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Sex steroids deficiency impairs glucose transporter 4 expression and its translocation through defective Akt phosphorylation in target tissues of adult male rat

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Abstract

There is a substantial body of evidence suggesting that altered level of sex steroids in male is associated with insulin resistance and type 2 diabetes mellitus. However, the mechanism of this effect is not apparent. Our recent study indicated that testosterone deprivation decreases insulin receptor expression and glucose oxidation in insulin target tissues. The present study was designed to assess the impact of deficiency of testosterone and estradiol on Akt phosphorylation, glucose transporter expression, and glucose uptake in skeletal muscle, adipose tissue, and liver of adult male rat. Adult male albino rats of Wistar strain were orchidectomized and supplemented with testosterone (100 μg/100 g body weight per day), estradiol (5 μg/100 g body weight per day), and their combination (100 μg testosterone plus 5 μg estradiol per 100 g body weight per day) for 15 days from the 11th day postorchidectomy. On the day after the last treatment, animals were perfused; and blood was collected for the assay of plasma glucose, serum insulin, testosterone, and estradiol. Gastrocnemius muscle, adipose tissue, and liver were dissected out and used for the assay of various parameters such as Akt phosphorylation, glucose transporter (GLUT) 2 and 4 expression, glucose uptake, and glycogenic and glycogenolytic enzymes activity. Castration elevated the blood glucose level, which was accompanied by inhibitory effect on serum insulin, Akt phosphorylation, GLUT4 expression and its plasma membrane population, glucose uptake, glycogen and glycogen synthase activity, and stimulatory effect on GLUT2 expression and glycogen phosphorylase activity in tissues studied. After testosterone and its combination with estradiol supplementation to castrated rats, a normal pattern of all these parameters was restored. Estradiol administration to castrated rats increased the Akt phosphorylation without altering other parameters studied. It is concluded from the present study that sex steroids deficiency–induced defective glucose uptake in skeletal muscle and adipose tissue is mediated through defective Akt phosphorylation and GLUT4 expression in plasma membrane.

1. Introduction

The modalities of androgen-deprivation therapy (ADT) include surgery (orchidectomy) or medical therapy (gonadotropin-releasing hormone agonists or antagonists), and it has been the cornerstone for the treatment of advanced and metastatic prostate cancer [1]. The aim of ADT is to achieve serum testosterone levels as low as possible [2], but it is associated with insulin resistance [3]. Male hypogonadism is associated with increased fat mass, decreased libido, impotence, decreased muscle strength, decreased quality of life, and osteoporosis [4]. Although these complications of hypogonadism are well established, newer complications have recently surfaced. In recent years, it has become evident that one of the complications of male hypogonadism is insulin resistance and type 2 diabetes mellitus [5,6]. Epidemiologic studies have shown that low testosterone levels predict the development of insulin resistance, type 2 diabetes mellitus, and metabolic syndrome in men [7-9]. Studies have also confirmed a direct relationship between serum testosterone and insulin sensitivity [10,11]. These findings are further supported by interventional studies showing an improvement in insulin sensitivity with testosterone replacement in hypogonadal obese men [12-14]. These observations are significant considering the fact that men undergoing ADT have low levels of testosterone that may put them at a higher risk of developing

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these complications. Experimental study also indicated that testosterone deprivation due to castration in male rat induces insulin resistance, whereas testosterone replacement improves the insulin sensitivity [15]. Furthermore, Morimoto et al [16] have shown through in vivo and in vitro studies that testosterone has a direct effect upon pancreatic islet function by favoring insulin gene expression and release. At physiologic levels, testosterone is suggested to be involved in maintaining normal insulin sensitivity [17]; and outside the "physiologic window," this steroid hormone promotes insulin resistance [18].

Several lines of evidence suggest that estradiol may play a role in mediating the effects of testosterone on insulin action. Ingestion of anabolic steroids, which are of nonaromatizable 17α-alkylated androgens, induces insulin resistance in men [19,20]. Similarly, administration of dihydrotestosterone, another nonaromatizable androgen, has no effect on insulin sensitivity in men with central obesity in contrast to the beneficial effects of testosterone in the same population [21]. In addition, insulin resistance is part of the phenotype of congenital estrogen deficiency due to mutations in either the aromatase [22,23] or estrogen receptor (ERα) genes [24,25] in both human and mouse models. Moreover, estrogen therapy causes a significant improvement in insulin sensitivity in these models [22,25,26]. Therefore, studies using selective suppression of testosterone and estradiol are needed to determine their relative importance in influencing insulin action.

Both testosterone and estradiol affect insulin action, but the molecular mechanisms involved are not known completely. We have recently reported that castration-mediated impairment in glucose oxidation is coupled with decreased serum insulin and its receptor expression in adipose tissue, liver, and skeletal muscles [27]. Moreover, defects at the level of insulin receptor, IRS, PI3K, Akt, glucose transporter (GLUT) 4 expression, and its translocation to plasma membrane have been observed in skeletal muscles and adipose tissue of subjects with type 2 diabetes mellitus [28,29]. Thus, receptor and postreceptor defects in insulin target tissues contribute to whole-body insulin resistance in type 2 diabetes mellitus [29,30]. Hence, the present study was undertaken to identify Akt phosphorylation, GLUT2 and GLUT4 expression, GLUT4 translocation to plasma membrane, and glucose uptake in adipose tissue, liver, and skeletal muscle of castrated and hormone-replaced rats.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in the present study were of molecular and analytical grade; and they were purchased from Sigma Chemical, St. Louis, MO; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; and Sisco Research Laboratories, Mumbai, India. Glucose estimation kit was supplied by Linear Chemicals, Barcelona, Spain. ^{14}C-2-deoxyglucose was purchased from the Board of Radiation and Isotope Technology, Mumbai, India. Radioimmunoassay (RIA) kit for the assay of insulin, testosterone, and estradiol was obtained from Diasorin, Saluggia, Italy. Total RNA isolation reagent (TRIR) was purchased from ABgene, Epsom, United Kingdom. Omniscript Reverse-Transcription and Real-Time qPCR SYBR Green I kits were obtained from Qiagen, Hilden, Germany, and Eurogentec, Seraing, Belgium, respectively. The GLUT2 and GLUT4 primers and the β-actin monoclonal antibody were purchased from Sigma Chemical. Polyclonal GLUT4 and monoclonal GLUT2 antibodies were generously gifted by Prof Geoffrey D Holman, Department of Biology and Biochemistry, University of Bath, United Kingdom, and Prof Bernard Thorens, Institut De Pharmacologie Et De Toxicologie, Universite De Lausanne, Switzerland, respectively. Monoclonal Akt and phospho-Akt antibodies were purchased from Cell Signaling Technology, Danvers, MA.

