高度氧化之低密度脂蛋白抑制人類血小板活性之機轉研究

Mechanisms Involved in the Antiplatelet Activity of Highly Oxidized Low Density Lipoprotein in Human Platelets

中文摘要

氧化低密度脂蛋白對於血小板的細胞內訊息路徑尚未完全知曉，其過去的發現是具爭議性的。因此本實驗的目的在探討氧化低密度脂蛋白抑制血小板凝集的作用。在本實驗結果顯示氧化低密度脂蛋白具濃度〈20~100 μg/ml〉相關性可抑制人類血小板懸浮液被 collagen〈1 μg/ml〉及 arachidonic acid〈60 μM〉引起的凝集作用，然對 thrombin〈0.02 U/ml〉引起的凝集作用卻沒有影響。類似的結果也發生在人類富含血小板之血漿的凝集作用。此外，氧化低密度脂蛋白的抑制血小板凝集作用會隨著氧化時間之延長而加強〈24h-oxLDL>12h-oxLDL〉。另 24h-oxLDL 抑制 collagen 所刺激引起的細胞內鈣離子的移動及 thromboxane B2 的形成也具濃度相關性。另外，由結果顯示 24h-oxLDL〈40 and 80 μg/ml〉對血小板之 cyclic AMP 的形成明顯增加，同時也發現及其誘導 vasodilator-stimulated phosphoprotein〈VASP〉 Ser157 磷酸化反應。但是對 cyclic GMP 及 nitrate 的形成卻沒有影響。在電子順磁共振〈ESR〉的實驗中，24h-oxLDL 明顯降低由 collagen〈2 μg/ml〉刺激血小板或是由 Fenton 反應產生的氫氧自由基〈OH●〉的 ESR 訊號強度。而氧化低密度脂蛋白〈40 and 80 μg/ml〉明顯抑制血小板細胞膜所標示 diphenylhexatriene 的螢光強度。PDBu〈150 nM〉能迅速將蛋白磷酸化? C〈protein kinase C〉活化的指標蛋白 Mr 47,000〈P47〉磷酸化。此一磷酸化反應能被氧化低密度脂蛋白〈40 and 80 μg/ml〉明顯抑制。另外，氧化低密度脂蛋白〈40 and 80 μg/ml〉能明顯抑制由 thrombin 引起的細胞內 pH 值增加。這些結果均顯示氧化低密度脂蛋白抑制血小板活性可能是經由下列途徑：〈1〉氧化低密度脂蛋白其衍生的脂質自由基與血小板活化產生的自由基〈例如氫氧自由基〉發生自由基與自由基終止反應，導致抑制細胞內鈣離子的移動，進一步抑制 thromboxane B2 的形成，最終抑制血小板之的凝集作用。〈2〉氧化低密度脂蛋白可能引起血小板細胞膜的結構改變，導致 protein kinase C 活化的抑制，接著抑制蛋白〈P47〉磷酸化及細胞內鈣離子的流動。〈3〉氧化低密度脂蛋白也會增加血小板之 cyclic AMP 及其所誘導之血管擴張劑刺激之磷酸蛋白〈vasodilator-stimulated phosphoprotein〉〈VASP〉Ser157 磷酸化反應，導致鈉氫交換的抑制；進而減少細胞內鈣的流動，最終抑制血小板之的凝集作用。這些研究對低濃度之高度氧化低密度脂蛋白在血小板的凝集作用上提供新的解說。

在研究 oxLDL 降低由 collagen〈2 μg/ml〉刺激血小板產生的氫氧自由基〈OH

●〉的 ESR 訊號強度的同時，我們發現以 DMPO 當自由基捕捉劑，collagen 除了刺激血小板產生一個四線的典型氫氧自由基之外，還產生一個長半衰期 g = 2.005 的自由基。此一四線的氫氧自由基慢慢消失後接著 g = 2.005 的自由基才生成，並在 5 分鐘時達到最大訊號強度。我們試圖進一步分析這 g = 2.005 的自由基，發現 thrombin，AA 及一些過氧化物也能刺激血小板產生 g = 2.005 的自由基。以 DMPO 及 PBN 當自由基捕捉劑的 ESR 圖譜顯示此自由基是一個不可移動的氮氧化物。將 DMPO 及 PBN 與自由基的生成物以酵素分解蛋白質後衍生出一個以碳爲中心的自由基。Potassium cyanide 及 desferroxamine 能抑制此一自由基形成。過氧化物會促進這 g = 2.005 的自由基形成，並能抑制 AA 引起的血小板凝集作用。前列腺素 H 合成?的 co-substrates 能增加此一自由基的訊號強度，但是卻抑制 AA 引起的血小板凝集作用。另外，S-nitro-L-glutathione 及 reduced glutathione 都能去除此 g = 2.005 的自由基，但卻不能恢復血小板的凝集活性。這些結果認爲此一碳爲中心的自由基是來自人類血小板中的前列腺素 H 合成?因被過氧化物破壞的自我毀滅性的自由基。另一方面，我們發現低度氧化的低密度脂蛋白〈moxLDL〉能引起 g = 2.005 的自由基的形成，而高度氧化的低密度脂蛋白並不具此一作用。我們的實驗結果認爲低度氧化的低密度脂蛋白對 collagen 引起血小板凝集反應的刺激作用可能是〈最少部分是〉經由活化前列腺素 H 合成?中的過氧化?而達成。
結論認爲氧化的低密度脂蛋白對血小板的淨反應決定於它的氧化程度。此一不同的氧化程度可以解釋爲什麼文獻中關於氧化的低密度脂蛋白對血小板凝集反應的結論不一致原因。

