Effect of metformin on renal microsomal proteins, lipid peroxidation and antioxidant status in dexamethasone-induced type-2 diabetic mice

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An SDS-PAGE analysis of renal microsomal fraction of albino mice was performed to study the involvement of proteins in dexamethasone-induced type-2 diabetes mellitus (DM) and their alterations by metformin, a widely accepted oral antidiabetic drug. In addition, changes in renal lipid peroxidation (LPO), activities of superoxide dismutase (SOD) and catalase (CAT), reduced glutathione (GSH) content, as well as renal somatic index (RSI) and daily rate of water consumption were also investigated. While dexamethasone administration (1.0 mg/kg for 21 days) expressed two renal proteins (43 kDa and 63.23 kDa), in addition to the increased fasting serum levels of glucose and insulin, renal LPO, RSI and daily rate of water consumption, a parallel decrease in renal SOD, CAT and GSH was also observed. Treatment with metformin normalized these alterations including the renal proteins and LPO, confirming its efficacy in ameliorating dexamethasone-induced type-2 DM and also the association of two proteins with type-2 DM.

Keywords: Dexamethsone, Lipid peroxidation, Metformin, Mice, SDS-PAGE

Diabetes mellitus (DM) is a disorder of carbohydrate, lipid and protein metabolism, caused either by lack in the secretion of insulin or loss of its function on target tissues\(^1\). It is primarily characterized by an inefficient utilization of circulating glucose, leading to consistent hyperglycemic condition\(^2\). The world health organization (WHO) recognized three main types of diabetes, viz. insulin-dependent DM (IDDM; type-1), non-insulin-dependent DM (NIDDM; type-2), and malnutrition-related DM (MRDM). Of these, type-2 comprises 85-90% of the total diabetic population. The etiology of type-2 DM, in which beta cells of pancreas are usually functional often involves hormonal imbalance\(^3,4\).

The hormonal diabetes results from either due to the hypo or hyper functioning of other endocrine glands, most commonly pituitary, thyroid and adrenal\(^3,4\). Among these, the hormones of adrenal cortex are well known for their diabetogenic effects\(^4\). In the treatment of type-2 DM, several oral hypoglycemic agents, including that of biguanides and sulphonylureas are commonly used and among biguanides, metformin is considered as a drug of choice\(^5\). Earlier study has clearly indicated that in DM kidney tissues are invariably affected\(^6\). Also, increased rate of urinary protein excretion in diabetic subjects has been observed\(^7\). However, influence of hyperglycemia on renal protein profile in corticosteroid-induced type-2 diabetes has not been reported till date.

In the present study, the alterations in the renal microsomal proteins expression and their possible reversal by an anti-diabetic drug metformin in dexamethasone-induced type-2 diabetic mouse model have been investigated. The changes in the concentrations of serum insulin and glucose, renal lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities, glutathione (GSH) and protein contents, renal somatic index (RSI) and daily rate of water consumption have also been investigated to correlate with the altered hyperglycemic condition.
Materials and Methods

Chemicals

Ellman’s reagent (DTNB), glycine, ammonium persulphate (APS) and m-phosphoric acid were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), EDTA, Tris-HCl, Coomassie blue, acrylamide, bisacrylamide, tetramethyleyldiamine (TEMED) were purchased from E. Merck Ltd., Mumbai, India. The test drug metformin (Glyciphage®, Franco-Indian, Pvt. Ltd., India) and dexamethasone (Decdan®, Merind Co., Mumbai, India) were purchased from a registered local medical store. Protein molecular weight marker mixture for SDS-PAGE was obtained from Bangalore Genei, India. Alpha Innotech Digi Doc 1200 gel documentation system was used for the estimation of molecular weight. All other chemicals were of reagent grade and obtained from Loba Chemie, Mumbai, India.

