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Suppression of hypercholesterolemic atherosclerosis by pentoxifylline and its mechanism

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Abstract

Reactive oxygen species (ROS) have been implicated in the development of hypercholesterolemic atherosclerosis. Hypercholesterolemia increases the levels of platelet activating factor (PAF) and cytokines which are known to stimulate granulocytes and endothelial cells to produce ROS. Pentoxifylline (PTX) is an inhibitor of cytokines and PAF and would reduce the generation of ROS by granulocytes and endothelial cells. PTX therefore would be expected to reduce the development of hypercholesterolemic atherosclerosis.

New Zealand white female rabbits were assigned to four groups: Group I ($n = 12$), control; Group II (n = 5), PTX control (40 mg/kg body weight daily orally); Group III ($n = 13$), 0.5% cholesterol; Group IV ($n = 9$), 0.5% cholesterol + PTX (40 mg/kg body weight daily orally). Blood samples were collected before (0 time) and after 1 and 2 months on experimental diets for measurement of serum triglycerides (TG), total cholesterol (TC), LDL-C, HDL-C and serum malondialdehyde (MDA), a lipid peroxidation product. At the end of 2 months the aorta was removed for measurement of atherosclerotic plaques, MDA, and aortic tissue chemiluminescence (Ao-CL), a marker for antioxidant reserve.

Rabbits in Group III developed atherosclerosis ($56.61 \pm 6.90\%$ of the intimal surface of aorta was covered with atherosclerotic plaques) which was associated with an increase in the serum TG, TC, LDL-C, HDL-C, TC/HDL-C, MDA and aortic MDA and antioxidant reserve. PTX reduced the development of atherosclerosis by 38.1% and this was associated with decreases in serum MDA by 32%, aortic MDA by 37%, and antioxidant reserve by 17.3% without changes in the serum lipids.

These results suggest that ROS generated during hypercholesterolemia via cytokines and PAF may in part contribute to the development of hypercholesterolemic atherosclerosis and that suppression of production and activity of cytokines and PAF may reduce the development of hypercholesterolemic atherosclerosis.

Keywords: Atherosclerosis; Hypercholesterolemia; Pentoxifylline; Oxygen radicals; Cytokines; Antioxidant reserve; Aortic chemiluminescence

1. Introduction

Reactive oxygen species (ROS) have been implicated in the development of hypercholesterolemic atherosclerosis [1–5]. Hypercholesterolemia could increase the levels of ROS through stimulation of polymorphonuclear leukocytes (PMNLs) and activation of endothelial cells [1,6]. Plasma levels of platelet activating factor (PAF) are elevated in hypercholesterolemia [7]. PAF is known to stimulate granulocytes to release cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) [8,9]. Tumor necrosis factor-alpha (TNF-α) [10–12], interferon-gamma (IFN-γ) [13,14] and interleukin (IL-1, IL-6) [12,13] are elevated in hypercholesterolemia. In addition TNF-α could induce production of IL-6 [14]. IL-1, IL-8 and IL-6 [15–17], TNF-α [15–17] and IFN-γ [18,19] are known to activate granulocytes to generate ROS. TNF-α [20,21] stimulates NADPH-oxidase in the endothelial cells to generate ROS.

Pentoxifylline (PTX) is a potent inhibitor of IL-1, IL-2, IL-6, TNF-α and IFN-γ [22–24]. PTX down-regulates the release of IL-1β, IL-6, IL-8 and TNF-α [25]. It also down-regulates the production of TNF-α and IFN-γ [26,27]. PTX inhibits the action of PAF on neutrophils, [28] and production of PAF [29].

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It is hypothesized that PTX may suppress the development of hypercholesterolemic atherosclerosis by inhibiting the production and action of cytokines and PAF on leukocytes and endothelial cells, hence reducing the levels of ROS (oxidative stress). The main objectives are to determine: (i) if PTX suppresses the development of hypercholesterolemic atherosclerosis and (ii) if suppression of atherosclerosis is associated with a decrease in the oxidative stress.