2.2. Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethical Committee (no. 03/016/05). Healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180 to 200 g (100 days old) were purchased from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India, and used in the present study. Animals were housed in polypropylene cages under specific humidity (65% ± 5%) and temperature (21°C ± 2°C) with constant 12-hour light and 12-hour dark schedule. They were fed with standard rat pelleted diet (Lipton India, Mumbai, India), and clean drinking water was made available ad libitum.

2.3. Experimental design

Rats were divided into 5 groups each consisting of 6 animals, as follows: group I—control (sham-operated rats received vehicle and were used as intact control); group II—orchidectomized (ORD) rats were treated with vehicle (orchidectomy was performed under ether anesthesia via the scrotal route); group III—orchidectomized rats treated with testosterone (ORD + T) (dissolved in propylene glycol) after 10 days of orchidectomy at a dose of 100 μg/100 g body weight per day subcutaneously in 2 equally divided doses at 8:00 AM and 6:00 PM for 15 days; group IV—orchidectomized rats treated with estradiol (ORD + E) (dissolved in propylene glycol) after 10 days of orchidectomy at a dose of 5 μg/100 g body weight per day subcutaneously in 2 equally divided doses at 8:00 AM and 6:00 PM for 15 days; and group V—orchidectomized rats treated with testosterone plus estradiol at a dose as given for group III and group IV animals. Testosterone [31] and estradiol [32] doses were selected based on previous studies. At the end of treatment, animals were anesthetized with ether, blood was collected through incision in the right atrium, sera were separated and stored at −80°C until the

assay of hormones, and 20 mL of isotonic sodium chloride solution was perfused through the left ventricle to clear blood from the liver [33]. Skeletal muscle (gastrocnemius), liver, and subcutaneous white adipose tissue from abdominal region were quickly excised and used for the assay of various parameters. In the rat, fast-twitch oxidative glycolytic and fast-twitch glycolytic fibers were located in both the medial and lateral regions of the gastrocnemius muscle [34], which has high glucose utilization capacity. Therefore, gastrocnemius muscle was considered for the present study. Subcutaneous white adipose tissue is abundant in the abdomen; and excessive central adiposity (especially intraabdominal) has been linked to the metabolic syndrome, which includes insulin resistance, dyslipidemia, and increased risk of cardiovascular diseases [35,36]. Therefore, subcutaneous white adipose tissue was also considered.

2.4. Plasma glucose

One day before the last injection, blood samples were collected after overnight fasting in microfuge tubes containing EDTA by puncturing the orbital sinus with the help of heparinized microhematocrit capillary tubes [37]. Plasma was separated by centrifugation for 10 minutes at 800*g* at 4°C within 30 minutes to prevent autoglycolysis by leukocytes. Plasma glucose was estimated by glucose oxidase-peroxidase method (CPC Diagnostics). The coefficient of variation was 1.8%. Results are expressed as milligrams per deciliter.

2.5. Radioimmunoassay

Serum testosterone was assayed using solid-phase RIA kit obtained from Diasorin. The sensitivity of the assay was 0.05 ng/mL. The percentage cross-reactivity of the testosterone antiserum to other steroids such as 5α-dihydrotestosterone and androstenedione is 6.9% and 1.1%, respectively. Intraassay coefficient of variation (CV) was less than 8%, and interassay CV was less than 7.6%. Results are expressed as nanograms per milliliter.

Serum estradiol was assayed using solid-phase RIA kit obtained from Diasorin. The sensitivity of the assay was 2 pg/mL. The percentage cross-reactivity of the estradiol antiserum to other steroids such as estrone and estriol is 0.0063% and 0.0065%, respectively. Intraassay CV was less than 2.6%, and interassay CV was less than 6.1%. Results are expressed as picograms per milliliter.

Serum insulin was assayed using ^{125}I-labeled RIA kit obtained from Diasorin. The sensitivity of the assay was 4 μU/mL. The percentage cross-reactivity of insulin antibody to C-peptide was less than 0.01%. Intraassay CV was less than 10.6%, and interassay CV was less than 10.8%. Results are expressed as microunits per milliliter.

2.6. Isolation of total RNA

Total RNA was isolated from control and experimental samples using TRIR kit from ABgene. Briefly, 100 mg of fresh tissue was homogenized with 1 mL TRIR; and the homogenates were transferred immediately to a microfuge tube and kept at 4°C for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Afterward, 0.2 mL of chloroform was added, vortexed vigorously for 15 seconds, and placed on ice at 4°C for 10 minutes. The homogenates were centrifuged at 12 000*g* for 15 minutes at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube, and an equal volume of isopropanol was added and stored for 10 minutes at 4°C. The samples were centrifuged at 12 000*g* for 10 minutes. The supernatant was removed, and RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 minutes at 7500*g* (4°C). The RNA pellets were mixed with 50 μL of autoclaved Milli-Q water (Millipore, Danvers, MA). The concentration and purity of RNA were determined spectrophotometrically at $A_{260/280}$ nm. The purity of RNA obtained was greater than 1.75. The yield of RNA is expressed in micrograms.

2.7. Reverse transcription real-time polymerase chain reaction

Total RNA (2 μg) was reverse-transcribed using final volume of 1 μmol/L Oligo-dT primer, 0.5 μmol/L dNTPs, 10 U ribonuclease inhibitor, and 4 U Omniscript reverse transcriptase (Qiagen). The reaction was carried out in an Eppendorf (Hamburg, Germany) thermocycler at 37°C for 60 minutes. The resulting complementary DNAs were diluted with Milli-Q water. Real-time polymerase chain reaction (PCR) was carried out on Mx 3000p Multiplex quantitative PCR system (Stratagene, La Jolla, CA). The following specific oligonucleotide primers were used for the real-time PCR. One 21-mer oligonucleotide primer and one 18-mer oligonucleotide primer for rat GLUT4 (Pubmed nucleotide accession no. NM_012751) were selected as previously described by Liu et al [38]. The sense primer is 5′-GGG CTG TGA GTG AGT GCT TTC-3′, and the antisense primer is 5′-CAG CGA GGC AAG GCT AGA-3′. The predicted size of the amplified fragment by real-time PCR is 150 base pairs (bp). Two 24-mer oligonucleotide primers for rat GLUT2 (Pubmed nucleotide accession no. NM_012879) were selected as previously described by Houghton et al [39]. The sense primer is 5′-CTC GGG CCT TAC GTG TTC TTC CTT-3′, and the antisense primer is 5′-TGG TTC CCT TCT GGT CTG TTC CTG-3′. The predicted size of the amplified fragment by real-time PCR is 238 bp. One 22-mer oligonucleotide primer and one 21-mer oligonucleotide primer for β-actin (Pubmed nucleotide accession no. V01217; J00691) were selected as described previously [40]. The sense primer is 5′-AAG TCC CTC ACC CTC CCA AAA G-3′, and the antisense is 5′-AAG CAA TGC TGT CAC CTT CCC-3′. The predicted size of the amplified fragment by real-time PCR is 96 bp. Reaction was performed in 20 μL using 100 nmol/L of specific primers, a diluted complementary DNA, and 2× SYBR Green I PCR Master mix (Eurogentec) according to the manufacturer's instructions. The thermal cycling protocol was as follows: 95°C for

