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PURIFICATION AND PARTIAL CHARACTERIZATION OF A BIOACTIVE SUBSTANCE FROM RAT'S VESSEL WALL INDEPENDENT OF PROSTACYCLIN PRODUCTION. A.C. Kempfer (1), N. Maugeri (1), C. Farías (1), E. Bermejo (1), M. Gimeno (2), M. Lazzari (1). Department of Hemostasis and Thrombosis, Academia Nacional de Medicina (1) and CEFAPRYM (2), Buenos Aires, Argentina.

Our previous observations provided evidence that a bioactive substance (BAS) with inhibitory effect on platelet aggregation and with inotropic activity on smooth muscle preparations is present in aortic wall of rats treated previously with indomethacin. The ability to inhibit platelet aggregation was used for monitoring its purification and partial characterization.
The original sample was extracted by rinsing aortic rings (1.5 mg dried rings) in buffer Krebs (300 ul) for 40 minutes at room temperature. The substance was purified by gel filtration (Bio-gel P-30, Sephadex G-100, Sephadex G-75) and ion exchange chromagraphy (DEAE cellulose). In the last method, salt gradient elution was performed. Further purification by Sephadex G-50 resulted in removal of 90% of the contaminating substances without a loss of inhibitory activity. The main peak of both chromatographic procedures was analyzed on SDS-PAGE and PAGE with denaturing solvents. The substance was evident by Coomassie Brilliant Blue and periodic acid Schiff staining.
In order to determine if the BAS was susceptible to proteolysis, an aliquot of the original sample (29 ug total protein) was incubated with trypsin (final concentration 0.3 ug/ml) and with chymotrypsin (final concentration 3 ug/ml). The BAS activity was not detected. An aliquot of the same original sample was incubated with neuraminidase (final concentration 1.2 units). The BAS activity was detected.
The substance appeared to be stable for at least 18 hours at room temperature and 2 hours at 37°C. In addition it was stable over a pH range between 6.8 to 8.6, showing an anionic behaviour.
The protein concentration of this substance determined by the method of Lowry was 1 ug/ml.
Partial characterization supports the conclusion that the substance present in aortic wall of rats is a homogeneous protein, which has a molecular size estimated at 55-65 kDa.

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INHIBITORY EFFECTS OF ITP SERA ON BINDING OF ANTI-PLATELET GLYCOPROTEIN (GP) IIb/IIIa MONOCLONAL ANTIBODIES TO HUMAN UMBILICAL VASCULAR ENDOTHELIAL CELLS (HUVE). T. Nakajima, T. Koyama, Y. Nishida, H. Tanaka, E. Kakishita and K. Nagai. The Second Department of Internal Medicine, Hyogo College of Medicine, Hyogo, JAPAN.

Some ITP patients have specific autoantibodies to platelet GP IIb/IIIa. On the other hand, HUVE were shown to synthesize platelet GP IIb/IIIa like substances. Therefore, we studied the binding of ITP sera to HUVE by showing the inhibitory effect of ITP sera on the binding of anti-platelet GP IIb/IIIa monoclonal antibodies to HUVE. HUVE were cultured according to the method of Jaffe et al. 125-I-anti-platelet GP IIb/IIIa monoclonal antibody (125-I-Anti-GP) (40.3 mCi/mg), 40 µl, was added to a cell suspension of HUVE (1.5×10^4/500 µl) in a plastic RIA tube. After incubation for 30 min. at 4°C and centrifugation of 10,000 xg for 3 min., the radioactivity of the cell pellet was measured. Specific binding was determined by determining the difference between cell-bound radioactivity in the absence and presence of an excess amount of unlabelled ligand at 100 x concentrations. Scatchard analysis using 125-I-Anti-GP showed that the maximum binding capacity was 8×10^4/cell and Kd was 40.2 nM. The binding rate of 125-I-Anti-GP to HUVE treated with ITP (high PAIgG) sera (n=6) was 15.2±3.3%, compared with 24.0±7.5%, observed for HUVE treated with normal sera (n=10). Treatment of ITP sera to HUVE significantly lowered the binding of 125-I-Anti-GP to HUVE ($P<0.05$). A combined analysis of SDS-PAGE and Western blotting of washed platelet and endothelial cell lysates shows that two proteins from each cells had similar or identical molecular masses to GP IIb/IIIa.

