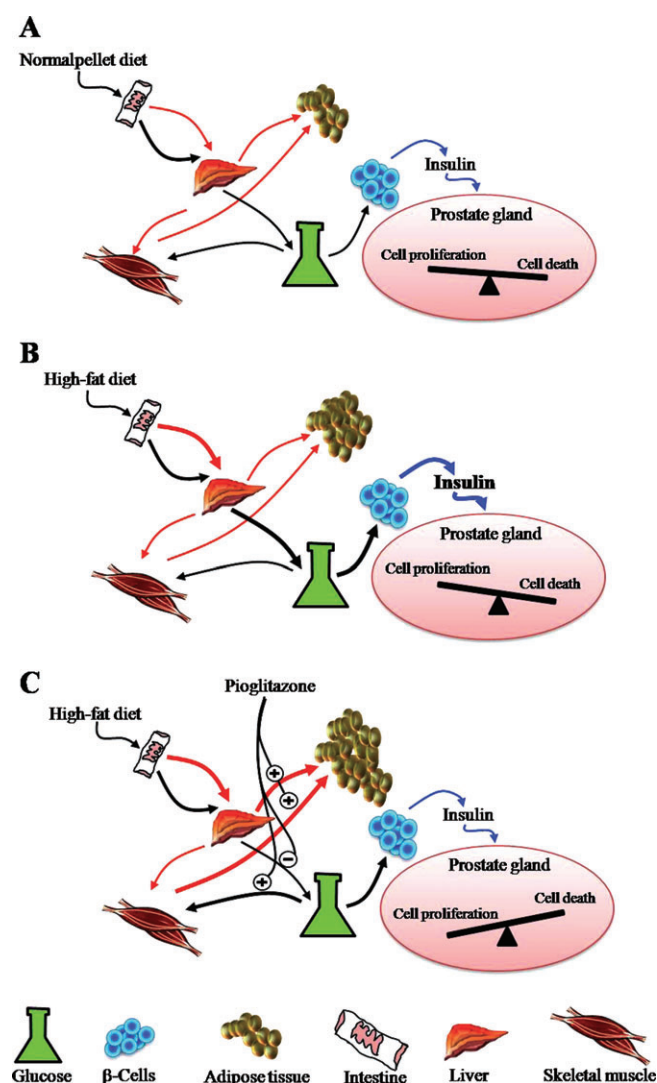

Figure 6

Schematic model of pioglitazone induced reversal of augmented cell proliferation and enlargement of prostate gland in diet-induced insulin-resistant rats. Diet rich in fat causes deposition of fat in liver and muscle apart from adipose tissues. Increased hepatic glucose production and/or decreased uptake by muscle leads to hypergly-caemia and compensatory hyperinsulinaemia. Hyperinsulinaemia directly or indirectly destabilizes cellular equilibrium and results in augmented cell proliferation and enlargement of the prostate gland. Pioglitazone redirects distribution of lipid from liver and muscle to the adipose tissue as well as improving plasma lipid levels. Redistri-bution of fat from non-adipose tissue to the adipose tissue causes further increase in the adiposity of HFD-fed rats receiving pioglita-zone treatment. Pioglitazone treatment restores fasting glucose and insulin level. Decreased insulin level causes compensatory increase in the apoptosis, decrease in the cell proliferation and weight of the prostate gland.

Insulin resistance is accompanied by a compensatory rise in the plasma insulin level, which is known to have growth-promoting effects. Pioglitazone treatment impedes the development of hyperglycaemia, hyperinsulinaemia and insulin resistance in the HFD-fed rats (Figure 1). Although the exact mechanism(s) of action of pioglitazone is not well understood, it is known to decrease free fatty acid levels and insulin gene transcription, to remodel lipid distribution and to improve glucose disposal in insulin-resistant individuals (Lebovitz and Banerji, 2001; Kubota *et al*., 2006; Quinn *et al*., 2008; Rizos *et al*., 2008; Schinner *et al*., 2009). At the molecular level, it acts as a ligand for PPARγ, a nuclear receptor of the NR1C family, expressed predominantly in adipose tissue. Pioglitazone treatment almost completely restored fasting plasma glucose and insulin level, while partially restored glucose intolerance and insulin induced glucose disappearance in the HFD-fed rats. The beneficial effects of pioglitazone on different metabolic parameters were in accordance with the previous findings (Takatori *et al*., 2008; Moritoh *et al*., 2009; Collino *et al*., 2010).

