ONLINE DATA SUPPLEMENT

Effects of Human C-Reactive Protein on Pathogenesis of Features of the Metabolic Syndrome

Running title: CRP and the metabolic syndrome

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Supplementary Methods

Oral glucose tolerance testing

Oral glucose tolerance tests were performed using a glucose load of 300 mg/100 g body weight after overnight fasting. Blood was drawn from the tail without anesthesia before the glucose load (0 min time point) and at 30, 60, and 120 min thereafter.

Skeletal muscle insulin sensitivity

Insulin stimulated nonoxidative glucose metabolism was determined in isolated soleus muscle by measuring the incorporation of ^{14}C-U glucose into glycogen. The soleus muscles were attached to a stainless steel frame in situ at in vivo length by special clips and separated from other muscles and tendons and immediately incubated for 2 h in Krebs-Ringer bicarbonate buffer, pH 7.4, gaseous phase 95% O_2 and 5% CO_2 that contained 5.5 mM unlabeled glucose, 0.5 μCi/ml of ^{14}C-U glucose, and 3 mg/ml bovine serum albumin (Armour, Fraction V) with or without 250 μunits/ml insulin. After 2 h incubation, glycogen was extracted and glucose incorporation into glycogen determined.

Biochemical analyses

Rat serum CRP and human serum CRP were measured using ELISA kits (Alpha Diagnostics International, San Antonio, U.S.A.). Blood glucose levels were measured by the glucose oxidase assay (Pliva-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5% trichloracetic acid and promptly centrifuged. NEFA levels were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). Serum triglyceride concentrations were measured by standard enzymatic methods (Pliva-Lachema, Brno, Czech Republic). Serum insulin concentrations were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum adiponectin was determined with a rat ELISA kit (B-Bridge International, Inc., Moutain View, U.S.A.). Serum IL6 and TNFα were measured by rat ELISA kits (BioSource International, Inc., Camarillo, U.S.A.).

Tissue triglyceride measurements

For determination of triglycerides in liver and soleus muscle, tissues were powdered under liquid N_2 and extracted for 16 h in chloroform:methanol, after which 2% KH_2PO_4 was added and the solution was centrifuged. The organic phase was removed and evaporated under N_2. The resulting pellet was dissolved in isopropyl alcohol, and triglyceride content was determined by enzymatic assay (Pliva-Lachema, Brno, Czech Republic).

Blood pressure measurement

Arterial blood pressures were measured continuously by radiotelemetry in paired experiments between conscious, unrestrained male rats. All rats were allowed to recover for at least 7 days after surgical implantation of radiotelemetry transducers before the start of blood pressure recordings. Pulsatile pressures were recorded in 5-second bursts every 10 minutes throughout the day and night, and 24-hour averages for systolic and diastolic arterial blood pressure were calculated for each rat for 1 week periods. After measuring blood pressure for two weeks, all rats were given 1% NaCl for drinking instead of tap water for an additional week of blood pressure measurements to test for effects of human CRP on blood pressure salt-sensitivity. The results from each rat in the same group were then averaged to obtain the group means.

Parameters of oxidative stress

The activity of superoxide dismutase (SOD) was analyzed using the reaction of blocking nitrotetrazolium blue reduction and nitroformazan formation. Catalase (CAT) activity measurement was based on the ability of H_2O_2 to produce with ammonium molybdate a color complex detected spectrophotometrically. The activity of seleno-dependent glutathione peroxidase (GSH-Px) was monitored by oxidation of gluthathione by Ellman reagent (0.01M solution of 5,5'-dythiobis-2 nitrobenzoic acid). The level of GSH was determined in the reaction of SH-groups using Ellman reagent. Glutathione reductase (GR) activity was measured by the decrease of absorbance at 340 nm using a millimolar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADPH (using Sigma assay kit). The levels of conjugated dienes (CD) were analyzed by extraction in the media (heptane:isopropanol = 2:1) and measured spectrophotometrically in the heptane layer. The levels of thiobarbituric acid reactive substances (TBARS) were determined by the reaction with thiobarbituric acid.

Histopathological examination.

Organs from each of three males of SHR-CRP and SHR strains, four months of age were collected immediately after euthanasia with ether overdose. The tissues were fixed in 10% buffered formalin. Samples were processed by the common paraffin technique and histological slices 5 μm thick were stained with hematoxylin and eosin, with blue trichrome and with PAS reaction as indicated.