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THROMBIN INDUCED PLATELET ADHESION TO ENDOTHELIUM IS MODIFIED BY ENDOTHELIAL DERIVED RELAXING FACTOR (EDRF)

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We investigated the hypothesis that thrombin-induced attachment of platelets to endothelial cells is modulated by EDRF. Thrombin significantly increased binding of radiolabelled platelets to cultured endothelium and to an intact pulmonary vasculature under flow conditions. These increases in binding were potentiated with hemoglobin (HB) and inhibited by superoxide dismutase (SOD) in both systems. We suggest that thrombin, in addition to enhancing platelet adhesion, elicits EDRF release from endothelial cells and that EDRF serves an antithrombotic function in the down regulation of platelet adhesion.

We (1,2) and others (3,4) have shown that thrombin augments platelet adhesion to cultured endothelial cells, to segments of sheep thoracic aorta, and to isolated perfused rat lungs under flow conditions. To more completely understand this interaction, it is necessary to evaluate how endothelial derived substances that modify platelet function affect platelet adhesion to endothelial cells. Especially pertinent are those endothelial derived substances in which synthesis or secretion are modulated by thrombin.

Thrombin is a potent platelet aggregator and has myriad effects on endothelial cells, some of which modulate antiplatelet activity. Thrombin decreases intracellular levels of 13-hydroxyoctadecadienoic acid (13-HODE) (5) and enhances endothelial release of both EDRF (6) and prostacyclin (7).

Recently, several investigators have demonstrated that bradykinin, which elicits EDRF release, and exogenous nitric oxide can inhibit thrombin stimulated platelet adhesion to endothelium (8-10). EDRF bioactivity can be inhibited by HB (11) and potentiated by SOD (12). In this study we have used these

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pharmacological modulators to test the hypothesis that EDRF has a role in thrombin-mediated platelet adherence in vitro and in an isolated perfused lung.

MATERIALS AND METHODS

PLATELET ISOLATION

Platelets were isolated from citrated rat or sheep blood using a modification of the method of Corash et al. (13) as described previously (14). Briefly, platelet-rich plasma (PRP) was prepared and incubated with ^{51}Cr or ^{111}In then layered over a discontinuous gradient consisting of 4 ml of 20% isoosmotic arabinogalactan solution (Champion Int. Corp.; Tacoma WA) and 3ml of 10% arabinogalactan in a 15 ml conical tube. The tubes were centrifuged for 22 min at 1,450 x g. The platelet layer was removed and resuspended in Tyrodes solution and washed twice. This method of platelet isolation effectively removes all plasma proteins while causing minimal damage to platelets.

ENDOTHELIAL CELL MONOLAYERS

Primary cultures of sheep aortic endothelial cells were isolated and grown in our laboratory using established techniques (15,16). The cultures were grown free of contamination of other cells and were identified as endothelial cells by the presence of Factor VIII antigen and morphology (17). Endothelial cells at three to ten population doublings were seeded ($2x10^5$ cells/well) onto 24 well plates (Corning; Chicago, Ill) and grown to confluence in DMEM supplemented with 20% bovine serum, non-essential amino acids and 50 ug/mL gentamicin sulfate in 5% CO_2 at 37°C. Confluent endothelial cell monolayers were used for platelet attachment assays.

IN VITRO ADHERENCE ASSAY

The adherence assay consisted of incubation of 4×10^7 ^{111}In labelled sheep platelets added to washed (3 times with HBSS) sheep pulmonary endothelial cell monolayers and incubated for 60 minutes at 37°C at 5% CO_2 in a humidified incubator. In some experiments, thrombin was added to platelets and endothelial cells at a final concentration of 2 U/mL (or 1.75×10^{-8} M) for the duration of the 60 min incubation. In other experiments, HB (10 uM) or SOD (66U/mL) was added to the wells in addition to thrombin. After incubation, the supernatant was aspirated and the monolayers were washed three times with HBSS to remove non-adherent platelets. 1N NaOH was added to hydrolyze the cells and quantitated for ^{111}In. Platelet adherence was expressed as per cent of platelets added.

