Material and Methods
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1. 5-HT-Induced Hyperglycemia and Receptor Characterization

Male Wistar rats in the body weight range of 200-250g were injected intraperitoneally with 5-HT at the dose of 1 and 2mg/kg. Blood samples were collected through tail vein prior to 5-HT injection and subsequent samples were collected at 30, 60 and 120 min after injection. These samples were subjected to glucose analysis. 5-HT$_1$ receptor agonist buspirone (15mg/kg), 5-HT$_2$ receptor agonist $\alpha$-methyl 5-HT (2mg/kg), 5-HT$_3$ receptor agonist 1-phenyl biguanide (2mg/kg), 5-HT$_4$ agonist cisapride (2mg/kg), 5-HT$_1$ receptor antagonist pindolol (5mg/kg), 5-HT$_2A$ receptor antagonist sarpogrelate (1mg/kg), 5-HT$_2A/2C$ receptor antagonist mianserin (10mg/kg), 5-HT$_3$ receptor antagonist ondansetron (0.5mg/kg) were used to study their interaction with 5-HT-induced changes in serum glucose and thereby to study the role of various 5-HT receptors. We also studied the interaction of 5-HT reuptake inhibitor fluoxetine (5mg/kg) and $\alpha_2$ adrenergic blocker yohimbine (2mg/kg) with 5-HT-induced changes in serum glucose. To study the involvement of adrenergic system, rats were adrenalectomized bilaterally and 5-HT (1mg/kg) was injected 72 hrs after the surgery. All the drugs were given intraperitoneally and were injected 30 min prior to 5-HT injection in separate studies. Each treatment group consisted of six rats.

2. Dopamine-Induced Hyperglycemia and Receptor Characterization

Male Wistar rats in the body weight range of 200-250g were injected intraperitoneally with dopamine at the dose of 1 and 2mg/kg. Blood samples were collected through tail vein prior to dopamine injection and subsequent samples were collected at 30, 60 and 120 min after injection. These samples were subjected to glucose analysis. DA$_1$ receptor agonists fenoldopam (1mg/kg) and SKF38393
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(1mg/kg), DA₂ receptor agonist bromocriptine (2mg/kg) and DA₁ receptor antagonist butaclamol (2mg/kg), DA₂ receptor antagonist sulpiride (2mg/kg) were used to study their interaction with DA-induced changes in serum glucose and thereby to study the role of peripheral dopamine receptors. All the drugs were given intraperitoneally and were injected 30 min prior to DA injection in separate studies. To study the role of central dopaminergic system, levodopa (100 and 300 mg/kg) was given orally and blood samples were drawn at 0, 30, 60 and 120 min time interval. We studied the interaction of Carbidopa (10mg/kg, orally) with levodopa in a separate study to make sure the effects of levodopa is strictly central. Each treatment group consisted of six rats.

3. Induction of Streptozotocin (STZ)-Induced Type 1 Diabetes Mellitus in Rats

Wistar/Sprague Dawley rats of either sex weighing 200-250 g were used in the present study. Diabetes was induced by injection of STZ (45mg/kg) dissolved in the saline through tail vein. Animals showing glucosuria more than 2% (Diastix, Bayer Diagnostics, India) or blood glucose level >140mg/dl 48 hr after STZ injection were selected for the experiment. Animals were divided into four groups with six animals in each group: non-diabetic control (NDCon), non-diabetic treated (NDTr), diabetic control (DCon) and diabetic treated (DTr).

Treatment groups received sarpogrelate/ondansetron/fenoldopam (1mg/kg, i.p.) dissolved in distilled water daily for six weeks.

4. Induction of Streptozotocin (STZ)-Induced Type 2 Diabetes Mellitus in Rats

STZ at the dose of 90 mg/kg dissolved in normal saline was injected intraperitoneally to two day old Wistar/Sprague Dawley pups. Pups were weaned at
three weeks of age and differentiated by sex. Female pups were selected for the study and housed in separate cages. Food and water was provided ad libitum till ten weeks of age. Glucose level was estimated in 18 hr fasted rats and animals showing blood glucose >140 mg/dl were selected for study. Age matched Wistar/Sprague Dawley female rats were maintained as non-diabetic controls. Four groups were made as: non-diabetic control (NDCon), non-diabetic treated (NDTr), diabetic control (DCon) and diabetic treated (DTr).

