A hypolipidemic agent, probucol lowers plasma and liver levels of lipids in mice fed a high-fat diet

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Abstract

Problems in multiple risk factor syndromes such as syndrome X, deadly quartet visceral fat syndrome and metabolic syndrome have been increasing all over the world. Non-alcoholic fatty liver disease is a new clinicopathological status of emerging importance. Obesity is the most significant single risk factor for the development of fatty liver. Probucol, 4, 4'-isopropylidene-dithiobis-［2, 6-di-t-butyl-phenol］, is a hypolipidemic agent effective in animals and humans. This agent is transported and incorporated into endothelial cell membranes to act as a radical-trapping antioxidant, protecting the endothelial cells against oxidative stress, and lowers an incidence of ischemic heart disease. In the present study, we investigated the effects of probucol in young male mice fed a high-fat diet. The body growth was slightly lowered by additional probucol-treatment in mice fed a high-fat diet. Liver weights in probucol-group were markedly lowered to 89.2％ of that of the high-fat diet group. Histological examination showed that the hepatic lipid deposits appeared as vacuoles of small size within the cytoplasm of liver cells in mice fed the high-fat diet compared with the normal-control. However, additional treatment with probucol markedly reduced the number of the fatty droplets in the cytoplasm of liver cells. Plasma levels of lipids（total cholesterol, free cholesterol, phospholipids and triglyceride）were markedly lowered by probucol -treatment in mice fed a high-fat diet. In conclusion, the activities of anti-obesity, anti-hyperlipidemia and anti-hyperlipids in liver cells of probucol seem effective in reducing body mass, liver lipids and decreasing the plasma lipid level, when used carefully.

Key words —— Metabolic syndrome, fatty liver, probucol, high-fat diet, mice

Introduction

Problems in multiple risk factor syndromes such as syndrome X[1], deadly quartet visceral fat syndrome[2] and metabolic syndrome[3] have been increasing all over the world. A definition for metabolic syndrome incorporates thresholds for 5 easily measured variables. i.e. waist circumference, triglyceride, HDL-cholesterol, fasting plasma glucose concentration and blood pressure, and this classification is triggered when predefined limits of only 3 of the above 5 criteria are exceeded. Non-alcoholic fatty liver disease（NASH）is a new clinicopathological status of emerging importance, now recognized as the most common cause of abnormal liver. It is characterized by a wide spectrum of liver damage, i.e. simple steatosis may progress to advanced fibrosis and to cryptogenic cirrhosis *via* steatohepatitis, and ultimately to hepatocellular carcinoma. Obesity is the most significant

single risk factor for the development of fatty liver, i.e. obesity is also predictive of the presence of fibrosis, potentially progressing to advanced liver diseases. From a histopathological point of view, insulin resistance plays a central role in the accumulation of triglycerides within the hepatocytes and in the initiation of the inflammatory cascade.

Probucol (PC), 4,4'-isopropylidene-dithiobis [2,6-di-*t*-butylphenol], is a hypocholesterolemic agent effective in animals and humans[4]. This agent is transported and incorporated into endothelial cell membranes to act as a radical-trapping antioxidant, protecting the endothelial cells against oxidative stress[5], and lowers an incidence of ischemic heart disease[6].

In the present study, we investigated the effects of probucol on body growth, organ weights, and plasma and liver lipids in young male mice fed a high-fat diet.

Materials and Methods

The effects of hypocholesterolemic agent on plasma levels of lipids and liver in mice fed a high-fat diet were evaluated. A kind of medicine was used; i.e. probucol ($C_{31}H_{48}O_2S_2$, MW 516.84, Lorelco™, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan).

Male ICR mice (Charles River Japan, Yokohama, Japan), 8 weeks of age, were used in the present study. They were housed in plastic cages with wood shavings under controlled conditions (24 ± 0.5 ℃ and 12 h of light from 06.00 to 18.00 h), in accordance with the principles outlined in the Guide for Animal Care and Use of the Committee of Tokyo Medical and Dental University, Japan. All mice had free access to a commercial normal-diet (AIN-76A; Oriental Yeast Co., Ltd., Tokyo, Japan), which is a purified diet based on casein as the sole source of protein[7] and tap water *ad libitum*. The daily food intake was approximately 3.0 ± 0.5 g/mouse. The mice were divided into three groups of 15 at the age of 10 weeks. The animals in the high-fat diet group (HF-group) were each fed 3.0 g of a commercial high-fat diet (F2HFD2; Oriental Yeast Co., Ltd.), which consisted of 58％ lard (wt/wt), 30％ fish powder, 10％ skim milk and a 2％ vitamin and mineral mixture (equivalent to 7.5％ carbohydrate, 24.5％ protein and 60％ fat)[8], daily for