ISOLATED PERFUSED RAT LUNG

Male rats (250-300 g) were anesthetized by i.p. injection of sodium pentobarbital (Fort Dodge Laboratories, Fort Dodge, IO) and tracheotomized. The animals were exsanguinated from the abdominal aorta, and the thorax was opened. The lungs and heart were removed and suspended in the perfusion apparatus from the trachea tube. Ventilation with room air by means of a small animal respirator (Harvard Rodent Respirator, Model 683; Millis, MA) was begun and maintained at 70 breaths per min and 1.5 ml per breath for the duration of the experiment. The pulmonary artery and the left atrium were cannulated. Perfusion of Hank's Balanced Salt Solution (Gibco; Grand Island, N.Y) with 0.5% bovine serum albumin (Sigma; St. Louis, MO) (HBSS-A) at pH 7.4 was begun within three minutes of thoracotomy using a peristaltic roller pump (Harvard Apparatus Model 1215; Millis, MA) at a flow rate 14 ml/min. HBSS-A perfusate was maintained in a water bath at 37°C for the duration of the experiment. The lung was regularly bathed with warm saline.

After a ten minute washout perfusion, 1.75 x 10^{-8} M alpha thrombin (a generous gift of Dr. John Fenton, Wadsworth Laboratories, Albany, N.Y.) in HBSS-A was reperfused for five minutes. Fluid phase thrombin was washed from the system for two minutes by circulation of HBSS-A. Next, 7 x 10^8 ^{51}Cr labelled rat platelets were recirculated through the lung for five minutes. In some experiments, 66 U/mL SOD (Sigma Chemical Co.; St Louis, MO) or HB (10 uM), prepared as described (11), were added to the post-thrombin wash and platelet recirculation. Finally, the lungs were perfused for an additional two minutes with HBSS-A to remove any unattached platelets from the lung. Residual binding of platelets to the perfusion apparatus, which consisted entirely of silastic tubing, was minimal.

After dissecting away the heart, the lung was minced and radioactivity was assessed in a gamma counter (Packard Model 500, Packard Inst. Co.; Sterling, VA). Per cent adherence of platelets was calculated as the radioactivity of the sample divided by the total counts in the original 7 x 10^8 platelets added to the lung, times 100. All lung perfusions were confined to less than 25 minutes.

RESULTS

IN VITRO

The adhesion of unstimulated platelets to endothelial monolayers (Figure 1) was 3.44 ± 0.34% (mean ± SEM) (n=16 with each determination the average of 4 wells) of total platelets added. In coincubation with 2 U/mL thrombin, adherence increased significantly to 13.01 ± 1.17% (n=16). The addition of HB (10 uM) to the platelet-endothelial adhesion assay increased platelet binding to 19.00 ± 2.76% (n=10). Conversely,in the presence of 66 U/mL SOD, platelet adhesion to endothelium decreased to 6.77 ±

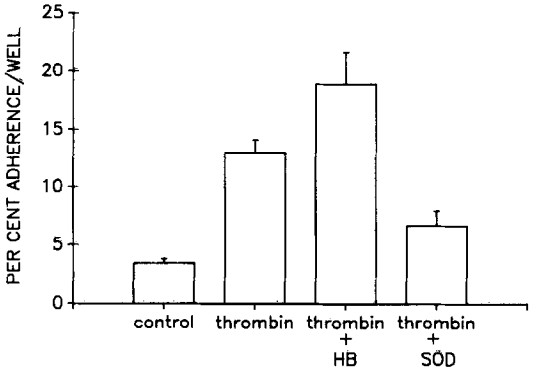

FIGURE 1:

^{111}In labelled sheep platelet adherence (5x10^8 cells/well) to confluent sheep pulmonary artery endothelial cells in 24 well culture plates coincubated with 2 U/mL alpha thrombin for one hour. In addition 10 uM hemoglobin (HB) or 66 U/mL superoxide dismutase (SOD) was added as indicated. Data is expressed as means ± SEM for 16 trials for control and thrombin, 10 for HB and 8 for SOD.

