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ENHANCED THROMBIN-INDUCED AGGREGATION AND INOSITOL TRISPHOSPHATE FORMATION OF PLATELETS FROM SPONTANEOUSLY HYPERCHOLESTEROLEMIC RATS. P.D. Winocour, M.L. Rand, J.D. Vickers, R.L. Kinlough-Rathbone, and J.F. Mustard. Department of Pathology, McMaster University, Hamilton, Ontario, Canada.

Platelets from rats with diet-induced hypercholesterolemia are hypersensitive to thrombin through a pathway independent of released ADP or thromboxane A₂ (TXA₂) formation. We examined if platelets from rats with spontaneous hypercholesterolemia (HC) are similarly hypersensitive. HC rats (plasma cholesterol: 130±4 mg/dl, n=15) were compared with their normocholesterolemic genetic controls (NC) (87±4 mg/dl, p<0.001, n=16). Total cholesterol/10⁹ platelets was not different between the groups (HC: 0.314±0.032 μmole, n=7; NC: 0.357±0.046 μmole, n=7). Washed platelets were prelabelled with ¹⁴C-serotonin. In the presence of aspirin (to inhibit TXA₂ formation) and creatine phosphate/creatine phosphokinase (CP/CPK) (to remove released ADP), HC platelets aggregated more (22±2%, n=11) than NC platelets (10±4%, n=12, p<0.01) in response to thrombin (0.065 U/ml); ¹⁴C release was not different. Thrombin causes inositol mono-, bis-, and trisphosphate (IP, IP₂, IP₃) formation from phosphoinositides (PI, PIP, PIP₂ respectively) in rabbit platelets; IP₃ may be involved in Ca⁺⁺ mobilization and the release of granule contents. We examined if enhanced inositol phosphate formation was associated with hypersensitivity of HC platelets. Platelets were prelabelled with ³H-inositol and stimulated with thrombin (0.057 U/ml) for 30 sec in the presence of aspirin and CP/CPK. Li⁺ (20 mM) was used to prevent degradation of inositol phosphates to inositol. ³H-IP, IP₂ and IP₃ were isolated by ion-exchange chromatography. The increase in radioactivity (dpm/10⁹ platelets) in IP₂ and IP₃ following thrombin stimulation was greater in HC platelets (IP₂: 2210±160, n=4; IP₃: 1430±180, n=4) than in NC platelets (IP₂: 660±150, n=4, p<0.001; IP₃: 490±100, n=4, p<0.01); IP was not different.

Thus platelets from spontaneously HC rats are hypersensitive to thrombin independently of released ADP or TXA₂ formation. This hypersensitivity is associated with only moderate increases in plasma cholesterol and no detectable increase in total platelet cholesterol. Enhanced labelling of IP₃ may indicate that enhanced activity of the pathways leading to IP₃ formation is associated with this hypersensitivity.

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IMPAIRMENT OF PLATELET PHOSPHOINOSITIDE METABOLISM IN PRIMARY HYPERTENSION. S Koutouzov, A Remmal, P Marche, P Meyer. U7 INSERM, Dept de Pharmacologie, Hôp. Necker, 75015 Paris, FRANCE.

Blood platelets from hypertensive patients and spontaneously hypertensive rats (SHR) display multiple abnormalities when compared with cells from normotensive controls. The major features of the modified platelet profile are an enhanced rate of adhesion/aggregation in response to many stimuli, a greater sensitivity for thrombin and adrenaline to produce increases in cytoplasmic free Ca²⁺, and an exaggerated release reaction. Furthermore, the resting levels of cytosolic free Ca²⁺ ions are specifically and constantly increased. Since phosphoinositides are involved in the stimulus-response coupling mediated by intracellular Ca²⁺ mobilization, the metabolism of these lipids was investigated in platelets of SHR and compared with those of normotensive Wistar-Kyoto rats (WKY). Following ³²P-labelling of quiescent platelets, labeled lipids were analyzed both in platelets at rest and after thrombin stimulation. In resting platelets, the ³²P associated with each of the phosphoinositides and phosphatidic acid (PA) was similar in SHR and WKY indicating that both the pool size of the various lipids and their basal turnover did not differ between the two strains. By contrast, within the first seconds after thrombin stimulation (10-60 sec), the dose-response and time-course curves of agonist-induced increase in ³²P-PA were markedly shifted to the left and reached higher equilibrium levels in SHR. Since thrombin-induced ³²P-PA formation is held as the most sensitive index of phospholipase C activity, our results indicate that this enzyme displays hyperreactivity in SHR (vs WKY). It is therefore likely that in SHR, the enhanced physiological responses (serotonin secretion, aggregation) that we observed under the same experimental conditions may be related to an increased formation of Phospholipase C products (inositoltriphosphate and diacylglycerol) which are the two second messengers responsible for internal Ca²⁺ mobilization and activation of protein kinase C, respectively. Therefore, these data suggest that the hypersensitivity of Phospholipase C may be involved in the overall alteration of cell calcium handling and hence in the SHR platelet responses.

