Chapter-2

Methodology

METHODOLOGY

Ethical Clearance

The Institutional Review Board, Human & Animal Research Ethics Committee, Sinha Institute of Medical Science & Technology, Kolkata and Vidyasagar University approved the study on the condition that followed the approved Human Ethics Protocol, strictly in accordance with 1964 Helsinki declaration and there was no deviation in the study protocol without the permission of the ethics board. AMI and ACS patient volunteers who participated in the study, their age ranges were about 43 to 62 years. Mentally retarded, prisoner or pregnant women were not included in the study. Volunteers signed the consent form before join to the study and they were informed detailing about the study experiment. Selection of ACS and AMI patients were performed for the study under the direction of a cardiologist. Their details blood report was intensely investigated. Minimal blood was drawn from the participated patient in presence of physician and nurses.

The maintenance of animals and all experiments were performed by the guidelines of the Ethics Committee for Animal Experimentation at institutional review board, human & animal research ethics committee, Sinha Institute of Medical Science and Technology, Kolkata, India and Department of Biochemistry, Vidyasagar University. Veterinarian inspected the animals before the study, according to existed protocol (14B) of the institute. A sterile water and standard diet were provided ad libitum. And it was strictly ensured that the animals were not to be disturbed or harmed or any pain during study period and all the study were performed in presence of an Animal Right Group member.

Chemicals

Goat anti–rabbit immunoglobulin G-alkaline phosphatase, human IgG, l-epinephrine, collagen, thrombin and insulin were purchased from Sigma Aldrich. DEA-NONOate from Sigma, Enzyme-linked immunosorbent assay (ELISA) Maxisorb plates were from Nunc, Roskilde, Denmark. Aspirin was obtained from Medica Zydus Healthcare. All other chemicals used were of analytical grade.

Selection of AMI patients

Patients (n=115; M=92; F=23) with longer duration (median 120 minutes) chest pain between the ages of 43 to 62 years, were presented characteristics ACS (it was found that the patients who had chest pain <5minuts, had good outcome within 30 days and recurrence was not found, on the other hand, the selected long lasting chest pain had very bad prognosis and so, we choose the patients with perennial chest pain) to the Intensive Coronary Care Unit (ICCU) of the Calcutta Medical College and Hospital, Kolkata.

Diagnosis of AMI

The suspected AMI patients with long duration chest pain were selected and ECG (electrocardiography) to all the selected patients was done and both ST elevated myocardial infarction (STEMI, n=47) and non-ST elevated myocardial infarction (non-STEMI, n = 68) were included in the experiment. And the patients who had chest pain, but the confirmatory occurrence of STEMI or non-STEMI were not found by (ECG) electrocardiography they were excluded from the study. We included those STEMI and non-STEMI subjects who had Q wave manifestation in ECG graph. And lastly, we measured the plasma Troponin-I level in those Q-wave patients within 2h hours of hospitalization and those were included in the following experiment.

The selection of patients with ischemic and hemorrhagic strokes

At presentation, all patients had neurologic disability affecting one or both limbs or were in semi or unconscious state and in most of the cases were feverish. Total numbers of patients (n = 48, Ischemic stroke = 22 and Hemorrhagic stroke = 26; M = 28, F = 20) between the age of 35 to 83 years participated in the study.

Preparation of platelet-rich plasma

The platelet-rich plasma (PRP) from the blood samples from either AMI subjects or normal volunteers were prepared by centrifugation as described before (Chakraborty *et al*., 2003). The platelet-free plasma (PFP) was prepared by centrifuging PRP at 10,000 g for 30 min as described before (Chakraborty *et al*., 2003).

Inhibition of platelet aggregation by acetyl salicylic acid (aspirin)

If not otherwise indicated, the inhibition of the ADP induced platelet aggregation was studied by incubating PRP with 80 µM aspirin for 30 minutes at 37°C before the aggregation of the platelets was initiated by adding 2.0 µM ADP to the PRP.

Preparation of aspirin solution

Aspirin (acetyl salicylic acid) was dissolved in de-ionized water. The pH was adjusted to 7.0 by adding 0.1 M NaHCO3 just before use and discarded after use.

Determination of nitric oxide

The amount of Nitric oxide (NO) was measured by methemoglobin method (Karmohapatra *et al*. 2007) by determining the spectral changes of the absorption maxima at 575 and 630 nm in a Spectrophotometer (Beckman, Model DU) as described early (Sinha *et al*. 1999).