Treatment groups received sarpogrelate/ondansetron/fenoldopam (1mg/kg, i.p.) dissolved in distilled water daily for six weeks.

5. Blood Sampling and Biochemical Analysis

At the end of six-week treatment, blood samples were collected from the tail vein into centrifuge tubes and allowed to clot for 30 minutes at room temperature. Blood samples were centrifuged at 3000 rpm for 20 minutes. Serum samples were analyzed spectrophotometrically for glucose, cholesterol, triglyceride, creatinine, and urea. Serum insulin levels were estimated by radioimmunoassay method using the kit from Bhabha Atomic Research center, Mumbai, India.

6. Oral Glucose Tolerance Test (OGTT)

Rats were fasted for 18 hr and glucose was administered at the dose of 1.5g/kg orally. Blood samples were collected at 0, 30, 60 and 120 min. Serum was separated immediately and analyzed for glucose and insulin. The concentrations of glucose and insulin were plotted against time in different graphs. The results of OGTT are expressed as integrated areas under the curves (AUCs) over a period of 0-120 min. AUCs were calculated using formula: \[\text{AUC} = \frac{(C_1+C_2)}{2} \times (t_2-t_1)\].
7. Insulin Sensitivity Index (K_{ITT})

Insulin sensitivity index was calculated as per the method of Alford et al. (1971). Rats were fasted for 6 hours and human insulin (Actrapid, Novo Nordisk, India) was injected through tail vein at the dose of 0.2 IU/100g body weight. Blood samples were collected at 10, 20, 30 and 60 min time interval. Blood glucose was estimated and plotted against time on semi log paper to calculate $t_{1/2}$. Insulin sensitivity index was calculated as:

$$K_{ITT} = (0.693/t_{1/2}) \times 100$$

8. Measurement of Blood Pressure

Blood pressure was recorded by tail-cuff method using Harvard blood pressure monitor (Kent, UK). Trained rats were restrained and tail cuff introduced. Tail cuff pressure was first raised to 200 mmHg and then slowly released. During the decline in pressure, the point at which there was an increase in magnitude of deflection of the pulse analyzer was recorded as the systolic blood pressure.

9. Assessment of Renal Function

Urine samples were collected over a period of 12 hours by placing animals in metabolic cages. Urinary sodium content was measured by using flame photometry. After the six-week treatment, the animals were sacrificed by cervical dislocation. Kidneys were quickly excised from all groups and washed with cold saline to remove as much of the blood as possible. Kidneys were dried on filter paper and dry weight was recorded immediately. Kidneys were used for estimation of DNA and RNA.
10. Effect of Sarpogrelate on Heart Function and Cardiac Membrane Glucose Transporters in STZ-Induced Type 1 Diabetic Rats

A) Preparation of Diabetic Animals

Male Sprague-Dawley rats weighing (225-250) were made diabetic by injecting STZ (65mg/kg) into the tail vein. Blood glucose levels were measured after 48 hrs and the animals showing fed blood glucose >180mg/dl were considered as diabetic. The animals were divided into four groups: control (NDCon), diabetic control (DCon), diabetic treated with insulin (10unit/kg, s.c.) (DIns) and diabetic treated with sarpogrelate (5mg/kg, p.o.) (DSar). Each group consisted of eight animals. Treatments were started after one week and continued till the end of experiment at 7th week. All the animals were maintained on normal chow diet and water ad libitum.

B) Biochemical Parameters

At the end of seven weeks blood samples were drawn from tail vein. Serum was separated and used for glucose, insulin, cholesterol and triglyceride estimation. Insulin was measured by radio immunoassay using insulin kit from Linco Inc., USA. Serum glucose, cholesterol and triglycerides were measured spectrophotometrically using kits from Boerenger-Manheim, USA.

