149-152

10 Andrews HE, Bruckdorfer KR, Dunn RC, Jacobs M Low-density lipoproteins inhibit endothelium-dependent relaxation in rabbit aorta Nature 1987; 327: 237 - 9

11 Prasad K, Kalra J Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E Am Heart J 1993; 125: 958 - 73

高胆固醇血症家兔 NO 合成酶抑制物含量与脂质过氧化的关系

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高脂血病 一氧化氮合成酶

关键词 胸主动脉; 血管舒张; 维生素 E; 动脉粥样硬化; 脂质过氧化; 二甲基精氨酸

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目的: 探讨高脂饲养家兔血中 NO 合成酶抑制物二甲基精氨酸(DMA)含量变化与脂质过氧化的关系. **方法**: 检测高脂饲养家兔血清总胆固醇、甘油三脂、丙二醛(MDA)及 DMA 含量, 并观察离体胸主动脉内皮依赖性舒张反应. **结果**: 高脂饲养家兔血脂、血清 MDA 和 DMA 含量比正常组增加(MDA 为 2.88 ± s 0.20 *vs* 1.54 ± 0.13 nmol·L^{-1}, $P < 0.01$, DMA 为 1.51 ± 0.07 *vs* 0.75 ± 0.13 μmol·L^{-1}, $P < 0.01$), 胸主动脉舒张反应降低(最大舒张% 为 45.59 ± 3.1 *vs* 76.93 ± 5.68 %). 维生素 E 抑制 MDA 升高的同时降低 DMA 含量及改善内皮舒张功能. **结论**: 高脂血症家兔血清 DMA 含量的升高可能与脂质过氧化的增加有关.

R589.2

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 Mar; 17 (2): 152 - 155

Heterogeneity of human platelet density subpopulations in aggregation, secretion of ATP, and cytosolic-free calcium concentration

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KEY WORDS blood platelets; platelet aggregation; calcium; adenosine diphosphate; adenosine triphosphate; thrombin; serotonin

AIM: To investigate thrombin (500 U·L^{-1})-, ADP (0.1 - 30 μmol·L^{-1})-, and 5-hydroxytryptamine (5-HT, 3 μmol·L^{-1})-induced aggregation, secretion of ATP and cytosolic-free calcium mobilization in density subpopulations of human washed platelets. **METHODS**: Using Percoll discontinuous gradient. **RESULTS**: The human platelets were separated into high density (HD), intermediate density (ID), and low density (LD) subpopulations, and their sizes were diminished with decreasing density ($r = 0.978$, $P < 0.01$). The magnitude of aggregations by thrombin, ADP, and 5-HT was more significant in HD platelets than that in LD platelets ($P < 0.01$). The amount of secretion of ATP induced by thrombin and ADP in HD platelets was also much higher than that in LD platelets ($P < 0.01$), except for 5-HT which did not cause the ensuring release reaction in any subpopulation of human platelets. Thrombin (1500 U·L^{-1})-, ADP (μmol·L^{-1})-, and 5-HT (3 μmol·L^{-1})-induced cytosolic-free calcium mobilization was evaluated as well. Results showed that the resting level of cytosolic-free calcium concentration ($[Ca^{2+}]_i$) was the same in all subpopulations, about 80 - 90 nmol·L^{-1}. However, the level of $[Ca^{2+}]_i$ mobilization was entirely different, heightened with increasing density. **CONCLUSION**: The function of HD platelets was much stronger and more active than that of LD platelets in human.

Received 1994-03-15 Accepted 1994-11-21

The previous studies suggested that platelet function was different with platelet density in rabbits[1,2] and the density was depends on the composition of subcellular components[3], especially on the

concentrations of platelet specific granules[4], including dense and α-granules. Some clinical evidences showed that the high density platelet were significant increased in myocardial infarction[5] and decreased in thrombocytopenia due to sepsis[6]. To know whether or not the heterogeneity could also be found in density subpopulations of human platelets and the number of high density platelets was closely associated with pathological processes mentioned above, the human platelets were tested in this experiment to evaluate the ability of aggregation and ATP release induced by thrombin, adenosine diphosphate (ADP), and serotonin (5-HT). In addition, the intracellular calcium was thought to be very important in signal transduction system, including the binding of collagen to its receptors, glycoprotein Ib on platelet membrane[7], the formation of fibrinogen and glycoprotein IIb/IIIa/Ca^{2+} complex[8], platelet shape change[9], platelet aggregation[10], and platelet release reaction[11]. So, the intracellular calcium mobilization was also investigated in single washed platelets loaded with Fura-2 from healthy volunteers in this research by using fluorescence and imaging techniques.

