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# Probucol inhibits lipid peroxidation of macrophage and affects its secretory properties[1]

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**KEY WORDS** probucol; macrophages; lipid peroxidation; secretin

## ABSTRACT

**AIM**: To investigate the mechanisms of anti-atherogenic actions of probucol. **METHODS**: Human peripheral blood monocytes were cultured, and treated by copper ion (10 μmol/L) and/or probucol (PBC). Lipid peroxidation was measured by assaying malondialdehyde (MDA). The cytokine interleukin-1 beta (IL-1 beta) and apolipoprotein E (apo E) secreted by monocyte were assayed by enzyme linked immunoassay (ELISA). **RESULTS**: PBC 10 - 80 μmol/L inhibited copper ion-induced cellular lipid peroxidation from 15.30 to 7.74 μmol MDA/g cell protein. PBC 40 μmol/L inhibited oxidized macrophage-mediated oxidation of LDL from 5.18 to 1.65 μmol MDA/g cell protein, and attenuated secretory properties of monocytes induced by copper ion. The release of apo E, which is involved in reverse cholesterol transport, increased by 65 %. And the release of IL-1 beta, which was shown to enhance vascular smooth muscle cell proliferation, decreased by 45 %. **CONCLUSION**: Probucol inhibits lipid peroxidation of macrophages and affects their secretory properties.

## INTRODUCTION

Extensive oxidative modification of low density lipoprotein (LDL) may occur in an antioxidant-depleted subendothelial microenvironment[1]. Cells of the arterial wall, including endothelial cells, smooth muscle cells, and macrophages, can oxidize LDL *in vitro* in the presence of catalytic amounts of transition metal ions. Recently Fuhrman *et al* have shown that macrophages subjected to oxidative stress can oxidize LDL even in the absence of metal ions[2], which may be mediated by lipid peroxidation of macrophages.

Probucol (PBC) possesses properties of a superoxide free radical scavenger *in vitro*[3,4], and can inhibit oxidative modification of LDL[5] and monocyte adhesion to endothelial cells[6]. Another study showed that PBC inhibited macrophage accumulation in atherosclerotic plaque and restenotic vascular endothelium. These studies suggest that PBC is involved in regulation of macrophage functioning, but its mechanism is not known. Secretion of interleukin-1 beta (IL-1 beta) and apolipoprotein E (apo E) by macrophages is very important in formation of atherosclerotic lesion. IL-1 beta[7,8] may serve as an important regulatory factor in the development of atherosclerosis by stimulating the proliferation of VSMC and their transformation to the synthetic state for the formation of the atherosclerotic lesion. Monocyte-macrophage apo E in the vessel wall and HDL-apo E probably induces a high cholesterol efflux from the macrophages[9]. Therefore, we investigated whether PBC can inhibit lipid oxidation of macrophages and influence secretion of IL-1 beta and apo E by macrophages *in vitro*.

## MATERIALS AND METHODS

**Preparation of LDL** Native LDL (D = 1.019 - 1.063 kg/L) was isolated from plasma of normolipidemic fasting volunteers by sequential ultracentrifugation. Then it was dialyzed in buffer A (NaCl 0.9 % + edetic acid 0.5 % w/v). The amounts and concentrations of LDL were expressed in terms of protein[9].

**Isolation and culture of monocytes** According to our previous[6] procedure, human peripheral blood monocytes were obtained from heparinized blood by the following steps: i) conventional Ficoll-Hypaque density

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gradient separation of whole mononuclear cell fraction; ii) incubation of mononuclear cells on treated plastic dishes in medium 199 (M199) plus 20 % fetal calf serum (FCS) (v/v) to isolate monocytes by adherence; iii) after 1 h of culture, during which most lymphocytes spontaneously detached, the monolayer was washed and the monocytes were detached by incubation at 4 ℃ in PBS containing 0.2 % edetic acid (w/v) and 5 % FCS; iv) suspension of monocytes in M199 plus 1 % FCS (cell count: $0.5 \times 10^9 - 1.2 \times 10^9$ cell/L). More than 90 % of the purified cells were viable as identified by 0.4 % trypan blue exclusion test.