C) Hemodynamic Studies

After collecting the blood samples, the animals were anesthetized with a mixture of ketamine (60mg/kg) and xylazine (10mg/kg). The carotid artery was dissected out and catheter sensor (Millar, MIKRO-TIP® catheter Model SDR 249, Millar instrument Inc., Houston, TX, USA) was inserted into the left ventricle through a small incision on carotid artery. The sensor was connected to the pressure transducer (Model 1050BP; BIOPAC System Inc., Goleta, CA, USA) for recording the left
ventricular developed pressure. Transducer was calibrated at 10 mmHg at the beginning of experiment and the rate of ventricular pressure development (+dP/dt) and the rate of ventricular pressure decline (-dP/dt) were determined using the Acknowledge 3.03 software for windows (BIOPAC Systems Inc., Goleta, CA, USA).

Once the hemodynamic studies were done, rats were sacrificed by the overdose of anesthetic. Hearts separated and dried on filter paper. Whole heart weights and ventricle weights were recorded to calculate whole heart/body weight and left ventricle/body weight ratios.

D) Estimation of Glucose Transporters in Cardiac Membrane

I. Membrane and Cytosol Preparation

Immediately after the weights of separated hearts were recorded the tissue was frozen in liquid nitrogen (-20°C). Frozen cardiac tissue (approx. 700 g) was homogenized in a solution containing 10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 1 mM ethylenediamine tetra acetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using polytron at 12000 rpm. This homogenate was centrifuged at 1000 g (Beckman, USA, rotor JA-20) at 0°C. Supernatant was subjected again to centrifugation at 10,000 g at 0°C. Supernatant from this was finally subjected to centrifugation at 40,000 g at 0°C. Pellets obtained from this final centrifugation are rich in crude membrane fraction and the supernatant are rich in cytosol. Pellets were resuspended in a solution containing 10 mM histidine and 250 mM Sucrose (pH 7.0). Protein estimation for the suspension was done using Lowry’s method and equal amount of protein (2mg/ml) was loaded by diluting with Laemmli buffer for the gel electrophoresis.
II. Western Blotting

Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% resolving gel. Each sample was run in duplicate. Gels were run at 200 V ~ 45 min. Prestained molecular weight standards (Sigma, USA) were run in one corner well, whereas Laemmli buffer was added to another corner well to ensure the evenness of the electrophoresis. Electrophoretic transfer of proteins was done to PVDF membranes (Amersham, USA) using Bio-Rad assembly at 100 V~2 hr. Then the membranes were incubated in (Tris)-buffered saline containing 50 mM Tris and 750 mM NaCl (pH 7.4) with 5% non fat dry milk for 1 hr at room temperature. The membranes were washed with TBS, added with GLUT 1 (antirabbit) or GLUT 4 (antigoat) antibody (Santa Cruz Biotechnology Inc, USA) in 1:750 dilution in TBS containing 5% milk. These were incubated overnight at 4°C. Membranes were then washed with TBS containing 0.1% Tween 20 (TBST) the next day. Appropriate second antibody (Santa Cruz Biotechnology Inc, USA) was added in 1: 3000 dilution in TBS with 5% milk and incubated for 1 hr at room temperature, washed again with TBST and then finally the membranes were incubated with anti-rabbit horseradish-peroxidase labeled antibody for 30 min at room temperature. After washings with TBST the membranes were dipped in chemiluminescence solution (Amersham, USA) for 1 min and then immediately exposed on the Kodak X-ray film for duration of 1-2 min. The relative levels of proteins were quantified by densitometry using Bio-Rad device and analyzed by using Molecular Analyst Software. The results are presented as percentage of control values.
11. Effect of Restoring Insulin Sensitivity on Renal Dopamine Function Using Rosiglitazone Treatment in Zucker fa/fa rats

A) Animals and Drug Treatment

Eight to nine weeks old male obese Zucker rats and age matched male lean Zucker rats (Harlan Sprague-Dawley Inc., Indianapolis, USA) were housed in plastic cages and fed normal rodent chow and tap water ad libitum. The obese rats were separated into two groups- obese controls (ObCon) and obese treated with rosiglitazone (ObTr). Treatment group received rosiglitazone maleate (10mg/kg) suspended in 1% carboxy methyl cellulose in distilled water by oral route for four weeks. Obese control group received 1% carboxy methyl cellulose. Lean Zucker rats (Lean) received no treatment. Each group consisted of 6 rats.