MATERIALS AND METHODS

Agents A series of concentrations of Percoll solution (Pharmacia Co, Ltd, Tokyo) was prepared and stored at 4 ℃, and shook thoroughly before use. Lucifferin-luciferase (Chrono-Log Corp, Havertown PA, USA) for measuring the secretion of ATP was dissolved in saline (40 g·L^{-1}) before use and stored at 4 ℃ and avoided the light. ATP and ADP (Sigma Co, Ltd, St Louis MO) were dissolved in distilled water and stored at −20 ℃ and diluted with saline before use. Thrombin (Topical, Bovine Origin, Parke-Davis Co, Morris Plains NJ) was kept at −20 ℃ and dissolved with HEPES-buffer before use. 5-HT (Wako Pure Chemicals, Osaka) dissolved with HEPES-buffer, the stock solution was kept at 4 ℃. Fura 2-AM [acetoxymethyl ester of Fura-2, Wako Pure Chemicals, Osaka (licensed under USA patent *No.* 4,603,209)] was used for platelet loading.

Healthy volunteers Six healthy men (33 − 46 years of age) with free consent were recruited. Criteria for inclusion was nonsmoker, absence of alcoholism, normal hematology, and no history of taking medicines, eg, indometacin and aspirin, 1 month prior to blood sampling.

Total population of platelets Blood (60 mL) taken from antecubital vein was immediately mixed with 10 mL of acid citrate-dextrose solution (ACD, sodium citrate 85, citric acid 71 and glucose 110 mmol·L^{-1}) was centrifuged at 200 × *g* at 25 ℃ for 15 min to obtain the total population of washed platelets.

Density subpopulations of platelets[12] The platelet pellet was resuspended in HEPES buffer to a final concentration of 6 × 10^8 platelets·L^{-1} for aggregation and release reaction experiments or for Fura 2-AM loading.

Platelet aggregation and release reaction[2,12,13]

Fura-2 loading As described in our previous study[12], to achieve a satisfactory intensity of flourescence, 3 subpopulations of platelets were prepared to a final concentration of 6 × 10^8 platelets·L^{-1} and loaded with Fura 2-AM 2 μmol·L^{-1} (final concentration) at 37 ℃ for 15 min.

Cytosolic-free calcium Our modified method[12] was used.

Size determination of platelet The size of washed platelets was assayed by an area directly measured from video images of sample[12]

Statistical analysis All data were calculated by Statview SE + Graphics software and Excel 3.0 running in Apple Macintosh LC Ⅲ personal computer, and expressed as $\bar{x} \pm s$. The statistical significances were evaluated by *t* test.

RESULTS

Density subpopulations The characteristics of platelet subpopulations, including density, size, and proportion of each in the total population, were well correlated with its size ($r = 0.978$, $P < 0.01$) (Tab 1). In the following experiments, HD, ID, and LD platelets were employed to scrutinize the aggregation, release reaction, and cytosolic-free calcium mobilization by thrombin, ADP, and 5-HT.

Tab 1. Normal parameters of density subpopulations of human platelets. $n = 45$ cells in 12 samples from 6 healthy men. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* LD.

	Density	Size	Proportion
HD	over 1.062	836 ± 46[c]	27.3 ± 4.3 %
ID	1.057	513 ± 75[b]	41.4 ± 5.2 %
LD	lower 1.051	374 ± 34	31.2 ± 6.4 %

HD: high density; ID: intermediate density; LD: lowdensity

Aggregation Thrombin-, ADP- and 5-HT-induced aggregation in HD, ID, LD, and total population were investigated in this experiment. 5-HT-induced aggregation was usually weak and reversible, and the aggregations by thrombin, ADP, and 5-HT in LD subpopulation were markedly lower

than those of in HD, ID, and the total population (Tab 2, 3, and Fig 1A).

Tab 2. Thrombin-induced aggregation and ATP release of various subpopulations. $n = 11 - 14$ samples from 6 healthy men. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* LD.