10 minutes, followed by 37 cycles of PCR at 95°C for 30 seconds and 60°C for 1 minute. All reactions were performed in triplicate with no-template control. Melting curve analysis was performed on each sample to determine the presence of multiple amplicons, nonspecific products, and contaminants. Random samples of PCR product were resolved on a 2% agarose gel (Amersham Biosciences) and visualized with the use of ethidium bromide (Amersham Biosciences) as a quality control. Agarose gel was compared with melting curves for the presence of the appropriate-sized amplicon as well as the presence of a single PCR product. The results were analyzed using Mx 3000p software (Stratagene). As the invariant control, the present study used rat β-actin.

2.8. Western blot analysis

2.8.1. Isolation of plasma membrane and cytosolic fractions

For the determination of GLUT4 translocation, 2 different fractions were used according to the method published by Dombrowski et al [41] and Nevado et al [42]. Briefly, tissues were homogenized in buffer A containing 10 mmol/L $NaHCO_3$ (pH 7.0), 250 mmol/L sucrose, 5 mmol/L NaN_3, protease inhibitor cocktail, and 100 μmol/L phenylmethylsulfonyl fluoride using a Polytron-equipped homogenizer (Model PT 3000, Kinematica, Littau, Switzerland) at a precise low setting on ice. The resulting homogenate was clarified at 1300*g* for 10 minutes at 4°C. The resultant supernatant was centrifuged at 20 000*g* for 30 minutes at 4°C. The supernatant was used for cytosolic fraction; and the pellet was resuspended in buffer A, applied on discontinuous sucrose gradients (25%, 32%, and 35%, wt/wt), and centrifuged at 150 000*g* for 16 hours at 4°C. Plasma membrane at the 25% to 32% interface was recovered, diluted with sucrose-free buffer A, and centrifuged at 190 000*g* for 1 hour at 4°C. Pellets were resuspended in buffer A, and protein concentration was estimated [43] using bovine serum albumin (BSA) as a standard. The GLUT4 protein level was measured in both plasma membrane and cytosolic fractions, and translocation was evaluated by the difference in protein levels in cytosol and membrane fractions.

2.8.2. Preparation of tissue lysate

Tissues were homogenized in buffer containing 20 mmol/L Tris-HCl (pH 7.8), 300 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L dithiothreitol, 2% NP-40, 0.2% sodium dodecyl sulfate (SDS), 0.2% sodium deoxycholate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 25 mmol/L sodium pyrophosphate, 40 mmol/L β-glycerophosphate, 2 mmol/L Na_3VO_4, and protease inhibitor cocktail (Sigma-Aldrich, Bangalore, India) using a Polytron-equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 1300*g* for 10 minutes at 4°C. The supernatant was centrifuged at 12 000*g* for 15 minutes at 4°C. The resultant supernatant was sampled as a total protein for Akt, phosphorylated Akt, and GLUT2; and then the protein concentration was estimated [43] using BSA as a standard. Briefly, each sample (25 μg) was subjected to heat denaturation at 96°C for 5 minutes with Laemmli buffer. The proteins were resolved by SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was treated with blocking buffer containing 5% blocking reagent (Amersham Biosciences) in Tris-buffered saline with Tween-20 (TBS-T) for 1 hour at room temperature followed by incubation with primary antibody to GLUT2/GLUT4/Akt/phospho-Akt at a dilution of 1:1000 in TBS-T at room temperature for 1 hour. The membrane was washed in 3 times with TBS-T and then incubated for 1 hour in horseradish peroxidase–conjugated mouse/rabbit secondary antibody, which was diluted 1:7500 with TBS-T. The membrane was washed 3 times with TBS-T, and targeted protein was detected using enhanced chemiluminescence reagents (ECL, Amersham Biosciences). The protein bands were captured using Chemidoc and quantified by Quantity One image analysis system (Bio-Rad Laboratories, Hercules, CA). Later, the membranes were incubated in stripping buffer (50 mL containing 62.5 mmol/L Tris-HCl [pH 6.8], 1 g SDS, and 0.34 mL β-mercaptoethanol) at 55°C for 40 minutes. After this, the membrane was reprobed using a β-actin antibody (1:2000). As the invariant control, the present study used rat β-actin.

2.9. Glucose uptake

^{14}C-2-deoxyglucose uptake in tissues was estimated by the methods of Valverde et al [44] and Nevado et al [42]. Briefly, after control and experimental rats were anesthetized, skeletal muscle, adipose tissue, and liver were dissected out and rapidly cut into pieces of 10 mg. The tissues were put into 12-well plate containing 2 mL Krebs-Ringer bicarbonate (KRB) buffer (119 mmol/L NaCl, 4.8 mmol/L KCl, 1 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 24 mmol/L $NaHCO_3$, 12 mmol/L HEPES, 0.1% BSA, and 2 mmol/L sodium pyruvate) supplemented with 8 mmol/L glucose and incubated at 37°C for 60 minutes. Afterward, tissues were incubated for 20 minutes using KRB buffer supplemented with (for measurement of insulin-stimulated glucose uptake) or without (for measurement of basal glucose uptake) insulin (2 μU/mL). Tissues were then rinsed using KRB buffer and further incubated for 20 minutes at 37°C in 2 mL KRB buffer that contained 8 mmol/L ^{14}C-2-deoxyglucose (0.05 μCi). Plates were supplied continuously with 95% O_2/5% CO_2 throughout the experiment, and insulin was present during the wash and for measuring insulin-stimulated glucose uptake. Tissues were then removed, rapidly rinsed in isotope-free KRB buffer, and solubilized with 1 N NaOH. Radioactivity was counted using liquid scintillation counter. Results are expressed as counts per minute (CPM) of ^{14}C-2-deoxyglucose uptake per 10 mg tissue.