**Effect of PBC on cellular lipid peroxidation** In order to eliminate the interference of the medium colour with the production of the pink chromophore during MDA determination in the medium, the cells were incubated in RPMI-1640 medium without phenol red supplemented with benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and 10 % heat inactivated (56 ℃ for 30 min) FCS with or without $CuSO_4$ 10 μmol/L and/or PBC 10, 20, 40, or 80 μmol/L. At the end of the incubation period, the cell monolayer was washed three times with cold PBS. Then the cells were scraped into 1 mL PBS and sonicated for 15 s at 4 ℃ at 80 W. Aliquots were taken from the cell sonicate for lipid peroxidation assays. Formation of lipid peroxides was assayed in the cell sonicate using a commercially available kit (Institute of Nanjing Jiang Cheng Biological Engineering). The toxicity of $CuSO_4$ to monocytes was assayed by 0.4 % trypan blue, and more than 80 % of cells were viable.

**Effect of PBC on oxidized macrophage-mediated oxidation of LDL** Macrophages were incubated at 37 ℃ for 12 h with or without $CuSO_4$ 10 μmol/L and/or PBC 40 μmol/L. Then the medium was removed, the cells were washed once with the medium and incubated with fresh medium containing LDL 100 $\mu g \cdot ml^{-1}$ at 37 ℃ for 18 h and sonicated at the end of the incubation. The extent of LDL oxidation was analyzed directly in the medium containing cells by measuring the amount of thiobarbituric acid reactive substances (TBARS). LDL oxidation was identified by electrophoresis mobility.

**Effect of PBC on macrophage secretion** IL-1 beta and apo E were determined by ELISA, using polystyrene microtiter wells precoated with an IL-1 beta antibody or apo-E monoclonal antibody. Briefly, microplates were coated with apo-E 10 μg or IL-1 beta monoclonal antibodies and blocked by 1 % BSA-PBS. Conditioned medium collected from control and oxidized cells was then added to the plates. After 4 h of incubation at room temperature, the plates were washed three times with PBS, then were added with relative mAbs and goat-antimouse HRP-GAM. OPD-$H_2O_2$ substrate was used.

**Statistical analysis** Results are expressed as $\bar{x} \pm s$, $n = 6$ experiments performed in triplicate. Statistical analysis was performed using $t$-test.

## RESULTS

**Inhibitory effect of PBC on macrophage lipid peroxidation** Cellular MDA was elevated 15-fold in human monocyte-derived macrophages treated with $CuSO_4$ 10 μmol/L at 37 ℃ for 12 h in comparison with control cells (Fig 1). PBC inhibited copper-induced macrophage lipid peroxidation, at 10, 20, 40, and 80 μmol/L, MDA values decreased from 15.30 to 13.20, 8.76, 9.48, and 7.74 μmol/g cell protein.

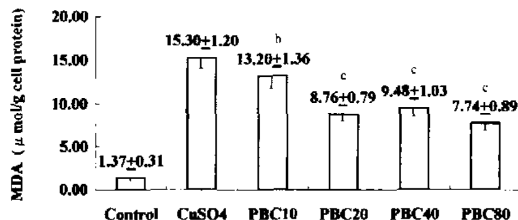


**Fig 1. Inhibitory effects of PBC on macrophage lipid peroxidation. $n = 6$ wells. $\bar{x} \pm s$. Control: control cells; $CuSO_4$: cells treated with $CuSO_4$ 10 μmol/L; PBC10: cells treated with $CuSO_4$ 10 μmol/L and PBC 10 μmol/L, and so on. $^bP < 0.05$, $^cP < 0.01$ vs $CuSO_4$.**

**Inhibitory effect of PBC on macrophage-mediated oxidation of LDL** Upon incubation of macrophages with LDL (100 mg/L) at 37 ℃ for 18 h in the presence of 10 μmol/L $CuSO_4$, a 4-fold increase in the cell-mediated oxidation of LDL was found in comparison with non-oxidized cells (Fig 2). Preincubation of macrophages with PBC 40 μmol/L resulted in 68 %

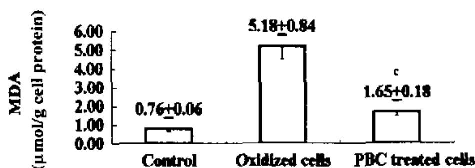


**Fig 2. Inhibitory effect of PBC on macrophage-mediated oxidation of LDL. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ vs oxidized cells.**

inhibition of copper-induced, cell-mediated oxidation of LDL ($P<0.01$).