Experimental animals

Swiss albino male mice weighing 30 ± 2 g, acclimated for a week before experimentation were maintained in polypropylene cages in a standard light (14 h light: 10 h dark cycle) and temperature (23 ± 2°C) controlled room with the provision of laboratory feed (Gold Mohur feed, Hindustan Lever Ltd., Mumbai, India) and water ad libitum. The animals were maintained in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Treatment

Drug administration was done by gastric intubation method between 1000 and 1100 h of the day to avoid circadian variation, if any. Twenty-eight healthy male mice were divided into four groups of seven each and the body weight of each animal was recorded. Group I animals receiving the vehicle normal saline (0.1 ml/animal/day, intramuscular) served as control. Groups II and III received a pre-standardized dose of dexamethasone (1.0 mg/kg, intramuscular) in saline for initial 7 days, as done earlier in our laboratory for inducing type-2 DM, whereas animals of group IV received saline only. From 8th day onwards of the study, animals of groups I and II continued to receive equivalent amounts of vehicle and dexamethasone respectively, while group III were administered with metformin (250 mg/kg/day), along with dexamethasone. Group IV received equivalent amount of metformin only. The experiment was continued for 2 weeks before termination. On the day of termination, final body weight of each animal was recorded and overnight fasted animals were sacrificed after exposing them to mild anesthesia. Blood from each animal was collected and serum was isolated for the estimation of insulin and glucose concentrations. After exsanguinations, the kidneys were removed quickly, freed from blood clots, weighed and processed for the protein and biochemical estimations.

Radioimmunoassay (RIA) of insulin

Total circulating insulin was estimated by RIA in serum samples, following the protocol provided in the RIA kit supplied by Bhabha Atomic Research Centre (BARC), Mumbai, India, as routinely done in our laboratory. In brief, antisera, hormone standards, radiolabeled hormone (125I insulin) and the control sera were reconstituted with assay buffer (tris hydroxyl-methyl amino methane, pH 8.6)/double-distilled water. The reaction mixture comprised of standard/sample, buffer and anti-serum in microcentrifuge tubes and incubated at 4°C for overnight after gentle mixing. Then the radio-labeled (125I insulin) hormone was mixed and the tubes were kept at room temperature for 3 h. After incubation, the second antibody (anti-guineapig rabbit IgG) was added. Finally, after adding PEG to each tube, the reaction was terminated and tubes were centrifuged 1500 g for 20 min. After decanting the supernatant, traces of liquid were removed with the help of filter paper wicks without disturbing the precipitate. The tubes were then subjected to radioactivity counting for one min (CPM) using an 125I Gamma counter. A set of quality control sera was also used with each assay.

Biochemical assays

Serum glucose concentration was measured by glucose oxidase/peroxidase method, as routinely followed in our laboratory. For the estimation of LPO, the kidneys were homogenized in phosphate-buffered saline (0.1 M, pH 7.4) and then centrifuged at 14,000 rpm for 30 min to obtain microsomal fraction. LPO was determined by the reaction of TBA with malondialdehyde (MDA), a major product formed due to the peroxidation of lipids. The
renal SOD and CAT activities were estimated as described previously. Tissue glutathione (GSH) content (reduced sulfhydryl groups) was estimated, following the protocol of Ellman. Renal somatic index (RSI) was expressed as kidney weight (g)/body weight (g) × 100.

Analysis of renal proteins
The total proteins in microsomal fraction were estimated by the method of Lowry et al using bovine serum albumin (BSA) as a standard. The renal microsomal proteins were separated by SDS-PAGE in reducing conditions on discontinuous slab-gels. The microsomal fraction was mixed with 20 µl of SDS sample solubilization solution [5% w/v SDS, 10% v/v glycerol, 0.03% bromophenol blue, 5% v/v 2-mercaptoethanol, 0.0625 M Tris-HCl, pH 6.8] and was incubated on a heating block (NEOLAB) for 3 min. SDS-PAGE was carried out on a 0.75 mm thick slab gel with 4.4 and 10% gels for condensation and separation, respectively. The amount of protein layered was 48 µg. Protein bands were stained by Coomassie brilliant blue R-250. The gel system was calibrated for molecular weight determination by measuring the migration of standard proteins (range 14.3-97.4 kDa) using Alpha Innotech Digi Doc 1200 gel documentation system.

Statistical analysis
Data were expressed as mean ± SEM. For statistical evaluation of the data, analysis of variance (ANOVA) and student t-test were used.