4 weeks. The animals in the probucol-treated group (PC-group) were each fed 3.0 g of the commercial high-fat diet (F2HFD2) with 3.5 mg of probucol; i.e. approximately 10-fold dose of the human dose (500 mg/day). The animals in the control group (NC-group) were each fed 3.0 g of the commercial normal-diet (AIN-76A; Oriental Yeast Co., Ltd., Tokyo, Japan) only according to the same procedure. All experimental procedures conformed to the regulations described in the Guide to the Care and Use of Laboratory Animals of the U. S. National Institute of Health (NIH). Five mice a cage were daily given 15.0 g of the diet (3.0 g of diet/day/mouse), and the body growth was checked weekly throughout the experiment. All mice were killed at 14 weeks of age by cervical dislocation after cardiac puncture under deep urethane anesthesia (1.5 g urethane/kg of body weight, Merck, Darmstadt, Germany) to measure the plasma levels of lipids and biochemical substances, while the sera obtained were later commercially measured (SRL, Inc., Tokyo, Japan). At autopsy, the removed testis, spleen, kidney and liver was weighed and recorded. Each liver obtained was immediately fixed in a 10％ formaldehyde buffer solution (pH 7.2), embedded in paraffin, prepared as 5 μm serial sections and stained with Mayer's hematoxylin and eosin for histological examination.

All parameters were expressed as the mean ± SEM. The significance of differences between groups was evaluated using the unpaired *t*-test and/or Wilcoxson's rank test. A p value less than 0.05 was considered statistically significant.

Results

There were no differences in the initial and final body weights and among groups (data not shown). The body growth in PC-group was lowered compared with that in HF-group, though not significantly (data not shown). Differences in the wet weights of testis, spleen and kidney were not observed among groups with or without the high-fat diet and PC. However, the high-fat diet markedly increased the liver weight to 48.3 ± 0.9 mg/g body weight, compared with that of the control (36.4 ± 0.7 mg/g body weight) ($p > 0.01$). On the other hand, liver weights in PC-group were markedly lowered

to 89.2% (p < 0.05) of that of the high-fat diet group (HF-group) (**Figure 1**).

Histological examination showed that the hepatic lipid deposits appeared as vacuoles of small size within the cytoplasm of liver cells in mice fed the high-fat diet (HF-group) (**Figure 2B**) compared with the normal-control (**Figure 2A**). However, additional treatment with probucol markedly reduced the number of the fatty droplets in the cytoplasm of liver cells in mice of PC-group (**Figure 2C**).

There were few differences in plasma levels of total protein and glucose (data not shown). Statistical differences were not noticed in activities of aspartate aminotransferase, alanine aminotransferase of which

values were different from those in humans, lactate dehydrogenase, alkaline phosphatase, and cholinesterase among groups. Plasma levels of blood urea nitrogen and creatinine did not differ among groups with or without high-fat diet and PC for only 4 weeks.

Although the high-fat diet significantly elevated the plasma levels of lipids, supplemented PC markedly reduced those levels, i.e. the plasma levels of total cholesterol (TC) were markedly increased to 124.6 ± 8.3 mg/dl by 4-week feeding with the high-fat diet, compared with that of the control (93.9 ± 5.1 mg/dl) (p < 0.01). However, an additive treatment using PC markedly decreased the levels to 19.5% of that of the HF-group (p < 0.01) (**Figure 3A**). The high-fat diet
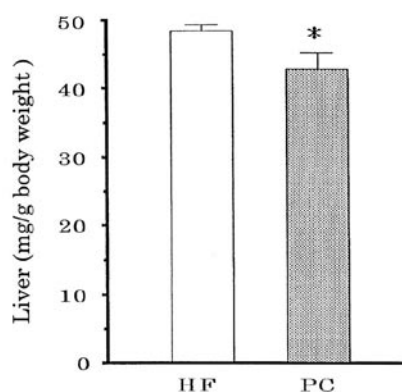