英文摘要

The intracellular mechanisms underlying oxidized low-density lipoprotein-signaling pathways in platelets remained obscure and findings had been controversial. Therefore, we examined the influence of oxLDL in human platelets. In this study, we found that highly oxidized LDL (oxLDL) concentration-dependently (20-100 μg/ml) inhibited platelet aggregation in human platelets suspensions stimulated by collagen (1 μg/ml) and arachidonic acid (AA) (60 μM), but not by thrombin (0.02 U/ml). Similar results were observed in human platelet-rich plasma stimulated by agonists. 24h-oxLDL showed more potent activity than that of 12h-oxLDL at inhibiting platelet aggregation. 24h-oxLDL concentration-dependently inhibited intracellular Ca+2 mobilization and thromboxane B2 formation in human platelets stimulated by collagen. In addition, 24h-oxLDL (40 and 80 μg/ml) markedly increased levels of cyclic AMP and cyclic AMP-induced vasodilator-stimulated phosphoprotein (VASP) Ser157 phosphorylation, but not cyclic GMP or nitrate. In the ESR study, 24h-oxLDL markedly reduced the ESR signal intensity of hydroxyl radicals (OH●) either in collagen (2 μg/ml)-activated platelets or in the Fenton

reaction (H2O2 + Fe2+). Moreover, oxLDL (40 and 80 μg/ml) markedly decreased the fluorescence intensity of platelet membranes tagged with diphenylhexatriene. Rapid phosphorylation of a protein of Mr 47,000 (P47), a marker of protein kinase C activation, was triggered by PDBu (150 nM). This phosphorylation was markedly inhibited by oxLDL (40 and 80 μg/ml) in phosphorus-32-labeled platelets. In addition, the thrombin-evoked increase in pHi was inhibited in the presence of oxLDL (40 and 80 μg/ml). These results indicate that the antiplatelet activity of oxLDL may involve the following pathways. (1) oxLDL may induce radical-radical termination reactions by oxLDL-derived lipid radical interactions with free radicals (such as hydroxyl radicals) released from activated platelets, with a resultant lowering of intracellular Ca+2 mobilization, followed by inhibition of thromboxane A2 formation, and finally inhibited platelet aggregation. (2) oxLDL may initially induce conformational changes in platelet membranes, leading to inhibition of the activation of protein kinase C, followed by inhibition of P47 protein phosphorylation, and intracellular Ca2+ mobilization. (3) oxLDL also activated formation of cyclic AMP and cyclic AMP-induced VASP Ser157 phosphorylation, resulting in inhibition of the Na+/H+ exchanger; this leads to reduced intracellular Ca2+ mobilization, and ultimately to inhibition of platelet aggregation. This study further provides new insights concerning the effects of low concentrations of oxLDL on platelet aggregation.

During the ESR study of the effects of oxLDL on the ESR signal intensity of hydroxyl radicals (OH●) in collagen (2 μg/ml)-activated platelets, we found that incubation of human platelets with collagen produced a typical four-line hydroxyl radical signal and a long-lived g = 2.005 radical detectable by spin trapper 5,5-dimethyl-1 pyrroline N-oxide (DMPO). This four-line signal slowly disappeared leading to the sequential generation of the g = 2.005 doublet signal with a maximum at 5 min. We attempted to further analyze this g = 2.005 doublet signal and found that g = 2.005 signal radical also formed from the incubation of thrombin, AA, and a variety of peroxides with human platelets. The ESR spectra obtained using DMPO and α-phenyl N-tert-butylnitron (PBN) were typical of an immobilized nitroxide. Extensive Pronase digestion of both DMPO and PBN adducts were deduced to be a carbon-centered radical. The formation of this radical was inhibited by potassium cyanide, and desferroxamine. Peroxides stimulated the g = 2.005 signal radical formation and inhibited platelet aggregation induced by AA. Prostaglandin H synthase (PGHS) co-substrates increased the intensity of the radical signal but inhibited platelet aggregation induced by AA. On the other hand, both S-nitro-L-glutathione and reduced glutathione quenched the g = 2.005 radical but could not restore platelet aggregatory activity. These results suggest that the

carbon-centered radical is a self-destructive free radical forms during peroxide-mediated deactivation of PGHS in human platelets. On the other hand, we found that mildly oxidized LDL (moxLDL), but not oxLDL, may induce the g = 2.005 radical formation. Our results suggest that the stimulatory effects of moxLDL on collagen-induced platelet aggregation seem to be mediated, at least partially, by activation of PGHS-peroxidase in human platelets.

In conclusion, we suggest that the net effect of oxidized LDL on platelets will depend on its extent of oxidation. Variations in oxidation methods may explain some of the contradictions cited in the literature concerning the effect of oxidized LDL on platelet aggregation