2.10. Glycogen synthase enzyme assay

Glycogen synthase enzyme activity was assayed following the method of Leloir and Goldemberg [45]. Briefly, tissues were homogenized in buffer A (pH 8.4) containing

0.25 mmol/L sucrose and 1 mmol/L EDTA using a Polytron-equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 25 000*g* for 15 minutes at 4°C. The pellet was washed twice in solution containing 0.15 mol/L KCl, 0.01 mol/L Tris buffer, 1 mmol/L EDTA, and 1 mmol/L glucose-6-phosphate and resuspended in buffer A. To determine the activity of glycogen synthase, to 100 μL of each extracted sample, 1.5 μL of 25 mmol/L uridine diphosphoglucose was added and incubated for 10 minutes at 37°C in a 190-μL incubation mixture (4% glycogen, 0.75 mol/L glycine, and 0.05 mol/L glucose-6-phosphate). After incubation, 10 μL of 0.01 mol/L phosphoenolpyruvate and pyruvate kinase (5 U/mL) were added and incubated for 15 minutes at 37°C. At the end of incubation, reaction was arrested by adding 10 μL of 0.1% dinitrophenylhydrazine. The content of the tubes was mixed well and allowed to stand for 5 minutes, and 10 N NaOH was added for the maximum development of color. Afterward, 20 μL ethanol was added and centrifuged for 15 minutes at 750*g*. The optical density of the supernatant was measured at 520 nm. The enzyme activity is expressed as micromoles of uridine diphosphate (UDP) formed per minute per milligram protein.

2.11. Glycogen phosphorylase enzyme assay

Glycogen phosphorylase enzyme activity was assayed following the method of Cornblath et al [46]. Briefly, tissues were homogenized in buffer (pH 6.8) containing 35 mmol/L glycerol-2-phosphate, 20 mmol/L NaF, and 1 mmol/L EDTA using a Polytron-equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 25 000*g* for 15 minutes at 4°C. The supernatant was used for enzyme assay. To determine the activity of glycogen phosphorylase, 100 μL of each extracted sample was incubated for 15 minutes at 37°C in a 190-μL incubation mixture (0.05 mol/L glucose-1-phosphate, 2% glycogen, 0.02 mol/L adenosine-5′-monophosphate). At the end of incubation, reaction was arrested by adding 10 μL of 10% trichloroacetic acid (TCA) and centrifuged at 2000*g* for 15 minutes at 37°C. To the supernatant, 10 μL of 0.1 mol/L ammonium molybdate and 0.3% 1-amino-2-naphto-4-sulfonic acid were added. After 10 minutes, the optical density of the blue color was measured at 680 nm. The enzyme activity is expressed as micromoles orthophosphate (Pi) liberated per minute per gram protein.

2.12. Estimation of glycogen

Glycogen was estimated by the method of Hassid and Abraham [47]. Briefly, tissue was digested with 30% KOH in a boiling water bath for 20 minutes. The contents were cooled; and 95% ethanol was added, thoroughly mixed, and gently brought to boil in a hot water bath. This was cooled and centrifuged at 750*g* for 15 minutes. The supernatant was decanted, and the tubes were allowed to drain on a filter paper for few minutes. The precipitate was redissolved in distilled water, reprecipitated with 95% ethanol, centrifuged, and drained as stated above. The precipitate and series of standard (D-glucose) were dissolved in distilled water, and 0.2% anthrone reagent (containing 95% sulfuric acid) was added under ice-cold conditions. The tubes were covered with glass marbles and heated for 10 minutes in a boiling water bath. The contents were cooled immediately, and the color was read at 680 nm. The amount of glycogen is expressed as milligrams per gram wet tissue.

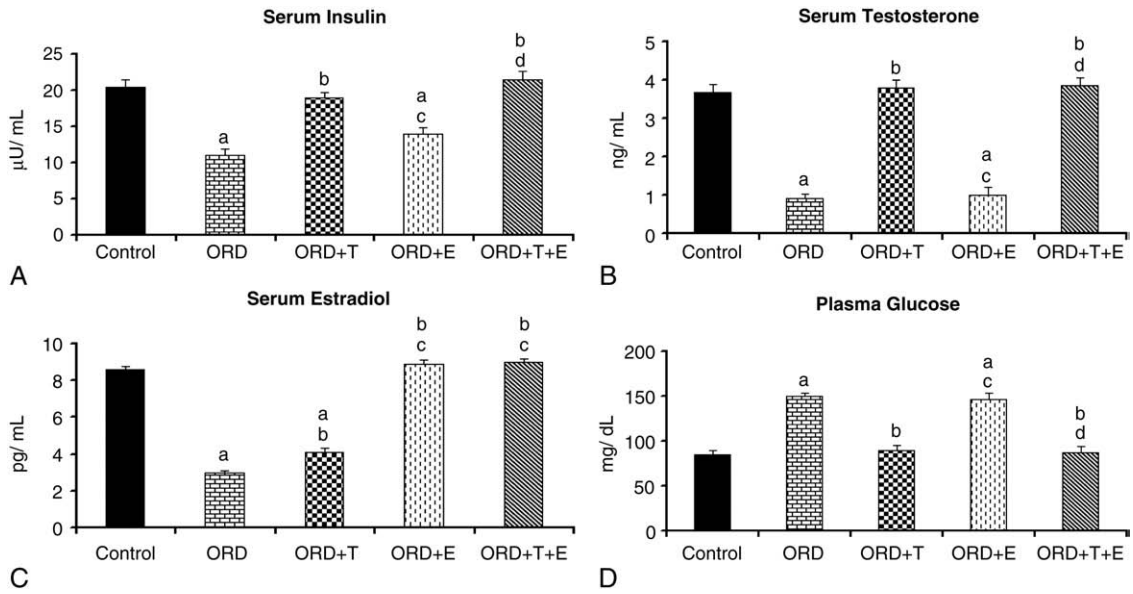

Fig. 1. Effects of castration and sex steroids replacement on serum insulin (A), testosterone (B), estradiol (C), and plasma glucose (D) in adult male rat. Results are expressed as mean ± SEM of 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD +E.

2.13. Statistical analysis

The data were subjected to statistical analysis using 1-way analysis of variance and Duncan multiple range test to assess the significance of individual variations between the control and treatment groups using a computer-based software (SPSS 7.5 for Windows student version; SPSS, Chicago, IL). In the Duncan test, the significance was considered at the level of $P < .05$.

3. Results

3.1. Plasma glucose level

Orchidectomy significantly ($P < .05$) diminished the serum insulin (Fig. 1A), testosterone (Fig. 1B), and estradiol (Fig. 1C). These hormonal changes resulted in significant ($P < .05$) increase in plasma glucose level (Fig. 1D). However, orchidectomized rats supplemented with physiologic doses of testosterone and its combination with estradiol exhibited the control level, whereas estradiol alone did not show any significant alteration.

