SUPPLEMENTAL MATERIAL

2. MATERIALS AND METHODS

2.1. Isolated Heart Preparations

Experiments were performed in accordance with the regulations of the university ethics committee as stablished by the Mexican Federal Law ZOO-1999 for use of experimental animals. Male Wistar rats (350-400 g) were anesthetized intra-peritoneally with Nembutal (50 mg/Kg) and heparin (500 IU). Animals were artificially ventilated and the heart was removed, the ascending aorta was cannulated and retrogradely perfused according to the method of Langendorff [22, 23]. Thereafter, we proceeded to biotinylation of VELM proteins.

2.2. Biotinylation of VELM Proteins

VELM proteins were labeled with biotin during control conditions. Sulfosuccinimidyl-6-biotinamido hexanoate (S-NHS-LC-biotin), an electrically charged and cell membrane impermeable agent was utilized to biotin label VELM proteins during control conditions. The coronary vasculature was perfused with phosphate-buffered saline (PBS, pH 7.4) to remove blood from the vascular bed. Coronary flow was kept constant at 8 ml/min. PBS containing S-NHS-LC-biotin 0.125 mg/ml was perfused. To restrict S-NHS-LC-biotin to the vascular lumen to label VELM proteins, it was perfused during brief periods of 5 sec duration followed by a 55 sec wash period with PBS. This cycle was repeated six times. At the end of the last cycle, free S-NHS-LC-biotin was washed out with 50 ml PBS containing 5 mg/ml glycine.

2.3. Immunohistochemistry of Biotin-labeled Proteins in Insitu Myocardium During Control

After labeling hearts were immersed for 24 hr in a solution of 10 % buffered paraformaldehyde. Ventricular tissue blocks were progressively dehydrated and embedded in paraffin and 4-5 μm sections were made. Paraffin removed and tissue sections endogenous peroxidase inactivated with 3% hydrogen peroxide and blocked with 2% bovine serum albumin and then incubated in 10 μg/ml horseradish peroxidase (HRP)-conjugated streptavidin at room temperature for 1h. 3, 3´-diaminobenzidine (DAB) was used to develop HRP color reaction. The sections were counterstained with hematoxylin and mounted for microscopic observation.

2.4. Control, Ischemia and Ischemia/ Reperfusion Protocols

After VELM biotin labeling during control period, hearts were perfused with PBS at a constant flow of 8 ml/min for 5 min. At this time 3 hearts were processed for tissue protein extraction (control group). Another group of 3 hearts was subjected to 25 min of no flow ischemia and at this point processed for tissue protein extraction (ischemia group). Another group of 3 hearts was subjected to 25 min ischemia, followed by a 30 min reperfusion period (I/R group), thereafter processed for tissue protein extraction. Proteins of the hearts of each group were pooled for analysis. This protocol was performed two times.

2.5. Processing and Fractionation of Tissue

The three ventricles of each group were dissected, weighed, minced and added to ice cold 5 vol (volume/weight, ml/mg) of PBS containing 1 mM of phenylmethylsulphonyl fluoride (PMSF), and homogenized with a Polytron (TP 18/10 S1 homogenizer). Sodium dodecyl sulphate (SDS) was added to the homogenate to a 5% final concentration and gently mixed for 1 h. The homogenate was centrifuged (20,000g, 4^{o}C) for 2h to remove any insoluble materials remaining in the supernant [33]. In some experiments aliquots of 20,000g supernatant were further centrifuged to 100,000g to insure that no biotin-labeled proteins remained in the supernatant, indicating the 20,000g fraction contains the cardiac membranes (CM) of the VELM [29]. To eliminate possible contamination of free biotin, the 20,000g supernatant was dialyzed (molecular weight cut-off = 10 kDa) against PBS overnight. Protein concentration was determined with the bicinchoninic acid kit.

To identify biotin labeled VELM proteins 2 different procedures were utilized. The dialyzed CM homogenate was divided into 2 aliquots. For procedure No 1, an aliquot of CM proteins was directly processed for SDS-PAGE 2D gel electrophoresis and Western blot [30] to identify biotin-labeled VELM proteins.

Procedure No 2, isolation of VELM protein fraction. The second CM aliquot was applied to the pre-equilibrated monomeric avidin affinity column (PIERCE). The non-biotin labeled proteins, unbound proteins, was washed off thoroughly from the column with PBS. The column bound biotinylated VELM proteins were eluted with 2 mM D-biotin in PBS, extensively dialyzed, and processed for SDS-PAGE. The results obtained with both procedures were the same.

2.6. Identification of Specific Antigens in VELM Protein Fraction by Immuno-Dot Blot

On VELM protein fraction Immuno-blots were performed. 3 μg of VELM protein were dot deposited in PVDF membrane and at room temperature, blocked with albumin, rinsed and incubated with a primary antibody (1:1000), rinsed, incubated with a secondary antibody coupled to HRP (1:10000), rinsed and DAB was used to develop HRP color reaction. Twelve specific antigens were identified.

2.7. SDS-PAGE Gel and Western Blotting

In all experiments an equal amount of protein was loaded in each well of the electrophoresis gel. Proteins were separated in 12% SDS-PAGE (80 x 75 x 1 mm) gels under reducing conditions at 10 mA/gel in a running buffer; 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS. For the Western blotting, a mini-gel was electro-transferred to nitrocellulose membranes (0.45-µm pore size) at 20 V for 45 min in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) using a semi-dry transfer system (Trans-blot SD semi-dry transfer cell) as described by Towbin [36] Nonspecific sites on the membrane were blocked at 4^{o}C for 1h in 3% BSA-TBS. After the membranes were washed with 0.05% Tween-TBS three times, they were incubated with horseradish peroxidase-streptavidin diluted 1/10,000 in TBS for 1 h at room temperature. Blots were subsequently washed with Tween-TBS and developed in 10 ml of Tween-TBS containing Diaminobenzidine (DAB) 5 mg and 2 µl of 30% hydrogen peroxide until color developed.

2.8. Two-Dimensional Gel Electrophoresis

Immobilized linear pH gradient (IPG) 11-cm pH 5-8L strips were rehydrated overnight with rehydration solution containing 400 µg of a CM protein fraction. IEF was then performed using the Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) at 20^{o}C at the 3 steps and hold voltage mode for a total of 17.6 kVh (500 V for 1 min, 4000 V for 1.5 h, and 8000 V for 1.5 h). IPG strips were treated with 2 ml of SDS equilibration buffer (50 mM Tris-HCl, pH 8.6, 6M urea, 30% glycerol, 2% SDS, bromophenol blue and 1% DTT added just prior use) for 15 min. 12% SDS-PAGE gels (133 x 87 x 1 mm) were used for the 2-D. After setting the IPG strip on the gel, the gel was run in running buffer at 10 mA/gel for 45 min and then for 2 h at 20 mA/gel. The gels were fixed overnight in fixation solution and stained with Sypro Ruby for 30 min at room temperature. Subsequently they were visualized and photographed on an ultraviolet transiluminator.

In a different CAM protein fraction 2D-SDS-PAGE was performed and run as descirbed above but not stained with Sypro Ruby. The 2-D proteins were blotted to nitrocellulose membranes at 20 V for 45 min in transfer buffer using a semi-dry transfer system. After transfer, the biotin-labeled VELM proteins in the gels were incubated with horseradish peroxidase-streptavidin diluted 1/10,000 in TBS for 1 h at room temperature. Blots were subsequently washed with Tween-TBS and developed in 10 ml of Tween-TBS containing Diaminobenzidine (DAB) 5 mg and 2 µl of 30% hydrogen peroxide until color developed.