**Secretory properties of macrophages exposed to PBC** The release of IL-1 beta from macrophages treated with 10 μmol/L $CuSO_4$ at 37 ℃ for 4 h increased by 5-fold in comparison with control non-oxidized cells (Tab 1). The release of apo E decreased by 60 %. PBC 40 μmol/L inhibited 45 % release of IL-1beta from macrophages and increased 65 % apo E release in comparison with oxidized cells.

**Tab 1. Effect of PBC on oxidized macrophage secretory properties. $n=6$. $\bar{x}\pm s$. $^cP<0.01$ vs control.**

| μg·g protein⁻¹ | Control | $CuSO_4$ 10 μmol/L | $CuSO_4$ 10 μmol/L + PBC 40 μmol/L |
|---|---|---|---|
| IL-1 beta | 70±10 | 351±43$^c$ | 171±22$^c$ |
| Apo E | 116±17 | 48±9$^c$ | 103±18$^c$ |

## DISCUSSION

Cells of the arterial wall, including macrophages, are shown to oxidize LDL by different mechanisms[1]. Fuhrman[10] *et al* reported that copper ion induced lipid oxidation of macrophages, and the oxidized macrophages increased oxidation of LDL, leading to the formation of atherogenic oxidized LDL. Probucol[11] has been used as an antiatherogenic drug, due to its antioxidant property. The mechanisms of its antioxidative actions are still unknown. Therefore, we investigated whether PBC can inhibit lipid oxidation of macrophage in the presence of copper ion and demonstrated for the first time that PBC can inhibit lipid oxidation of macrophages induced by copper ion *in vitro*. During atherogenesis, macrophage activation results in the production of oxygen reactive species through activation of NADPH-oxidase, xanthine-oxidase, lipoxygenases and cyclooxygenases. These can further contribute to the oxidation of the cellular lipid constituents. Peroxidation of endogenous lipids such as cell membrane lipids can subsequently cause oxidation of extracellular LDL-lipids[1,9,12], which contributes to hyperlipidemia-induced atherogenesis, perturbation of enzyme activities, and the release of several cellular factors. Our previous data[4] demonstrated that PBC protected endothelial cells against oxygen free radical damage, which may be due to its function as a superoxide free radical scavenger. The production of IL-1 beta in macrophages may contribute to the inflammatory responses in atherosclerotic tissue[8]. Letters[13] *et al* have shown that progression of atherosclerosis in apo E-/- mice is associated with increased aortic lipid peroxidation. All these suggest that IL-1 beta and apo E are involved in lipid oxidation in atherogenesis. We found that PBC attenuated secretory properties of copper ion-stimulated macrophages, which enhanced secretion of apo E in macrophages treated with copper ion, and decreased the cytokine secretion of IL-1 beta.

Taken together, PBC was observed to inhibit lipid peroxidation of macrophages, and attenuate secretory function of oxidant-induced macrophages.

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## 丙丁酚抑制巨噬细胞脂质过氧化并调节其分泌功能[1]

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**关键词** 丙丁酚; 巨噬细胞; 脂质过氧化; 分泌

**目的:** 研究丙丁酚(probucol, PBC)的抗动脉粥样硬化机理. **方法:** 采用 $Cu^{2+}$(10 μmol/L)处理巨噬细胞, 并观察了丙丁酚对巨噬细胞脂质过氧化及其介导的低密度脂蛋白氧化的抑制作用和对巨噬细胞分泌功能的影响. **结果:** 10–80 μmol/L PBC 能抑制 $Cu^{2+}$ 诱导的巨噬细胞脂质过氧化(MDA 从 15.30 抑制到 7.74 μmol/g cell protein, $P<0.01$), 且 PBC 处理的巨噬细胞介导的低密度脂蛋白氧化较对照组低(MDA 从 5.18 到 1.65 μmol/g cell protein, $P<0.05$). PBC 对 $Cu^{2+}$ 诱导的巨噬细胞分泌 IL-1β 抑制 45 %, apo E 分泌增加 65 %. **结论:** 丙丁酚在体外能抑制巨噬细胞脂质过氧化及其介导的低密度脂蛋白氧化, 并调节巨噬细胞的分泌功能.

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