3.2. Glucose uptake

Castration-induced elevation of the plasma glucose could occur by defect in insulin stimulated glucose uptake in target tissues. Therefore, to find out whether glucose uptake is intimately associated with plasma glucose, glucose transport was measured in fresh tissues from control and experimental rats by determining the incorporation of labeled [^{14}C]-2-deoxyglucose. As expected, castration caused a remarkable decrease in [^{14}C]-2-deoxyglucose uptake in skeletal muscle (Fig. 2A), adipose tissue (Fig. 2B), and liver (Fig. 2C), whereas this was fully restored as a result of testosterone and its combination with estradiol replacement, although estradiol replacement alone did not induce any appreciable change.

3.3. GLUT4 expression and its plasma membrane population

Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter form GLUT4 from intracellular sites to the cell surface. Likewise, disturbance of GLUT4 in skeletal muscle and adipose tissue resulted in impaired glucose uptake. The diminished rate of glucose uptake by sex steroids deficiency may transpire by defective GLUT4 expression, its plasma membrane translocation, or both. To assess sex steroids deprivation effects on GLUT4 expression and its plasma membrane translocation, the present study analyzed GLUT4 protein level in plasma membrane and cytosol fraction of skeletal muscle (Fig. 3A) and adipose tissue (Fig. 3B). Orchidectomy caused significant decrease in both cytosolic and plasma membrane GLUT4 content. Substitution of testosterone and its combination with estradiol to castrated rats restored the same to control level, whereas replacement with estradiol alone restored the GLUT4 only in adipose tissue.

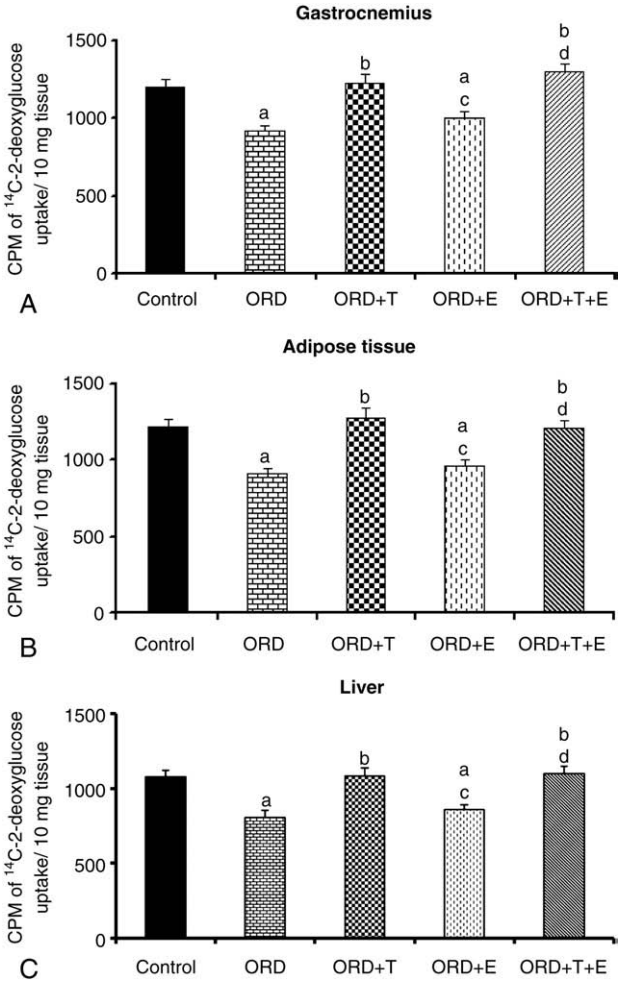

Fig. 2. Effects of castration and sex steroids replacement on ^{14}C-2-deoxyglucose uptake in gastrocnemius muscle (A), adipose tissue (B), and liver (C) of adult male rat. Results are expressed as mean ± SEM of 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

3.4. GLUT4 messenger RNA expression level

Castration-induced down-regulation of GLUT4 content in both cytosol and plasma membrane may be the result of its defective expression. Therefore, its messenger RNA (mRNA) expression was analyzed by real-time PCR. Similar to that of GLUT4 content, significant decrease in GLUT4 mRNA level was also recorded in skeletal muscle (Fig. 4A) and adipose tissue (Fig. 4B) as a result of orchidectomy. Despite the fact that administration of testosterone significantly increased the GLUT4 mRNA level in skeletal muscle, adipose tissue GLUT4 mRNA level was restored to control level. However, testosterone in combination with estradiol replacement fully restored the GLUT4 mRNA to control level. On the contrary, replacement with estradiol did not have any effect.

3.5. GLUT2 mRNA and its protein expression

GLUT2 up-regulation is more critical in glucose export from the liver to maintain glucose homeostasis than in

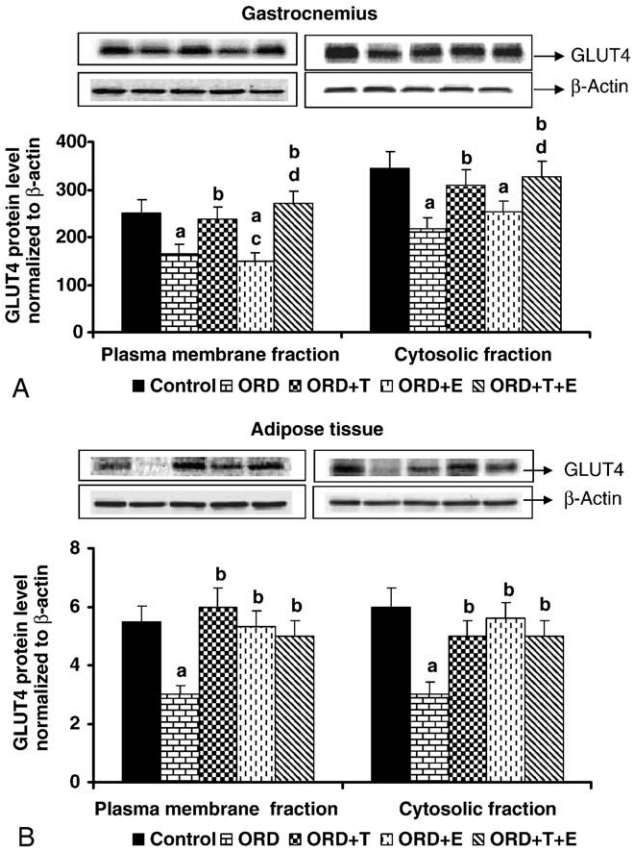

Fig. 3. Effects of castration and sex steroids replacement on GLUT4 protein in plasma membrane and cytosol fractions of gastrocnemius muscle (A) and adipose tissue (B) of adult male rat. Results are expressed as mean ± SEM of 3 separate assays representing 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