Dermcidin preparation

Dermcidin preparation was done by the isolated plasma from the ACS patients by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), followed by the PAGE omission of SDS of the protein (Fazekas *et al*. 1963). Then

the isolated protein was dialyzed against 0.9% NaCl at 4°C for 12hours as demonstrated before (Sarkar *et al*., 2013).

Production of dermcidin antibody

The dermcidin antibody (polyclonal) was developed in Newzeland rabbits by using dermcidin protein which was purified and isolated by electrophoresis as explained before (Arquilla, E. R. 1956).

Enzyme linked immunosorbant assay (ELISA) for dermcidin and insulin

Plasma concentration of dermcidin and insulin were measured by ELISA by using their respective antibodies have been described before (Engvall, E. & Perlmann, P. 1972).

Scatchard plot of the equilibrium binding of dermcidin to normal platelet suspension.

Gel filtered platelets (GFP) in Tyrod's buffer without Ca^{2+} was prepared at pH 7.4 by using 6B sepharose column as delineated previously in the paper (Dutta-Roy & Sinha 1987). Electrophoretically purified dermcidin at different concentrations were incubated with gel filtered platelets ($3X10^8$ platelets/mL) in Tyrod's buffer for 90 min (optimal time for the binding of dermcidin to platelets was determined from separate experiments). When incubation was completed, 0.5 mL of that mixture was perlocated over glass fibre membrane (GF/C, Sigma aldrich) by using filtration unit (Millipore) as demonstrated (Kahn, N. N. & Sinha, A. K. 1990). Then it was washed by the same buffer as prepared above. So, unbound components and dermcidin will pass through the membrane and simultaneously bound dermcidin were adhering to the membrane filter. And membrane bound dermcidin was eluted with 0.05% triton X-100. Then the dialyzed filtrate was used to check the amount of dermcidin present in the filtrate by ELISA as described above.

From the Scatchard plot analysis the binding characteristics, i.e. the dissociation constant (Kd), and the number of dermcidin binding (n) on the platelet was computed as described below. Platelet number was assessed by optical microscopy.

Synthesis of insulin in the liver cells from adult mice

From the liver of adult mice, hepatocyte cells were prepared as explained before (Bhattacharya *et al*. 2013). That freshly prepared homogenate cell suspension (10 mg protein/ml in tyrodes' buffer, pH 7.4) was incubated with glucose (0.02 M) and in the presence or absence of dermcidin or NAME [N^G –Nitro-L-arginine methyl ester] (0.1 mM), a blocker of nitric oxide synthase (NOS) for different periods of time at 37 °C. After incubation the synthesis of insulin in the reaction mixture was determined by enzyme linked immunosorbent assay described below.

Glucose activated nitric oxide synthase (GANOS) measurement in the liver cell homogenate

Typically, the liver homogenate suspended in tyrodes buffer pH 7.0 containing 2.0 mM $CaCl_2$ with or without 0.02M glucose for different periods of time at 37°C as described before (Bhattacharya *et al.,* 2013). The synthesis of NO was determined by the methemoglobin method as described below. In some of the experiments 0.14 μM DCN2 or 0.1 mM NAME was also added to the reaction mixture to inhibit the synthesis of NO.

Endothelial cells preparation from goat carotid arteries

Carotid arteries were collected from the fresh goats from the local slaughterhouse and the arteries were utilized for the experiment forthwith as described earlier (Chakraborty *et al*., 2009) and endothelial cells was isolated from the lumen of that carotid arteries by using a nylon brush (bottle brush) perfectly dispersed in buffer (tyrode's buffer, pH 7.4), and then the cell suspension in buffer was spun at 500g at 4ºC for 10 minutes.

Blood coagulation factor (factor V) was used to check the endothelial cells' property for the experiment.

Preparation of iso-sorbide di-nitrate, nitroglycerin and aspirin solution

Isosorbide dinitrate and nitroglycerin solution was made in de-ionized water. Solution of aspirin was also prepared in de-ionized water and 0.1(M) $NaHCO_3$ was used to gain the pH 7.4 and it was discarded after use.

Incubation of endothelial cell suspension with different "nitro" compounds, acetyl salicylic acid, insulin or glucose

Endothelial cells were incubated with these agents separately. The incubated cell suspension in each case was iced and defrosted repeatedly in liquid N_2 for 30 minutes. The cytosolic supernatant part was separated by centrifugation for 30 min at 30,000g at 0°C. And after cytosolic separation the pellet was treated with triton X-100 (0.05%) followed by the treated cell suspension was centrifuged at 30,000g for 60 min at 4°C to emancipate the membranous protein. On the other way, only stimulators were not added to the cells in the control, but the remaining other conditions were same for the experiment and the NOS activity was determined.