Figure 1 Wet weights of liver in mice fed a high-fat diet with or without probucol, respectively indicated as PC and HF.
Data are means ± SEM.
*Significantly different from that of HF-group; $p < 0.05$.
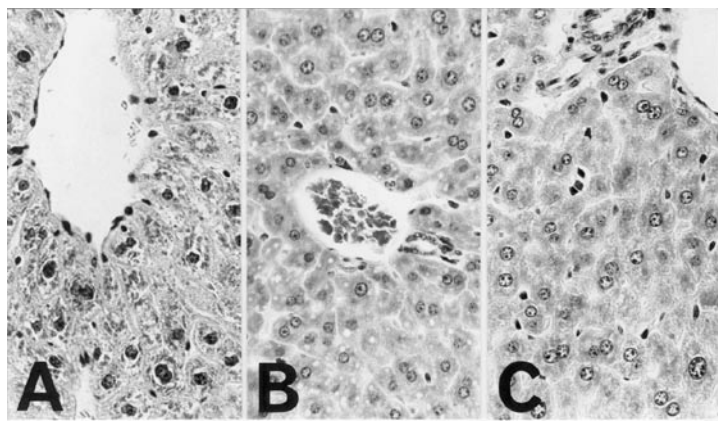

Figure 2 Histological structure of liver. (HE staining, original magnification 400 x).
A: from normal control group, B: from high-fat diet group, C: from probucol-treated group.

increased the plasma levels of free total cholesterol（FC）to 30. 9 ± 1. 8 mg/dl from 20. 2 ± 1. 3 mg/dl（NC-group）（p < 0. 01）, but the PC supplement decreased the levels to 12. 3 % of that of the HF-group （p < 0. 01）（**Figure 3B**）. Circulating phospholipids（PhL）were increased to 212 ± 12 mg/dl with the high-fat diet, compared with that of the control（160 ± 8 mg/dl）（p < 0. 01）. Supplemented PC decreased it to 24. 5 % of that of the HF-group （p < 0. 01）（**Figure 3C**）. The high-fat diet increased plasma levels of triglyceride（TG）to 48. 0 ± 8. 5 mg/dl from 36. 9 ± 3. 2 mg/dl（NC-group）, though not significantly, but PC supplement decreased the levels to 10. 0 % of that of HF-group （p < 0. 01）（**Figure 3D**）.

Discussion

Recently, an interest in multiple risk factor syndrome, in which clustering of diabetes mellitus, hyperlipidemia and hypertension is observed in each subject, is growing greater all over the world. The Adult Treatment Panel（ATP）III of the National Cholesterol Education Program（NCEP）has proposed a definition for metabolic syndrome to aid in the identification of individuals at risk for both coronary hear disease（CHD）and type 2 diabetes[9]. The definition incorporates thresholds for five easily measured variables linked to insulin resistance, as already stated in the section of introduction. The World Health organization（WHO）'s definition of metabolic syndrome is more complex and is focused on glucose dysregulation[10].

Thus, NCEP's definition of metabolic syndrome rather than WHO's definition is expected to help identify individuals who may receive particular benefits from life style measures to prevent CHD and diabetes. According to the modified criteria of the NCEP, the subjects with 4 pr 5 of the features are known to have a 3. 7-fold increase in risk for CHD and a 24. 5-fold increase for diabetes compared with normal controls[11]. The age-adjusted prevalence of obesity, whose body mass index（BMI）was more than 30, was 30. 5 % in 1999-2000 compared with 22. 9 % in 1988-1994 in USA[12]. The prevalence of overweight, whose BMI was more than 25, also increased during the same period from 55. 9 % to 64. 5 %. Extreme obesity, more than 40 in BMI, also increased in the population, from 2. 9 % to 4. 7 %.