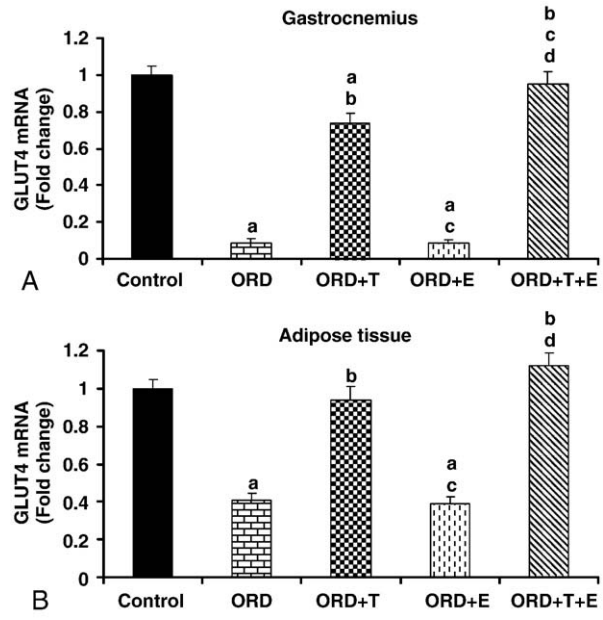

Fig. 4. Effects of castration and sex steroids replacement on GLUT4 mRNA in gastrocnemius muscle (A) and adipose tissue (B) of adult male rat. Results are expressed as mean ± SEM of 3 separate assays representing 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

import. To evaluate the effects of testosterone and estradiol deficiency on GLUT2, the present study analyzed both the mRNA and protein expression level in liver (Fig. 5A and B). GLUT2 mRNA and its protein level in liver were significantly increased because of castration. Supplementation of testosterone and its combination with estradiol brought back the same to normal level in castrated rats, whereas estradiol alone did not have any effect.

3.6. Akt phosphorylation level

Phosphorylation of Akt is essential for insulin-stimulated GLUT4 translocation to plasma membrane. In the present study, castration diminished Akt serine^{473} phosphorylation in gastrocnemius (Fig. 6A), adipose tissue (Fig. 6B), and liver (Fig. 6C), which was reversed as a result of testosterone in combination with estradiol replacement. Testosterone or estradiol treatment alone significantly increased the Akt phosphorylation in liver and adipose tissue compared with castrated animal, but skeletal muscle Akt phosphorylation was brought back to normal level.

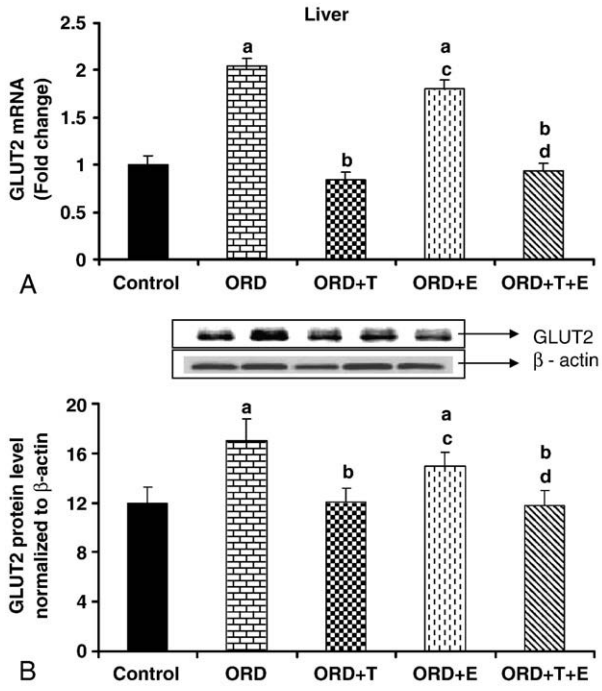

Fig. 5. Effects of castration and sex steroids replacement on GLUT2 protein and its mRNA in liver (A, B) of adult male rat. Results are expressed as mean ± SEM of 3 separate assays representing 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

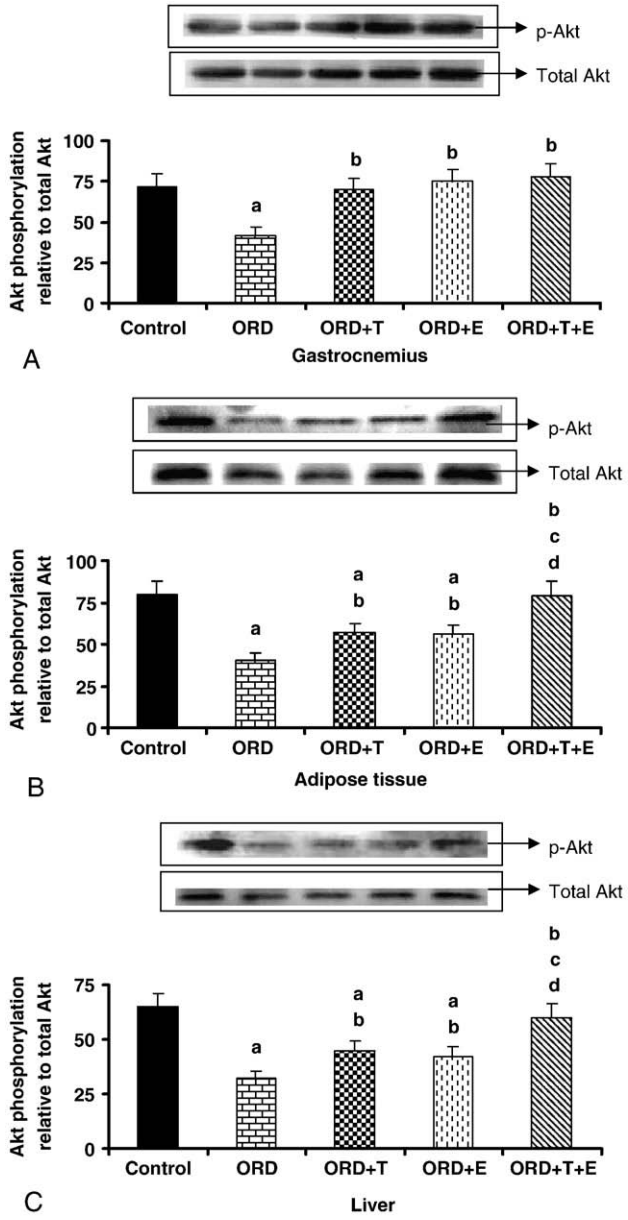

Fig. 6. Effects of castration and sex steroids replacement on Akt and phospho-Akt proteins in gastrocnemius muscle (A), adipose tissue (B), and liver (C) of adult male rat. Results are expressed as mean ± SEM of 3 separate assays representing 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

3.7. Glycogen level

Castration diminished glycogen content in gastrocnemius, adipose tissue, and liver; and this was fully reverted in testosterone and its combination with estradiol supplementation. On the contrary, estradiol alone did not have any effect (Fig. 7).