In addition to improving glucose disposal, pioglitazone favourably altered lipid distribution in the body. Deposition of fat in the non-adipose tissue such as liver and muscle has been implicated in the development of insulin resistance (Medina-Gomez *et al*., 2007). Fatty liver and hepatic triglyceride accumulation are strongly associated with obesity and insulin resistance (Weickert and Pfeiffer, 2006; Kirchhoff *et al*., 2007). To clearly understand the effect of pioglitazone on the lipid profile, triglyceride content was also measured in liver and muscle. Despite a comparable increase in the body weight and even an increase in the adiposity (Figure 1A,I), pioglitazone treatment markedly decreased fat deposition in the liver and muscle of HFD-fed rats (Figure 2). Excessive deposition of fat in the liver and muscle (which are not meant for the storage of fat) can affect their normal functioning such as insulin-associated glucose release and uptake. The PPARγ agonist pioglitazone is known to improve lipid distribution (Yang and Smith, 2007), insulin sensitivity and to suppress hepatic glucose production (Natali and Ferrannini, 2006). Ye *et al*. (2004) demonstrated that lipid sequestration was a possible mechanism by which rosiglitazone improved fatty acid-induced insulin resistance. In the present study, pioglitazone treatment might have redirected the distribution of lipid from the liver and muscle to the adipose tissue, resulting in the improved insulin sensitivity. Our results are consistent with those of Collino *et al*. (2010), who recently reported that pioglitazone treatment improved lipid and insulin level in overweight rats. Despite increased adiposity,

numerous foci with small adipocytes in the BAT indicate the formation of new adipocytes in the HFD-fed rats receiving pioglitazone treatment. Fat cell enlargement has been suggested as an independent marker of insulin resistance and hyperinsulinaemia (Lundgren *et al.*, 2007). Adipogenesis in the HFD-fed rats receiving pioglitazone treatment appears to be responsible for the clearance of fat from non-adipose tissues. Despite a decrease in the total cholesterol content, the increase in HDL-cholesterol and concomitant decrease in LDL-cholesterol in the HFD + PIO group indicate a valuable effect of pioglitazone treatment on the plasma lipid profile (Figure 2A,B). Further, pioglitazone improves insulin sensitivity in patients with impaired glucose tolerance through a reduction in the muscle lipid content and its redistribution into subcutaneous adipose tissue (Rasouli *et al.*, 2005). Based on these results, it can be concluded that the excessive fat deposition in liver and muscle might contribute to the increased cell proliferation and enlargement of the prostate gland by promoting the development of insulin resistance and ultimately hyperinsulinaemia.

To explore the effect of improved insulin sensitivity and reversal of compensatory hyperinsulinaemia, we assessed cell proliferation, apoptosis and different molecular elements in the prostate gland. Pioglitazone treatment led to decreased cell proliferation and increased cell death in the prostate of insulin-resistant rats as shown by corresponding changes in the PCNA level (Figure 3G–J) and frequency of TUNEL-positive cells (Figure 4). Cell proliferation and death is a continuous process and is sensitive to the hormonal microenvironment. Increased cell death with simultaneous decrease in cell proliferation in HFD-fed rats receiving pioglitazone treatment might be due to a decline in the insulin level. Decreased insulin level might also provide an explanation of the rare pattern of cell death observed only in the HFD-fed rats receiving pioglitazone treatment (Figure 4D). Further, to confirm the results obtained with TUNEL assay, caspase 3 level was examined in the prostate gland. Increase in the caspase 3/procaspase 3 was observed in the HFD + PIO group relative to the diet-matched controls (Figure 3L).

One pathway of insulin signalling that is dependent on insulin receptor substrate (IRS)/phosphatidyl inositol 3-kinase (PI3-kinase) is mainly concerned with the glucose uptake and metabolic effects, whereas MEK/ERK-dependent signalling is responsible for its growth-stimulating actions. During insulin resistance, the IRS/PI3-kinase pathway is impaired, whereas the MEK/ERK pathway remains unaffected (Jiang *et al.*, 1999).