Gel electrophoresis of membranous protein fraction treated with Triton X-100

Membrane protein (treated with triton-100) which showed NOS activity was run at 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of reducing agent. From the gel electrophoresis the molecular weight of the protein was found at 57 kDa. It was confirmed by nitric oxide activity of the sliced and triturated protein in 0.9% NaCl solution.

Protein sequencing analysis

Sequence analysis of protein was performed at the Harvard, Mass Spectrometry and proteomics Resource Laboratory, FAS Centre for Systems Biology, Northwest Bldg Room B247, 52 Oxford St, Cambridge MA by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

Stimulation of NOS activity of the electrophoretically purified 57 kDa protein by different stimulators

The purified protein (0.08mg/ml) was incubated with compounds like 10 μM isosorbide di-nitrate or 10 μM nitroglycerine or insulin 25μUnit /ml or with glucose (0.02M) or with 15 μM aspirin at pH 7.0 in the presence of 2.0mM $CaCl_2$ and *l*-arginine (varied concentration) in a total volume of 2.5 ml. After incubation of the reaction mixture for different times at 37°C, the synthesis of NO was determined. It was found that the endothelial NO was maximally stimulated after 30 minutes of the incubation at 37°C. Lineweaver-Burk plot was constructed from the rate of NO production during the maintenance of the first order reaction rate (i.e. within 1 min). The K_m and V_{max} were calculated from the Lineweaver-Burk plot.

The preparation of cell free plasma and leukocytes

The nucleic acids from the leukocyte preparation were immediately isolated by the Trizol method (Baelde *et al*., 2001).

The determination of the plasma dermcidin level

The dermcidin level in the cell free plasma preparation, as described above, was determined by enzyme linked immunosorbant assay (ELISA) using electrophoretically purified protein isolated from AIHD patients as described before (Ghosh *et al*., 2011).

The details of the determination by ELISA and the "precision" of the assay using electrophoretically pure dermcidin have been described before (Ghosh *et al*., 2011). The synthesis of dermcidin by *in vitro* translation of dermcidin mRNA separated from the isolated leukocytes as described above, was carried out in parallel experiments and quantitated by ELISA as immediately as possible without storing the samples at cold temperature in a freezer. The plasma insulin level was determined in the cell free plasma of the blood samples by ELISA as described before (Ghosh *et al*., 2011; Ghosh *et al*., 2012). The blood glucose level was determined by using a glucometer (Behringer).

mRNA extraction from muscle cells by the Trizol-chloform method

Skeletal muscle cells from mice were homogenized and equally allocated in centrifuge tubes. One ml of Trizol reagent was added per 0.5 ml of sample and vortexed thoroughly. Then, cells-trizol mixtures were again homogenized completely. The mixture was left to stand for 5min at room temperature to permit the complete dissociation of the nucleoprotein complex. After incubation 0.2ml of chloroform was added per 1ml of TRIzol reagent and was shaken vigorously for 15 seconds and left it at room temperature for 3min. Then the sample was centrifuged at 12,000g for 15min at 4ºC. The sample was separated into two distinct phases; the upper aqueous phase was pipetting out and placed in new tubes and 0.5ml of 100% isopropanol was mixed into the aqueous phase solution per 1ml of TRIzol reagent and it was incubated at room temperature for 10min. Then the sample was centrifuged at 12,000g for 10min at 4ºC. After centrifugation, the supernatant was removed and RNA pellet was in the tube. This RNA pellet was washed by 1ml of 75% ethanol per 1ml of TRIzol reagent used. Subsequently, the sample was vortexed briefly and centrifuged at 7500g for 5min at 4ºC and the wash was discarded. The RNA

pellet was air dried for 5-10 min and following that the RNA pellet was resuspended in RNase free water.

Separation of plant ribosomes

Bael leaves (Aegle marmelos) were washed to clean well by double distilled water. The leaves were homogenized and later the homogenized mixture was centrifuged at 5000rpm. Then, the supernatant was taken and centrifuged at 13,000g and the ribosome pellet was prepared as described (Cherry *et al*., 1974).