An investigation was therefore made of the effects of a high cholesterol diet in rabbits with and without treatment with PTX on the genesis of atherosclerosis, serum lipid profile [triglycerides (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C)] and oxidative stress parameters [aortic and serum malondialdehyde (MDA), a lipid peroxidation product, and aortic chemiluminescence (Ao-CL), a measure of antioxidant reserve]. It is not known if PTX possesses antioxidant activity in addition to its inhibitory effect on cytokines. Antioxidant activity of PTX was therefore also investigated using chemiluminescence (CL) of the oxidant hydrogen peroxide (H_2O_2). For comparison the effect of secoisolariciresinol diglucoside (SDG) a known antioxidant [30] was also used in this study. Weights of the rabbits were recorded at 0, 4 and 8 weeks on their respective diets.

2. Methods

New Zealand white female rabbits weighing between 1.2 and 1.5 kg (6–8 weeks old) were assigned to four groups (Table 1) after 1 week of adaptation. The rabbits in Group I were fed rabbit laboratory chow diet. The other groups received pentoxifylline with (Group IV) or without cholesterol (Group II), or cholesterol (Group III), in addition to rabbit chow diet. The diet was specially prepared by Purina. PTX at a dose of 40 mg/kg body weight daily was given orally in drinking water. Food and water were supplied ad libitum. The rabbits were housed in individual cages under a 12-h light:12-h dark cycle. The experimental protocols were approved by the Ethics Committee of the University of Saskatchewan and the animal care was according to the approved standards for Laboratory Animal Care. The rabbits were on their respective diets for 2 months. Blood samples (from ear marginal artery) were collected before (time 0) and after 1 and 2 months on their respective diets for measurement of TG, TC, LDL-C, HDL-C and MDA. At the end of the protocol (2 months on their respective diets), rabbits were anesthetized with pentobarbital sodium (40 mg/kg, intravenously) and aortas were removed for assessment of atherosclerotic changes, aortic MDA and antioxidant reserve (aortic-CL).

Table 1
Experimental diet groups

Group I ($n = 12$)	Control (rabbit chow diet)
Group II ($n = 5$)	Pentoxifylline control (rabbit chow diet supplemented with pentoxifylline 40 mg/kg body weight daily in drinking water)
Group III ($n = 13$)	Cholesterol diet (0.5% cholesterol in rabbit chow diet)
Group IV ($n = 9$)	Cholesterol diet + pentoxifylline (0.5% cholesterol diet supplemented with pentoxifylline 40 mg/kg body weight daily in drinking water)

2.1. Serum lipids

TG, TC and HDL-C were measured on an automated Beckman Synchron LX20 Clinical System Analyzer. LDL-C was calculated. Risk ratio was estimated using the quotient TC/HDLC.

2.2. Preparation of aortic tissue for measurement of MDA and aortic-CL

Aortas between the origin and bifurcation to iliac arteries were removed, cleaned of gross adventitial tissue and cut longitudinally into two halves. One half was used for assessment of atherosclerotic changes, and the other half was used to prepare supernatant by a previously described method [2] for measurement of MDA and aortic-CL.

2.3. Serum and aortic MDA (thiobarbituric acid-reactive substances)

MDA levels in the supernatant and serum were measured as thiobarbituric acid-reactive substances (TBARs) by a previously described method [31–33]. TBARs were extracted in a mixture of butanol: pyridine (15:1) which was separated by centrifugation. The fluorescence intensity of the butanol-pyridine solution was measured at 553 nm with excitation at 513 nm. The MDA content of the aorta was expressed as nmoles/mg protein, and that of serum as nmoles/mL.

2.4. Aortic tissue chemiluminescence (aortic-CL)

Antioxidant reserve is the amount of antioxidant present in the tissue at the time of the exposure of the tissue to oxidants. Aortic-CL, a measure of antioxidant reserve, was measured as previously described [32]. An increase in the aortic-CL indicates a decrease in the antioxidant reserve, and vice-versa. In short, 0.4 mL of aortic supernatant was added to a tube containing 0.2 mL of 2×10^{-4} M luminol, placed in luminometer AutoLumat LB953 (EG and G Berthold, Berthold GmbH & Co. KG. Bad Wildbad, Germany) at 37 °C and incubated for 5 min. Reaction was initiated by adding 0.1 mL of 200 mM *tert*-butyl hydroperoxide (t-BHP). The chemiluminescence was monitored every 6 sec for a period of 10 min. The unit of CL is the relative light unit (RLU). The aortic CL was expressed as RLU/mg protein.

2.5. Assessment of atherosclerotic changes in aorta

Assessment of atherosclerotic changes in the aorta was made by using Herxheimer's solution containing Sudan IV