Controlled regulation of MEK/ERK signalling is required for the cell proliferation and differentiation (O'Neill and Kolch, 2004; Shaul and Seger, 2007). Previously we have reported enhanced activation of ERK-1/2 during hyperinsulinaemia as a possible explanation for increased cellular proliferation and prostatic enlargement in hyperinsulinaemic rats (Vikram *et al.*, 2010a), and hence ERK-1/2 activation was determined. As expected, a significant increase in the p-ERK-1/2-to-ERK-1/2 ratio was observed in the HFD-fed rats and pioglitazone treatment prevented that diet-induced increase in ERK-1/2 activation. The results of the present study indicated that the decreased plasma insulin level blocked MEK/ERK signalling in the prostate gland and might be responsible for the decrease in the cell proliferation. Quantification of different molecular elements indicated mainly an increase in the expression of PCNA and p38 in the HFD-fed rats, compared to NPD-fed rats. Although pioglitazone treatment blocked the increased expression of PCNA, no change in the p38 level was observed in the HFD-fed rats. Evaluation of the role of increased p38 expression in the prostate of HFD-fed rats could lead to a better understanding of prostatic enlargement under hyperinsulinaemic conditions.

Compensatory hyperinsulinaemia under insulin-resistant conditions could also affect IGF and androgen signalling. IGFs and androgens have been known to play an important role in the normal as well as pathological growth of the prostate gland. Apart from increased IGF-1 and androgen level, hyperinsulinaemia has been associated with decreased level of plasma insulin-like growth factor-binding protein-1 and sex hormone-binding globulin (Nam *et al.*, 1997; Attia *et al.*, 1998). IGF-1 is a potent inducer of prostatic growth *in vivo* (Torring *et al.*, 1997), and a high level of plasma IGFs has been associated with a higher risk of BPH (Chokkalingam *et al.*, 2002). Recently, Fan *et al.* (2007) reported that IGF-1/insulin signalling activated androgen signalling through direct interaction of Foxo 1 with the androgen receptor. Direct involvement of Foxo 1, a substrate of Akt/PKB, suggests the involvement of IRS/PI3-kinase signalling in the HFD-fed hyperinsulinaemic rats. Further, to substantiate these findings and assess a possible role of androgen signalling, a separate study was performed with castration of the experimental animals. Dramatic decrease of the prostate gland in both NPD- and in HFD -fed rats, compared to non-castrated diet-matched control confirmed the crucial role of androgens in prostatic growth. Taking the results together, it appears that the hyperinsulinaemic condition can promote prostatic growth, without affecting the testosterone level, but the presence of

testosterone is critical. The hyperinsulinaemic condition might augment androgen signalling in the prostate through increased inactivation of Foxo 1 by the IRS/PI3-kinase branch of insulin signalling.

Dietary fatty acids can directly affect prostate growth, as a recent report showed prostatic enlargement and increased expression of androgen receptors and PPARγ in rats receiving saturated dietary fat (Escobar *et al.*, 2009). Previous studies (Cai *et al.*,

2001; Escobar *et al.*, 2009; Vikram *et al.*, 2010a) and the results of the present investigation provide substantial evidence for the prostatic growth-promoting effects of saturated dietary fat, involving distinct molecular mechanisms (Figure 7).

