Characterization of mRNA for SOD

Isolation of RNA

Hearts taken for RNA isolation were immediately frozen in liquid nitrogen after removal and then stored at -80 °C. Total RNA was isolated by a single-step acid guanidinium thiocyanate-phenol-chloroform extraction method.[12] Aliquots (15-20 g) of RNA were denatured with 48 formamide and 6.3 formaldehyde at 55 °C for 15 min and size-fractionated by electrophoresis in 1.2 agarose gels containing 2 formaldehyde and 0.5 μg/mL ethidium bromide (Sigma). RNA was transferred to Hybond N nylon membranes (Amersham, Buckinghamshire, UK) by Northern blot capillary transfer overnight using 2 volumes of saline sodium citrate (2X SSC) as the transfer medium. The transfer was controlled on an UV transilluminator and alternatively by staining the blot membrane with 0.05 methylene blue according to Herrin and Schmidt.[13] Filters were rapidly prehybridized at 65 °C in hybridization solution (Quikhyb, Stratagene, CA, USA).

cDNA Probes

Plasmids with cDNA of rat Mn-SOD and rat Cu, Zn-SOD were obtained as kind gifts from Dr. Ye-Shih Ho (Institute of Chemical Toxicology, Wayne State Univ., Detroit, MI, USA).Transformation in *Escherichia coli,* plasmid preparation, and cDNA purification were performed by standard molecular biology methods.[14] Radioactive probes were prepared by the multiprime DNA labeling system suggested by the supplier (Amersham). The prepared cDNA inserts and GAPDH probes were added directly into the prehybridization solution (Quikhyb, Stratagene) at a radioactivity of 1×10^6 CPM/mL.

Hybridization Procedure

Hybridization was performed at 60 °C for 1.5 h. After hybridization, blot membranes were washed twice for 15 min at room temperature with 2 SSC/0.1 SDS (2.5 mL/cm^2) followed by a 30-min high stringency wash with 0.1 SSC/0.1 SDS at 60 °C. Wet blot membranes were sealed in plastic foil and exposed to medium-sensitive medical x-ray film (Fuji, Tokyo, Japan) at -70 °C using intensifier screens. Exposure times were 2-3 days for Northern blots. The hybridization intensity of autoradiographic signals was measured using 2-dimensional densitometry. The obtained density (in optical units) was calculated versus the value of a slot blot for GAPDH to quantify the mRNA.

Western Blot Analysis

Moreover, we identified SOD enzymes in myocardium by Western blot analysis using polyclonal antibodies from Biodesign (Kennebunk, ME, USA). The obtained myocardium was lysed in buffer containing 1 Triton X-100. According to the previous method, protein samples were fractionated by gel electrophoresis run at 40 and 100 V at 4 °C during the stacking and separation steps, respectively. The separated proteins were blotted onto nitrocellulose.[15] After reaction with Cu,Zn-SOD antibodies (43 μg/mL) or Mn-SOD antibodies (33 μg/mL), immunostaining was performed by incubation in Trisbuffer (10 mmol/l; pH 7.4) for peroxidase activity using the enhanced chemiluminescence (ECL) development system (Amersham). This antibody is purified from human liver and was shown to be specific by the supplier using 2D-IEP and double diffusion. A response was observed at 25 kD for Mn-SOD and at 33 kD for Cu, Zn-SOD. The obtained immunoblots were then quantified by a laser densitometer.

Statistical Analysis

The number of experiments or experimental animals was 8 unless specified otherwise. Results are given as the mean ± SEM from the numbers of animals. Student's *t*-test was used to compare 2 mean values for paired and/or unpaired observations; a probability of 0.05 or less was considered significant. Where 2 or more treatment means were compared to 1 control mean, determination of the differences was carried out with Dunnett's multiple comparison.[16]

RESULTS

Blood Pressure in DOCA-salt-induced Hypertensive Rats and WKY Normotensive Rats

Figure 1 presents the systemic mean blood pres-