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PROTEIN C AND PROTEIN C INHIBITOR

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PURIFICATION AND CHARACTERIZATION OF A PROTEIN C ACTIVATOR FROM THE VENOM OF AGKISTRODON CONTORTRIX CONTORTRIX. Carolyn L. Ortner, Prabir Bhattacharya and Dudley K. Strickland. American Red Cross Laboratories, Rockville, MD 20855, U.S.A.

There are two recent reports on the purification and properties of a protein C activator (PCA) from the venom of the Southern copperhead snake. The purification of a 37,000 Mr nonenzymatic PCA (Martinoli and Stocker, *Thromb. Res.* 43, 253, 1976) as well as of a 20,000 Mr thrombin-like enzyme (Klein and Walker, *Biochem.* 25, 4175, 1986) have been described. The purpose of this investigation was to purify and further characterize the PCA(s) from this venom. A PCA has been isolated by sulphopropyl-Sephadex followed by gel filtration chromatography resulting in approximately a 100-fold purification with a 50% yield. PCA appeared as a single band on SDS-PAGE with an estimated Mr of 32,000 or 37,000 in the absence or presence of β-mercaptoethanol, respectively. High pressure gel permeation chromatography of PCA in Tris-buffered saline, pH 7.5 resulted in a single protein peak with a Mr of 39,000 which was coincident with activity. PCA was a potent activator of human protein C (PC) with a Km for PC of 0.6μM and a Vm of 0.02 sec⁻¹. In addition, PCA catalyzed the amidolysis of Tosyl-gly-pro-arg-p-nitroanilide (TGPRpNA) with a Km of 1.1 mM and a Vm of 66 sec⁻¹. The rate of amidolysis of five other peptidyl-arginyl-pNA substrates each tested at 1.0 mM was < 10% that of TGPRpNA. PCA was inhibited by nitrophenylguanidinobenzoate (NPGB), phenylmethylsulphonylfouride, D-phe-pro-arg-chloromethylketone (PPACK) and soybean trypsin inhibitor indicating that PCA is a serine protease. The active site concentration of PCA as measured by NPGB titration was 90% that of the protein concentration. Measurement of the rate of PCA inhibition at varying levels of PPACK indicated that it had a Ki of 34μM and an alkylation rate constant of 0.09 min⁻¹. PCA activation of PC was completely inhibited by CaCl₂ with an apparent Ki of 99μM. Since neither PCA amidolysis of TGPRpNA nor inhibition by PPACK was affected by Ca²⁺, the effect of this metal was likely on the substrate PC. In summary, a PCA has been purified to homogeneity and has properties which are distinct from those reported. PCA promises to be a useful enzyme in studies of PC and its activation.

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IMMUNOAFFINITY PURIFICATION OF PLASMA INHIBITOR FOR ACTIVATED PROTEIN C. M. Laurell (1), T. Carlsson (2), J. Stenflo (1). Department of Clinical Chemistry, Malmö General Hospital (1), Malmö, Sweden, Department of Pathology, School of Medicine (2), University of New Mexico, USA.

Activated protein C (APC) is an important regulator of blood coagulation in vivo. In plasma this serine protease is slowly inhibited by a specific inhibitor, activated protein C inhibitor (PCI), (Suzuki et al. (1983) *J.Biol.Chem.* 258, 163-168). We have now made monoclonal antibodies against PCI by immunizing with the APC-PCI complex. Positive clones were identified by solid phase immunoassay with ¹²⁵I labelled partially purified inhibitor. After subcloning and expansion in mice, one of the monoclonal antibodies was immobilized on Sepharose 4B and used in the purification of the inhibitor. A two step purification procedure was devised starting with passage of fresh human plasma over the column. Following extensive washing the inhibitor was eluted with 50 mM triethylamine-HCl, 0.5 M NaCl, pH 11.0, from the column together with a small amount of high molecular weight material. After gel filtration on a column packed with ACA 44 the inhibitor appeared homogenous on SDS - PAGE. Approximately 0.5 mg inhibitor was obtained from 200 ml of fresh plasma. The apparent M_r of the inhibitor was 57000 kDa on SDS - PAGE. The purified protein formed a complex (M_r ≈ 110000 kDa) with human APC. At the same time a band (M_r = 54000 kDa) appeared that represented the modified inhibitor. When analyzed on agarose gel electrophoresis (75mM barbital buffer, 2mM EDTA at pH 8.7) the PCI migrated to the β₂- region, whereas the modified inhibitor had a slightly more anodal mobility. The APC-PCI complex migrated to the α₂-region.

Two immunoradiometric assays were constructed with the monoclonal antibodies. One measured the complexes between APC and PCI while the other one measured the total amount of PCI present. These assays were used to study complex formation in buffer and plasma.