These findings show that there are GP IIb/IIIa on the HUVE, ITP sera from our patients may have antibodies to HUVE GP IIb/IIIa and that anti-platelet GP IIb/IIIa antibodies in the ITP sera may have become bound not only to some platelets, but also to the HUVE.

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MODULATION OF ANTITHROMBOTIC EFFECTS OF CULTURED HUMAN ENDOTHELIAL CELLS BY INHIBITORS OF CYCLOOXIGENASE OR PHOSPHODIESTERASE. T.H. Müller, K. Rühr, H.H. Callisen, W.G. Eisert. Department of Biological Research, Dr. Karl Thomae GmbH, 7950 Biberach, Federal Republic of Germany.

Intact endothelial cells are known to form a non-thrombogenic surface and to actively restrict the extent of thrombus formation on denuded vessel walls via such mechanisms as the binding of thrombin and activation of protein C, or the synthesis and release of prostacyclin. In an in vitro system, we have investigated how platelet inhibitors modulate the antithrombotic effects of human endothelial cells. Human endothelial cells isolated from umbilical veins were plated on one half of a subendothelial matrix (SEM) harvested from bovine cornea endothelial cells. The endothelial cells were preincubated with a drug and then exposed to anticoagulated whole blood from human donors in the presence or absence of the same drug and agitated for 15 min. The number and size of platelets interacting with the SEM were quantified by morphometric analysis.
In our in vitro system, platelet aggregates on SEM that was partially covered with human endothelial cells were significantly smaller than on uncovered SEM. No difference in platelet adhesion was observed. In the absence of endothelial cells, the cyclooxigenase inhibitors acetylsalicylic acid (ASA) and flurbiprofen strongly reduced the size of aggregates formed on the SEM. Pretreatment of only the endothelial cells with ASA increased the size of the aggregates, while ASA treatment of endothelial cells as well as the whole blood did not reduce the mean aggregate size below that of controls. In contrast, the platelet phosphodiesterase inhibitors AHP 719 and UDCG 212 strongly decreased platelet aggregation without reducing platelet adhesion not only in the absence but also in the presence of endothelial cells pretreated with the inhibitors.
Our results demonstrate that this in vitro model of a partially injured vessel wall is well suited to study the effects of endothelial cells on platelet function. Moreover, inhibitors of phosphodiesterase in contrast to ASA have profound antithrombotic effects in this model.

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ALTERATIONS IN VASCULAR ENDOTHELIAL CELL FUNCTION BY OXYGEN-FREE RADICALS. M. Shatos, J. Doherty, D. Allen and J. Hoak. Department of Medicine and SCOR in Thrombosis, University of Vermont, Burlington, VT, U.S.A.

The vascular endothelium is a target for oxidant-induced damage in many disease states including hyperoxia, inflammation, ischemia and reperfusion injury. However, little is known concerning oxidant injury to endothelial cells and its relationship to hemostasis. Our studies have focused on the ability of oxygen free radicals to injure and/or alter selected vascular endothelial cell functions pertinent to the regulation of hemostasis. Xanthine and xanthine oxidase, a well-characterized generating system for the production of the superoxide anion radical (O_2^-) was used to sublethally injure human umbilical vein endothelial cells (HUVE) in vitro. We examined the effects of a 15 min exposure of HUVE cells to xanthine (50µM), and xanthine oxidase (2.5-100mU) (previously determined to be non-toxic using trypan blue dye exclusion) on platelet adherence, and prostacyclin release using established assays. The antioxidant enzymes superoxide dismutase (SOD) 200µg/ml and catalase 50µg/ml were added to endothelium incubation systems to evaluate any protective effects upon O_2^--induced alterations. All experiments were conducted in a serum-free HEPES-Tyrode's buffer, pH 7.4 incubation system. Our results show that exposure of HUVE cells to sublethal concentrations of oxygen free radical generating systems causes significant enhancement of platelet adherence (65%) to injured endothelium. A 12-fold increase in prostacyclin release resulted after a 15 min treatment with xanthine and xanthine oxidase. The addition of exogenous PGI_2 (150nM) to platelet-endothelial systems did not completely prevent the enhanced platelet adherence suggesting that lack of prostacyclin was not completely responsible for the adherence of platelets to O_2^- injured cells. When SOD and catalase, scavengers of O_2^- and H_2O_2, were added to treated cells, platelet adherence decreased by 42-77% and prostacyclin release approached that of control cultures. These data implicate an active participation of activated metabolites of molecular oxygen in the alteration of vascular endothelial cell function.