***In-vitro* translation of dermcidin mRNA**

Different concentrations (w/v%) of AETL were incubated with homogenized skeletal muscle cells of mice for 2h and total mRNA was isolated from the incubated mixture by the Trizol method as described above. The isolated mRNA was mixed with ribosome particles (isolated from bael leaves), ATP and amino acid and incubated for 6h (Chakraborty *et al*., 2009). At every hour the whole mixture was shaken for 15 Sec. After 6h the reaction mixture was centrifuged at 10,000g for 10min at 0ºC. The supernatant was used to determine the amount of dermcidin by ELISA as described before (Engvall *et al*., 1972).

Neutrophil isolation

Two-three ml of blood was collected from normal volunteers in citrate solution (1:9) and neutrophil were isolated from the collected blood in HBSS buffer solution as described (Klock *et al*., 1972). The isolated neutrophils were observed by microscopy and then neutrophils were incubated with different conc. of nicotine for 2hr and mRNA was isolated by the TRIzol mRNA method (Cook L *et al*. 2000). Gene expression of mRNA was analyzed by the RT - PCR method.

Reverse transcription - Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by the above mentioned method. The two-step reverse transcription-polymerase chain reaction was performed - at first step 8ngm of isolated RNA, oligo d(T) (50µM), nuclease free water was added to a microcentrifuge tube (total vol. 8µL) and denature the RNA for 5min at 70ºC and it was spun briefly and put immediately on ice. Reverse transcriptase reaction buffer (8µL) and reverse transcriptase enzyme (2µL) was added to the RNA reaction mixture in the centrifuge tube and total 20µL cDNA synthesis reaction was incubated at 42ºC for 1h. Now, at the second step, the cDNA (2µL) was amplified by Taq polymerase using appropriate primers for dermcidin and dNTP mixtures. The PCR amplification of the cDNA was performed for 35cycles (denature at 94ºC for 30sec, annealing at 57ºC for 1min and primer extension at 72ºC for 30sec) conditions, before starting of the 35 cycles, the cDNA was heated at 94ºC for 5min and after completion of cycles the cDNA reaction mixture was kept at 72ºC for 5min. The amplification reaction products were resolved in 1% agarose/TAE gels, electrophoresed at 90mV and visualized by Ethidium Bromide (EtBr) staining.

Preparation of hypoxic animal model

Animals were subjected to hypoxia in a specially animal decompression chamber which were fabricated and the pressure was in the chamber equal to 282 Hg (condition at an altitude 7628m) and O_2 content ~8.5% as described previously (Singh *et al*. 2014). The temperature and humidity were 28ºC and 60±5% respectively. Control animals were not in the decompression chamber, but otherwise treated as same.

Binding of insulin was analyzed by Scatchard plot

Grated muscle cells were homogenized in Tyrod's buffer at pH 7.4. Muscle cells were incubated with dermcidin (0.35µM) and different concentration of insulin for 2h. In separate experiment, muscle cells were incubated with dermcidin along with insulin (30µUnits/mL) and acetyl salicylic acid (15µM) for 2h. After completion of incubation, 0.5mL of incubated mixture was filtered over glass fiber membrane (GF/C) by using Millipore filtration unit and washed by 3vol of buffer under mild vacuum conditions as described before (Kahn N.N. & Sinha A.K.,1990). After washing membrane filters were dried in the air and washed by 1vol of 0.05% TritonX-100. Membrane bound insulin then came into the Triton X solution and this solution was next dialyzed and amount of insulin in the solution was determined by ELISA by using the insulin antibody (Engvall *et al*. 1972).

Localization of GLUT-4 in muscle cells of mice by immunofluorescence

Muscle cells were prepared in very tiny parts by the use of cryostat. Then these were incubated with fluorescent tagged anti-goat-rabbit primary GLUT-4 antibody (1:200 dilutions). Following the incubation, the sample was washed with PBS (phosphate buffer solution). And the sections were imaged by fluorescent microscope attached with high resolution digital color camera (Coligan *et al*. 1992).

Probable interaction of dermcidin with other proteins by Bioinformatics analysis

STRING (*Search Tool for the Retrieval of Interacting Genes/Proteins*) is a biological database and web resource of known and predicted protein-protein interactions. String is a pre-computed database derived from experimental data, literature mining, analysis of co-expressed genes etc. String applies a unique scoring method based on the different types of associations against a common reference set and produces a single confidence score per prediction. Evidence based interactive or interrelated pattern dermcidin protein of *Macaca*

mulatta has been deduced by this online resource/analytical software (http://string-db.org/). *Macaca mulatta* is the metabolically closer to human and mostly used primate for medical research. Its dermcidin protein also has a large network of protein interaction pattern deduced by this software.