Density	Thrombin/U·L^{-1} 100	300	500
Aggregation/%			
Total	13.6 ± 4.9	56.3 ± 8.9	78.2 ± 6.5
HD	34.7 ± 13.5^c	72.4 ± 11.6^c	81.5 ± 9.5^c
ID	10.4 ± 5.6^a	48.7 ± 7.2^a	71.8 ± 5.3^b
LD	5.12 ± 2.7	39.8 ± 8.6	58.6 ± 6.8
Adenosine triphosphate release/μmol			
Total	0	0.7 ± 0.16	3.7 ± 0.8
HD	0.46 ± 0.12^c	2.6 ± 0.37^c	4.7 ± 1.2^c
ID	0	0 36 ± 0 12^a	2.4 ± 0.2^a
LD	0	0.2 ± 0.11	1.9 ± 0.8

Tab 3. ADP (3 μmol·L^{-1})-induced aggregation and release reaction in various subpopulations of human platelets. $n = 13$, $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* LD.

Density	Aggregation/%	ATP release/μmol
Total	64.5 ± 6.3	0.62 ± 0.1
HD	81.2 ± 5.7^c	0.86 ± 0.12^c
ID	54.6 ± 11.4^b	0.57 ± 0.2^b
LD	32.8 ± 8.5	0.26 ± 0.07

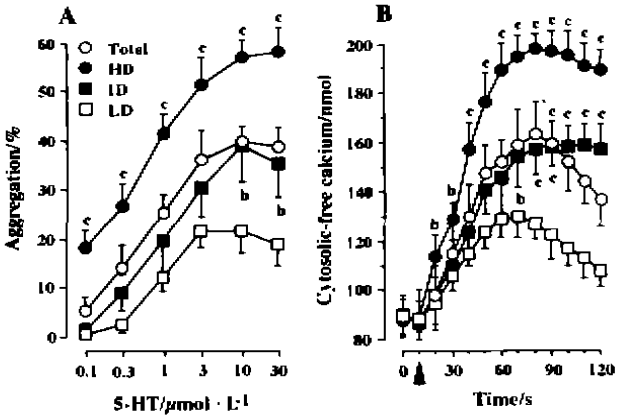

Fig 1. 5-HT-induced aggregation (A, $n = 10$) and cytosolic-free calcium mobilization (B, $n = 49 - 56$) in various density subpopulations of human platelets. arrow in Fig 1B: addition of 5-HT. $\bar{x} \pm s$.
$^bP < 0.05$, $^cP < 0.01$ *vs* LD.

Release reaction Apart from 5-HT, both thrombin and ADP caused release reaction simultaneously measured in the medium during aggregation, and the amount of ATP released in HD subpopulation was much more than that in LD subpopulation ($P < 0.01$) (Tab 2 and Tab 3). Similar result was found in ID subpopulation ($P < 0.05$).

Cytosolic-free calcium The basic levels of cytosolic-free calcium concentrations were almost identical in various density subpopulations and the total population, approximately 80 – 90 nmol·L^{-1}. After the addition of thrombin 1500 U·L^{-1} (Fig 1B), ADP 3 μmol·L^{-1} (Fig 2A) and 5-HT 3 μmol·L^{-1} (Fig 2B) to the samples, the cytosolic-free calcium mobilization was increased in HD and ID subpopulations *vs* LD, and the time of reversion was longer than that of others.

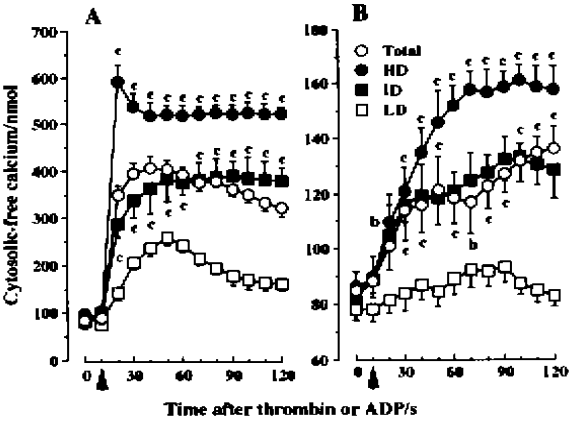

Fig 2. Cytosolic-free calcium mobilization in various density subpopulations by thrombin 1500 U·L^{-1} (A, $n = 60 - 74$) and ADP 3 μmol·L^{-1} (B, $n = 45 - 49$). Arrows: addition of thrombin or ADP. $\bar{x} \pm s$.
$^bP < 0.05$, $^cP < 0.01$ *vs* LD.