1.28% (n=8). Both these values differ significantly ($p < 0.05$) from that of thrombin alone.

When 10 uM HB or 66 U/mL SOD, but not thrombin, were added with platelets to endothelial monolayers, no significant change in platelet binding from control values was observed (5.06 ± 1.27% and 3.2 ± 2.58%, respectively).

EX VIVO

Isolated perfused rat lungs were perfused for 25 minutes and showed no signs of edema or leakage of buffer during this time. A basal attachment of 5.04 ± 0.71% platelets was seen in 10 control lungs. When thrombin was recirculated and fluid phase thrombin rinsed from the lung, a significant increase in platelet retention to 11.46 ± 0.94% was seen in 13 determinations. (Figure 2).

When 10 uM HB was perfused through the lung during platelet recirculation (n=6) a significant increase in platelet binding was observed (20.88 ± 1.10%). When SOD was added to the perfusion buffer at 66 U/mL, there was a significant decrease to 7.59 ± 1.18% platelets retained by the lung (n=6). When HB or SOD were perfused in the absence of thrombin there was no significant change in platelet adhesion from control levels (7.66 ± 0.73% and 8.22 ± 1.14%, respectively).

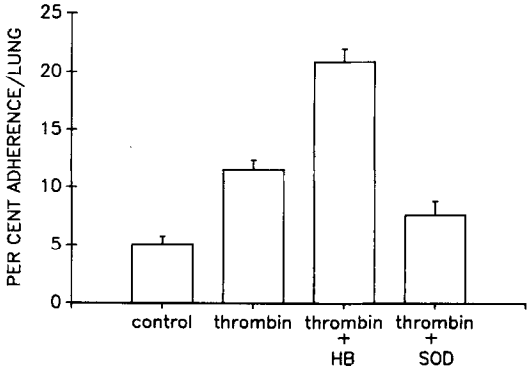

FIGURE 2:

Effect of thrombin pretreatment of isolated perfused rat lungs on adherence of ^{51}Cr labelled rat platelets. Lungs were cleared of blood for 10 minutes and thrombin (2 U/mL or 1.75×10^{8} M) was recirculated for five minutes. After a 2 min wash with buffer alone or with HB (10 uM) or SOD (66U/mL), 7×10^{8} platelets in buffer alone, HB (10uM) or SOD (66 U/mL) were recirculated for five minutes. The lung was rinsed with buffer for two minutes and radioactivity retained was measured and expressed as per cent adherence. Data is expressed as means ± SEM for 10 control, 13 thrombin, 6 HB and 6 SOD determinations.

DISCUSSION

Thrombin significantly increased levels of platelet adhesion to endothelium. This effect was potentiated by HB and inhibited by SOD, agents known to modify the bioactivity of EDRF (11,12). This is entirely consistent with our hypothesis that thrombin stimulated platelet adhesion to endothelium is modulated by EDRF.

The effects of HB and SOD were seen in an homologous sheep platelet-sheep pulmonary artery endothelial cell system. In addition, identical responses were seen in an homologous isolated perfused rat lung system in which thrombin was perfused and fluid phase thrombin washed from the lung before rat platelets were recirculated. This suggests that an intact vasculature under flow conditions employs EDRF as a modulator of platelet adherence.

Radomski et al. (10) and Sneddon and Vane (9) have demonstrated the inhibition of platelet adhesion by both bradykinin, a stimulator of EDRF release, and exogenously added nitric oxide. Our data suggests that thrombin, in addition to enhancing platelet adhesion, can down regulate thrombin-mediated platelet adhesion by increasing the release of EDRF.

The current study suggests that EDRF therefore contributes to the antithrombotic properties of the endothelium after treatment with thrombin. The relation of these findings to the development of thrombosis and vascular diseases such as atherosclerosis remains to be defined.

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