3.8. Enzyme activities

Castration significantly ($P < .05$) decreased glycogen synthase activity (Fig. 8A) and, in contrast, increased glycogen phosphorylase activity (Fig. 8B) in all tissues studied, whereas testosterone and its combination with estradiol replacement restored the same to normalcy. No significant alteration in the enzymes activity was evident in all the tissues after estradiol administration to castrated rats.

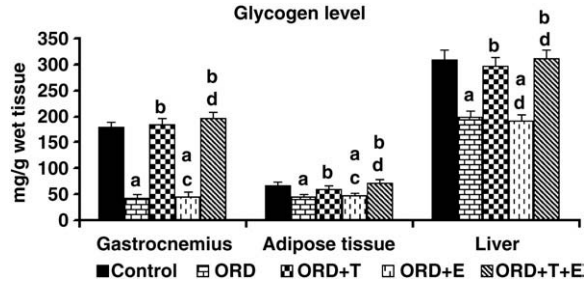

Fig. 7. Effects of castration and sex steroids replacement on glycogen level in gastrocnemius muscle, adipose tissue, and liver of adult male rat. Results are expressed as mean ± SEM of 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

4. Discussion

The present study examined the effects of sex steroids deprivation on glucose transporter expression and glucose uptake in insulin target tissues. To examine if perturbations

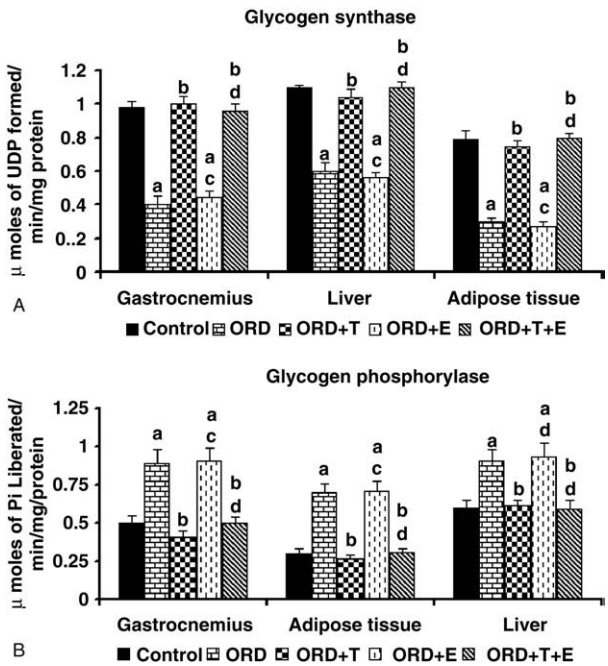

Fig. 8. Effects of castration and sex steroids replacement on glycogen synthase (A) and glycogen phosphorylase (B) in gastrocnemius muscle, adipose tissue, and liver of adult male rat. Results are expressed as mean ± SEM of 6 animals. Significance at $P < .05$. a, compared with control; b, compared with ORD; c, compared with ORD + T; d, compared with ORD + E.

observed in castrated animals are caused by deficiency of sex steroids, testosterone and estradiol were given in a dose aimed to substitute for the endogenous production. This resulted in maintenance of testosterone and estradiol concentration similar to that of control level. Administration of physiologic dose of testosterone and its combination with estradiol to castrated rats increased serum insulin when compared with castrated rats, whereas estradiol alone had no effect, suggesting that normal circulating level of testosterone is essential to maintain optimum insulin concentration in serum. In support of this, Morimoto et al [16] have shown that testosterone modifies serum insulin levels through direct effect on pancreatic islet function by favoring insulin gene expression and release.

Insulin-stimulated transport of glucose into muscle and fat cells is mediated by redistribution of GLUT4 from intracellular GLUT4 storage vesicles to plasma membrane [48]. In the present study, the testosterone deprivation–mediated decrease in the amount of GLUT4 protein in plasma membrane and the restoration of the same in rats supplemented with testosterone and its combination with estradiol imply the importance of physiologic level of testosterone to maintain optimum level of GLUT4 population in plasma membrane. Sato et al [49] also showed an increase in GLUT4 translocation to plasma membrane in skeletal muscle cells of neonatal rats by testosterone. In this regard, it is worth to recall the report of Rincon et al [50] that showed that the absence of sex hormones in female rats appears to decrease insulin-mediated whole-body glucose uptake via an impaired insulin-stimulated translocation of GLUT4 to the plasma membrane. The present study showed diminution in the rate of glucose uptake in skeletal muscle and adipose tissue of castrated rats that was restored to normal level by testosterone and its combination with estradiol. It is well known that glucose uptake is achieved by plasma membrane GLUT4 population [51,52]. Therefore, the observed changes in glucose uptake in skeletal muscle and adipose tissue of castrated and testosterone plus estradiol–replaced rats are the result of corresponding changes in GLUT4 population in plasma membrane. Studies have shown that the activation of Akt through PI3K is essential for insulin-stimulated GLUT4 translocation to plasma membrane [53,54]. Akt is fully active only when it is phosphorylated on the $Thr^{308/309}$ and $Ser^{473/474}$ residues [55,56]. In the present study, testosterone deprivation diminished Akt $serine^{473}$ phosphorylation in adipose tissue and skeletal muscle, which was reversed as a result of testosterone plus estradiol replacement. Testosterone enhances Akt phosphorylation through activation of PI3K, and the same has been established by the observation that inhibition of PI3K abolished testosterone-induced Akt activation in osteoblast cells and skeletal muscle cells of neonatal rat [49,57]. Therefore, alteration in GLUT4 population in the plasma membrane of skeletal muscle and adipose tissue is the result of changes in Akt activation.

Administration of estradiol to castrated rats shows increased Akt phosphorylation in all the tissues studied. It has been demonstrated that estradiol enhances phosphorylation of Akt through IRS/p85 of PI3K association in adipocytes [58] and cardiac muscle [59]. Enhanced Akt phosphorylation is associated with increased plasma membrane GLUT4 population but without increasing glucose uptake in adipose tissue. This could be due to defect at the level of glucose transporter intrinsic activity. Concurrently, enhanced Akt phosphorylation was not associated with plasma membrane GLUT4 in skeletal muscle from castrated rats in response to estradiol replacement. In support of these findings, Rogersa et al [60] have reported that ex vivo muscle stimulation with 17β-estradiol resulted in a rapid increase in the phosphorylation of Akt, adenosine monophosphate–activated protein kinase, and TBC1D1/4 without stimulation of glucose uptake. The differential response of adipose tissue and skeletal muscle indicates the existence of tissue-specific influence of estradiol.