DISCUSSION

In the present experiments, human platelets from 6 healthy volunteers were used to investigate the functional differences. The present data indicated that the density, size, and functions of human platelets were significantly different in various density subpopulations, the platelet size was increased and functions activated with increasing platelet density, the high density platelets were much sensitive to the aggregating agents and responses of aggregation and release reaction were significant. These results were similar to those of a previous study in

rabbits[12]. It has been known that cytosolic-free calcium ion as a second messenger plays a major role in transmembrane signal transduction, and results from the present study also demonstrated that $[Ca^{2+}]_i$ mobilization was closely correlated with the size, density and functions of the platelet. The functions in HD were more active and stronger than those in LD may be attributable to the fact that a great number of dense and α-granules was present in HD platelets, and that those granules were the most important determinant in platelet density[3,14]. Therefore, it is very easy to understand why the high density platelets were increased in myocardial infarction or decreased in thrombocytopinia, but the further studies of the mechanisms above the changes of the density and the number of platelets in those cases will be needed.

In conclusion, the present work strongly suggested the existence of a heterogeneous characters in human platelets and that the highest density platelets were always accompanied with the largest size and the most active functions revealed by platelet aggregation, secretion of ATP and cytosolic-free calcium mobilization.

REFERENCES

1 Haver VM, Gear ARL. Functional fractionation of platelets. J Lab Clin Med 1981; **97**: 187 - 204.

2 Li BY, Li WH, Bai Y. Relationship between platelet density and platelet aggregation, ATP release, and cytosolic-free calcium mobilization in rabits. Acta Pharmacol Sin 1995; **16**: 367 - 72.

3 Chamberlain KG, Seth P, Jones MK, Penington DG. Subcellular composition of platelet density subpopulations prepared using continuous Percoll gradients. Br J Haematol 1989; **72**: 199 - 207.

4 Corash L, Costa JL, Shafer B, Donlon JA, Murphy D. Heterogeneity of human whole blood platelet subpopulations III. Density-dependent differences in subcellular constituents. Blood 1984; **64**: 185 - 93.

5 Martin JF, Plumb J, Kilbey RS, Kishk YT. Changes in volume and density of platelets in myocardial infarction. Br Med J 1983; **287**: 456 - 9.

6 Bessman JD, Gardner FH. Platelet size in thrombocytopenia due to sepsis. Surg Gynecol Obstet 1983; **156**: 177 - 80.

7 Jenkins CSP, Phillips DR, Clemetson KJ, Meyer D, Larrieu MJ, Luscher EF. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation. Studies of the proteins from patients with Bernard-Soulier syndrome and von Willebrand's disease. J Clin Invest 1976; **57**: 112 - 24.

8 Plow EF, McEver RP, Collelr BS, Woods VL, Marguerie GA, Ginsberg MH. Related binding mechanisms for fibrinogen, fibronection, von Willebrand factor and thrombospondin on thrombin-stimulated human platelets. Blood 1985; **66**: 724 - 7.

9 Siess W. Molecular mechanisms of platelet activation. Physiol Rev 1989; **69**: 1459 - 63.

10 Rink TJ, Sage SO. Calcium signaling in human platelets. Annu Rev Physiol 1990; **52**: 431 - 49.

11 Rink TJ. Cytosolic calcium in platelets. Experientia 1988; **44**: 97 - 100.

12 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; **194**: 927 - 9.

13 Feinman RD, Lubowsky J, Charo I, Zahinski MP. The lumi-aggregometer: a new instrument for simultaneous measurement of secretion and aggregation. J Lab Clin Med 1977; **90**: 125 - 9.

14 Van Oost BA, Timmermans APM, Sixma JJ. Evidence that platelet density depends on α-granule content in platelets. Blood 1984; **63**: 482 - 5.

人血小板密度亚群在聚集反应，ATP 释放及细胞内游离钙浓度的异质性

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关键词 血小板；血小板聚集；钙；腺苷二磷酸；腺苷三磷酸；凝血酶；血清素

目的：研究人血小板密度亚群在聚集和释放反应及细胞内钙浓度的异质性。**方法**：Percoll 间断梯度离心法。**结果**：正常人血小板分为高(HD)，中(ID)和低(LD)密度三个亚群。血小板的大小随密度的降低而减小($r = 0.978$，$P < 0.01$)。HD 对凝血酶(Thr)，ADP 和血清素(5-HT)诱导的聚集反应明显强于 LD ($P < 0.01$)，ATP 释放反应，除 5-HT 无 ATP 释放外，与聚集反应的结果一致。尽管细胞内游离钙($[Ca^{2+}]_i$)的静息水平各亚群相同，但 Thr，ADP 和 5-HT 的 $[Ca^{2+}]_i$ 动员仍以 HD 最为明显。**结论**：人 HD 血小板的功能及活性均高于 LD 血小板。