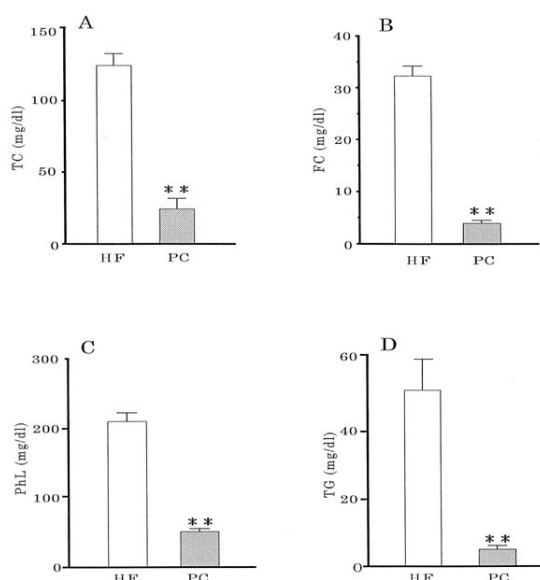Fatty liver disease is a new clinicopathological entity

Figure 3　Plasma levels of lipids in each group （HF & PC）.
A: total cholesterol（TC）, B: free total cholesterol（FC）, C: phospholipids（PhL）, D: triglyceride（TG）.
HF: high-fat diet group, PC: high-fat plus probucol diet group.
Data are means ± SEM.
** and *Significantly different from that of HF-group; p < 0.01 and 0.05.

of emerging importance, now recognized as the most common cause of abnormal liver. It is suggested that chronic hepatocellular injury, necroinflammation, stellate cell activation, progressive fibrosis and, ultimately, cirrhosis are initiated by the peroxidation of hepatic lipids and injury-related release of cytokines. Obesity is the single most significant risk factor for the development of fatty liver, both in children and in adults, as already stated in the section of introduction.

PC is a potent hypocholesterolemic agent and transported and incorporated into endothelial cell membranes to act as a radical-trapping antioxidant, protecting the endothelial cells against oxidative stress[5]. As previously reported, PC markedly lowered the serum levels of lipids resulting in the reduction of oxidative stress on the endothelial cells of blood vessels in the endometrium and myometrium, and then suppressed the incidence of uterine adenomyosis in pituitary-grafted mice compared with the control[13]. In the present study, PC markedly reduced the number of the fatty droplets in the cytoplasm of liver cells in mice with reduction of liver weight and plasma lipids, and affected the body growth though not significantly.

In conclusion, the activities of anti-obesity, anti-hyperlipidemia and anti-hyperlipids in liver cells of probucol seem effective in reducing body mass, liver lipids and decreasing the plasma lipid level, when used carefully.

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抗高脂血症剤プロブコールは高脂肪食摂取マウスの血漿・肝脂質を低下させる

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要旨

　シンドローム X，死の四重奏，内臓脂肪症候群そしてメタボリックシンドロームなどのマルチプルリスクファクター症候群における問題は世界中で増加しつつある。非アルコール性脂肪肝炎(NASH)は新たに重要な臨床的課題となってきた。肥満は脂肪肝発生の最も重大なリスクファクターである。

　プロブコール（4, 4'-iso-propylidene-dithiobis-［2, 6-di-t-butyl-phenol］）は抗高脂血症薬である。この薬剤は，血管内皮細胞膜に取り込まれラジカル除去による抗酸化剤として内皮細胞を酸化的ストレスから防御することにより，虚血性心疾患の発生を低下させることができる。

　今回，高脂肪食を摂取させた若い雄のマウスにおけるプロブコールの効果を検討した。実験には，正常対照群，高脂肪食摂取群，高脂肪食摂取・プロブコール投与群の 3 群を用いた。体重は，高脂肪食マウスにおいてプロブコールの投与によりわずかに低下したが，肝重量は，プロブコール投与群において高脂肪食マウスの 89.2 ％まで著しく低下した。病理学的所見では，正常対照群に比較すると高脂肪食マウスの肝細胞質内には蓄積した脂肪滴が多数みられた。しかしながら，プロブコール投与を行うことによって肝細胞質内の脂肪滴の数が著しく減少した。高脂肪食マウスにおける血漿脂質濃度（総コレステロール，遊離コレステロール, リン脂質，中性脂肪）は，プロブコール投与により著しく低下した。

　今回の実験から，注意深く使用すれば，抗高脂血症薬であるプロブコールは，体重減少，肝脂肪減少および血漿脂質濃度低下に効果的であることが示唆された。

キーワード

メタボリックシンドローム，脂肪肝，プロブコール，高脂肪食，マウス