GLUT4 synthesis can also contribute to the cell surface GLUT4 population [61]. The present study showed a decrease in the amount of cytosolic GLUT4 content in adipose tissue and skeletal muscle of castrated rats that was reversed by testosterone and its combination with estradiol replacement. In accordance with this, a previous study on myocytes and adipocytes also showed a comparable effect wherein a low dose of testosterone increased GLUT4 expression [62]. In general, the rate of synthesis of protein (mRNA translation) is directly related to the amount of encoding mRNA and the efficiency of translation of that specific mRNA [63]. In the present study, changes in GLUT4 in castrated rats and in rats supplemented with testosterone and its combination with estradiol were found to be associated with corresponding changes in GLUT4 mRNA expression, indicating the defective transcription of the gene as well as translation of mRNA. Therefore, it is suggested that castration-mediated down-regulation of GLUT4 expression may be one of the reasons for decreased GLUT4 population in plasma membrane. In this respect, it is worth to recall the report of Sato et al [49] that showed that addition of testosterone to cultured skeletal muscle induced an elevation of GLUT4 expression and translocation with increased Akt phosphorylation. These findings tempt to propose the involvement of testosterone in GLUT4 expression and its translocation to plasma membrane.

GLUT4 promoter contains DNA sequences responsive to insulin, indicating that this gene is regulated by insulin [64]. In the present study, castrated rats showed a significant decrease in serum insulin level, GLUT4 protein, and its mRNA levels in target tissues. Therefore, it is suggested that the castration-induced deficiency in insulin may be one of the reasons for impaired GLUT4 gene expression.

The presence of high plasma glucose level is considered as the marker for diabetes. In the present study, plasma glucose level was high in castrated rat, which appears to be

the result of testosterone deficiency–mediated impairment of glucose uptake in skeletal muscle and adipose tissue. In this regard, Holmang and Bjorntorp [15] have reported that castration was followed by marked insulin resistance in the clamp experiments. This was also paralleled by a diminished insulin stimulation of glucose incorporation into glycogen down to about 50% of control values. Substitution of castrated rats with testosterone (which is equivalent to the dose used in the present study) completely abolished these perturbations of insulin sensitivity. Furthermore, castration decreases glycogen synthase and increases glycogen phosphorylase; and these changes were reversed by testosterone and its combination with estradiol. Thus, it is evident that testosterone also maintains an optimum amount of glycogen reserve in skeletal muscle and adipose tissue by augmenting glycogenic enzyme on one side and inhibiting glycogenolytic enzyme on the other side [65]. Plasma glucose in animals supplemented with testosterone plus estradiol was similar to that of normal rats, reinforcing the importance of these steroids in glucose homeostasis. In this respect, it is worth to mention the report of Thomas et al [66] that showed a significant inverse correlation between testosterone and fasting blood glucose. These observations indicate the involvement of testosterone in the regulation of glucose homeostasis, which may explain the testosterone deficiency–induced impairment of glucose uptake in target tissues.

Rencurel et al [67] suggested that GLUT2 promoter contains glucose-responsive elements. This was demonstrated in mhAT3F cells transfected with plasmids containing −338-bp to +49-bp proximal region of GLUT2 promoter fused to the CAT gene. When the −338-bp to +49-bp proximal region of GLUT2 promoter was transfected, the reporter activity was 4-fold increased in the presence of glucose, indicating the regulatory role of glucose [67]. In the present study, castrated rats showed high plasma glucose level associated with up-regulation of GLUT2 and its mRNA levels in liver. It is therefore suggested that the castration-induced hyperglycemia may be one of the reasons for up-regulated GLUT2 expression. GLUT2 up-regulation is more critical in glucose export from the liver to maintain glucose homeostasis than in import [68]. In support of this study, Im et al [69] reported that, in diabetic states, GLUT2 in the liver is up-regulated, indicating that hyperglycemia may play a positive role in GLUT2 gene expression.

A significant reduction in hepatic glycogen level in castrated rat may be due to impaired glycogen synthesis and increased glycogenolysis [70]. Akt activation plays an important role in hepatic glucose production and glycogen synthesis [71,72], and it is established by the fact that Akt-deficient mice showed impaired glucose tolerance because of increased hepatic glucose production [73,74]. In the present study also, diminished hepatic Akt and its phosphorylation were recorded because of sex steroids deprivation, which were reverted by testosterone plus estradiol replacement. Taken together, it is suggested that testosterone could be involved in the maintenance of euglycemic state through activation of hepatic Akt, which is associated with up-regulation of glycogen synthesis [72] as a result of increased glycogen synthase and diminished glycogen phosphorylase. These interpretations attest to the involvement of testosterone in the regulation of hepatic glucose output, which may explain the testosterone deficiency–induced hyperglycemia.

Effect of testosterone on gene expression is known to be mediated through the activation of its receptor [75]. Insulin target tissues have receptor for androgen [76]. The fact that testosterone replacement was able to restore GLUT2, GLUT4, and their mRNA levels suggests that testosterone may have a direct effect on GLUT2 and GLUT4 transcription as well as translation of mRNA. Although the presence of androgen response element has not been identified in the promoter region, it is possible that androgen receptor may interact with basal transcription machinery [77] or that other coactivators that bind to the androgen receptor [78,79] could be involved in the mechanism by which testosterone increases GLUT2 and GLUT4 expression. In this regard, studies on the promoter region of GLUT2 and GLUT4 would be of great interest.

In addition to testosterone and estradiol, corticosterone was found to play an important role in glucose homeostasis. Although the circulating level of corticosterone was not measured in the present study, a previous study from our laboratory [65] showed that gonadectomy enhanced the serum corticosterone titer and that it was brought back to normalcy by testosterone replacement. Excess glucocorticoids have been shown to decrease the number of insulin receptors and their affinity in rat liver and adipocytes [80,81]. In view of these findings, it is proposed that castration-induced increase in corticosterone could have contributed to the changes recorded in the present study.

It is concluded from these findings that sex steroids deficiency–induced defective glucose uptake in skeletal muscle and adipose tissue is mediated through postreceptor defects in glucose transport system, including Akt and its phosphorylation, and GLUT4 expression and its translocation. In accordance with the present study, in vitro studies using 3T3 L1 adipocytes and skeletal muscle cells have also recorded such postreceptor defects at the level of Akt/PKC phosphorylations and translocation of GLUT4 [49,62]. In analogy with the results of the present study, our previous findings showed that castration-mediated impairment in glucose oxidation is coupled with decreased insulin receptor expression in adipose tissue, liver, and skeletal muscle [27]. Taken together, sex steroids deficiency induced a defect in glucose transport system that is linked to the loss of cell surface insulin receptors as well as a direct postreceptor defect.

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