Instability of the cellular equilibrium either due to augmented cell proliferation and/or decreased cell death is the key signature of prostatic hyperplasia, in contrast to the normal adult prostate. In
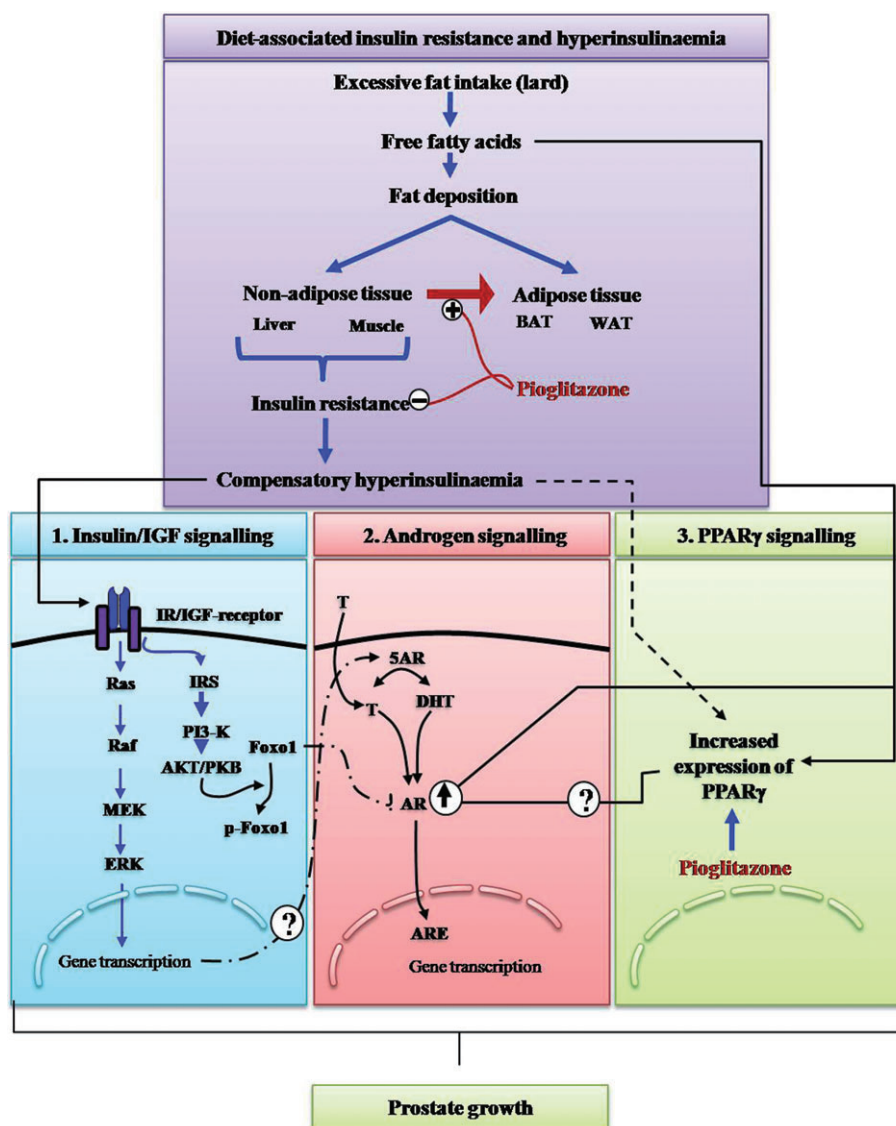

Figure 7

Possible interactions between insulin, androgen and PPARγ signalling: Enlargement of prostate gland in diet induced insulin-resistant rats. Excessive fat intake leads to increased free fatty acid level and accumulation of fat in the liver, muscle and adipose tissue, a condition implicated in the development of insulin resistance and hyperinsulinaemia. Pioglitazone redirects distribution of fat from non-adipose tissues to the adipose tissue, improves insulin sensitivity and thereby compensatory hyperinsulinaemia. Hyperinsulinaemia can directly augment prostatic growth through MEK/ERK signalling and can affect androgen signalling through IRS/PI3-kinase signalling. Saturated dietary fat affects expression of PPARγ, androgen receptors and prostatic growth. Pioglitazone can further affect prostatic growth owing to the increased expression of PPARγ. 5AR, 5-α reductase; AR, androgen receptor; ARE, androgen response element, BAT, brown adipose tissue; DHT, dihydrotestosterone; ERK, extracellular signal-regulated kinase; IGF, insulin-like growth factor; IR, insulin receptor; IRS, insulin receptor substrate; MEK, mitogen-activated protein kinkase; PI3-kinase, phosphatidyl inositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor-γ; T, testosterone; WAT, white adipose tissue.

the present investigation, we report that pioglitazone attenuated enhanced cell proliferation and enlargement of prostate gland in insulin-resistant rats. Increased cell death and concomitant decrease in the cell proliferation in prostate gland as well as improvement in the insulin sensitivity and lipid profile indicates that existing or new PPARγ agonists should be considered for the treatment of prostatic enlargement in patients with the metabolic syndrome. Our results show a previously unexplored therapeutic potential of pioglitazone in prostatic enlargement under insulin-resistant conditions and further suggest that targeting the re-distribution of lipid from non-adipose tissue such as liver and muscle to adipose tissue and the pathways of insulin signalling could be new strategies for the treatment of BPH.

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Conflict of interest

None.

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