B) General and Biochemical Parameters

Body weights were recorded at the beginning and at the end of the treatment. At the end of treatment, rats were placed in metabolic cages for 24 hours with free access to water. Urine samples were collected and analyzed for urinary sodium and potassium using flame photometry (Corning 480 Flame Photometer, Halstead, UK). For blood pressure measurement, the rats were anesthetized with sodium pentobarbital (50mg/kg body, i.p.). After midline incision the aorta was catheterized below kidneys and blood pressure was measured with a Statham pressure transducer and recorded on a Grass polygraph for 30 min. Blood samples were collected from aorta in EDTA coated tubes for measurement of blood glucose and plasma insulin. Blood glucose was measured with a glucose analyzer (Roche Diagnostic Inc., Indianapolis, USA). Plasma insulin was measured by radioimmunoassay using a commercial kit (Linco, USA).
C) Isolation and Enrichment of Proximal Tubules

After blood pressure was measured and blood samples were withdrawn, rats were used for the preparation of renal proximal tubules. Protein was measured with the use of a kit (Pierce, Rockford, IL, USA).

D) Na\(^+\), K\(^+\)-ATPase Assay

The proximal tubular suspension (1mg protein/ml) in Kreb's buffer was incubated with or without dopamine (1nmol/L to 1μmol/L) at 37°C for 20 minutes. After incubation, the tubules were permeabilized by rapid freezing in dry ice/acetone and thawing. Ouabain sensitive Na\(^+\), K\(^+\)-ATPase activity was measured and was expressed as nmoles P\(_i\)/mg protein/minute.

E) Na\(^+\), H\(^+\)-Exchanger Assay

Na\(^+\), H\(^+\)-exchanger assay in the proximal tubular suspension was performed fluorometrically. The renal proximal tubules (5mg/ml) were incubated with fluoroprobe 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyflurescein, acetoxymethyl ester (BCECF) 3μmol/L for 30 minute at 25°C with continuous oxygenation. Dopamine (1 nmol/L to 1μmol/L) was added to the fluoroprobe loaded tubules and the fluorescence was measured (excitation at 503 nm and emission at 525 nm) using LS 50 luminescence spectrometer (Perkin Elmer, Beaconsfield, UK).

F) Membrane Preparation

Proximal tubules were homogenized in a buffer containing (mmol/L) 10 Tris-HCl, 250 sucrose, 0.2 phenylmethylsulfonyl fluoride, protease inhibitor cocktail at pH 7.6, and centrifuged at 2500 g for 10 min. The supernatant was centrifuged at 24000 g for 20 min. The upper fluffy layer of the pellet was resuspended in the same buffer, dounced and centrifuged again at 24000 g for 20 min. The pellet was washed with washing buffer containing 100 KCl, 100 mannitol, 5 (N-[2-hydroxyethyl]piperazine-...
N'-[2-ethanesulfonic acid]), pH 7.2, and centrifuged at 34000 g for 30 min. Finally, the membrane fraction (containing both BBM and BLM) was resuspended in a small volume of homogenization buffer and stored frozen for further use.

G) \[^{3}H\] SCH 23390 Binding

Binding of \[^{3}H\]SCH 23390 to the proximal tubular membranes was performed. To generate saturation isotherm the ligand concentration was varied from 1-60 nmol/L. Cold SCH 23390 (10μmol/L) was used for determining the non-specific binding. The specific binding data were used to determine the Bmax and Kd values.

12. Statistical Analysis

To evaluate the results of 5-HT-induced hyperglycemia and its receptor characterization as well as DA-induced hyperglycemia and receptor characterization, Students t-test was applied and P<0.05 was considered significant. The results of treatments in diabetic animals were analyzed using one way ANOVA followed by Tukey’s multiple test to determine the level of significance. Value of P<0.05 was considered significant.