Structure retrieval

All the X-ray crystallographic structures of GLUT4 (2AL3), Dermcidin (2KSG), Insulin (1ZNI), Insulin ectodomain 1 (6CE7), Insulin ectodomain 2 (6CE9) were retrieved from Protein Data Bank (https://www.rcsb.org).

Protein-protein docking studies were performed using a PATCHDOCK server (Duhovny *et al*. 2002 and Schneidman-Duhovny *et al*. 2005), ClusPro (Kozakov *et al*. 2013, Vajda *et al.,* 2017, and Kozakov *et al*., 2017), ZDOCK server (Pierce *et al*., 2014) and SwarmDock server (Torchala *et al*., 2013a, 2013b and Torchala *et al*., 2014). The docking results were analyzed and represented using Autodock Tools 1.5.6 (Goodsell and Olson 1990) and PyMOL-2.0.7 (DeLano 2004).

Oral insulin preparation

One mL cow milk was heated for 2 minutes at 100 °C and then brought to room temperature. Next 100 µ lit of insulin (0.08 unit of insulin) was added to the cooled milk and incubated for two and half hour at room temperature (23°C). After incubation, the milk-insulin mixture was precipitated by 10 µ L (0.6 M) acetic acid or by lemon juice and let it stand in that condition for 45 min. After precipitation, the supernatant can be carefully decanted and added with the same amount of cooled and boiled milk to the milk-insulin precipitated mixture to make up the volume 1ml (final).

Preparation of alloxan induced diabetic mice: Alloxan (Sigma Aldrich) was injected (150 mg/kg) to the fasting mice. Then the mice were kept with sufficient food so that they could not hypoglycemic. After 72 h, experiments were done by the diabetic mice.

Determination of the distribution of insulin in orally fed milk-insulin from different parts of the diabetic mice

The milk-insulin preparation (0.4 mL) as described above was fed to the alloxan induced diabetic mice. After 3 h and 7 h blood sample (0.1 mL) were collected from tail vein, left and right superior vena cava, hepatic vein, and femoral vein. At the same time a blood sample was also taken from appropriate control diabetic mice where only insulin (i.e. not incubated with milk) was fed to the mice. To minimize the pain and discomfort of the mice, morphine was administered to the mice. The amounts of insulin were quantitated in each case by ELISA.

Preparations of intestinal loops filled up with milk-insulin preparation solution

For this experiment adult normal white mice (3–4 months) were taken and they were killed by cervical dislocation. Small intestine was taken out and cut into five pieces (same size, 1.5 inches). The inner portion of the intestine (each piece) was cleaned well by using Tyrode's buffer. Silk cord was used to tie one end of the each piece of intestine and milk-insulin complex solution as stated above was pipetted into the loop then the other open side was bound tightly. These piece intestinal loops were submerged separately in 2 mL of tyrod's buffer (glucose included) and both the intestinal loop and the external milieu (tyrod's buffer) were shaken mildly at 16 Hz at 37 °C and at different intervals (1, 2, 3, 4, 5, 6, 7 h) and a part of the (0.2 mL) of the milieu was withdrawn and the insulin in the milieu was determined by ELISA by using insulin antibody as described above.

Immunoblot analysis of milk-insulin mixture treated with trypsin

Presence of insulin in the trypsin treated milk-insulin mixture as described in the manuscript was analyzed by immunoblot using by insulin antibody. The transfer buffer used in the experiment contains Tris, Glycine in 20% Methanol while TBST (TBS with 0.1% Tween-20) was used for washing. Incubation buffer was contained Tris and NaCl (TBS). The membrane was blocked with 5%BSA in TBS. Ponceau (1 gm/100 mL) was used to check the transfer of protein from a gel to the membrane.

Statistical analysis

The results shown are mean ± standard deviation (SD); the significance (p) of the results was determined by student's ''t'' test. The coefficient of correlation (''r'') was determined by Pearson test. The dissociation constant (K_d) and the number of the dermcidin binding sites (Bmax) in the Scatchard plot analyses were determined by (Graphpad Prism software) and Microsoft Office Excel.

To demonstrate the comparison of two values, the Wilcoxon sign rank test was performed and Spearman's rank correlation was analyzed to see the correlation.