Results and Discussion
Analysis of renal microsomal proteins showed two new bands (Fig. 1) in dexamethasone-treated group (lane 3) having the molecular mass of 43 kDa and 63.23 kDa, as compared to the control mice (lane 2). Although the protein profiles in urine samples of diabetic patients have been analyzed earlier, the expression of different renal proteins in corticosteroid-induced type-2 DM was not reported. Therefore, the expression of two new proteins in the renal tissues, following dexamethasone administration appeared to be a new and interesting finding. We emphasize that the two new proteins are possibly involved in the prognosis of secondary renal complications (Fig. 2) and also in diabetic renal hypertrophy. The appearance of these proteins might be due to increased sugar levels, as hyperglycemic condition has been reported to cause renal hypertrophy and increased rate of tubular protein synthesis. Interestingly, metformin administration to diabetic animals resulted in the reversal of these changes (lane 4). These alterations in protein profile, following drug administration could be due to the restoration of normoglycemic condition. This was further supported by the unaltered microsomal proteins in drug-treated normoglycemic group (lane 5).

A significant increase in the concentrations of serum glucose and insulin (P<0.001 and P<0.01, respectively, Fig. 2), renal LPO (P<0.001) and in RSI (P<0.05), and decrease in SOD (P<0.01), CAT (P<0.001) and GSH (P<0.001) as well as protein

![Fig. 1—SDS-PAGE analysis of renal proteins following metformin treatment to dexamethasone (dexa)-induced hyperglycemic and normoglycemic male mice [Lane 1, protein molecular weight markers; lane 2, control; lane 3, dexa-treated; lane 4, dexa + metformin-treated; and lane 5, metformin-treated]]

![Fig. 2—Changes in concentrations of serum insulin (U/ml) and glucose (mg/dl) in control and dexamethasone (dexa)-induced hyperglycemic male mice, following metformin treatment [Each bar represents the mean ± SEM (n=7). * P<0.001; ** P<0.01, as compared to the respective control values. #, P<0.01, as compared to respective value of dexa-treated animals]]
content (P<0.05) were also observed, following dexamethasone treatment (Table 1). However, when metformin was administered to diabetic animals, it increased renal SOD, CAT and GSH (P<0.001 for all), and reduced the concentrations of serum glucose and insulin, as well as renal LPO (P<0.001 for all). In normoglycemic animals, it reduced renal LPO (P<0.01) with a concomitant increase in GSH content (P<0.05), clearly indicating the anti-peroxidative effects of the test drug.

The hormones of adrenal gland are known to positively influence the glucose metabolism, particularly when the gland remains hyperactive for a longer period4. Earlier findings from our laboratory have indicated that exogenous administration of an adrenal hormone dexamethasone results in type-2 DM. In the present investigation also, treatment with dexamethasone exhibited an increase in serum glucose concentration. However, in the animals treated with both dexamethasone and metformin nearly normal level of glucose was restored, suggesting that metformin therapy might also ameliorate corticosteroid-induced type-2 DM. The anti-hyperglycemic nature of metformin was further supported by the alteration in serum insulin level, which was enhanced by the administration of dexamethasone, as reported earlier10,12,20. On the other hand, metformin administration to normoglycemic animals did not significantly alter the insulin level. This was not surprising, as some other antidiabetic agents also did not influence insulin secretion, but enhanced its sensitivity in normoglycemic animals21.

This may be emphasized here that although metformin has been used in the treatment of type-2 DM since 1957 across the globe5, the findings of present study, for the first time indicated its potential use in corticosteroid-induced DM.

In the present study, the dexamethasone-induced increase in tissue LPO could be due to an elevated serum glucose concentration, which might have provided an oxidative environment for basement membrane lipids24,25. It has been reported that glucose at higher concentration exerts oxidative stress, which is ameliorated by reducing its level24. However, dexamethasone-induced increase in renal LPO was normalized by metformin therapy, suggesting the beneficial role of test drug even in the renal problems. The alteration in tissue LPO was further supported by the restoration of endogenous antioxidants, such as SOD, CAT and GSH in animals which received both dexamethasone and metformin.

In conclusion, the present findings suggest that two hitherto unknown proteins were downregulated by metformin administration which were involved in the etiology of corticosteroid-induced type-2 DM.

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