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Inhibition of Expression of P-selectin by Antioxidant in Cholesterol-fed Rats

Butylated hydroxytoluene (BHT) can inhibit experimental atherosclerosis in animals. Although the agent is an antioxidant, the exact mechanism of the reaction in atherosclerosis is still unknown. To investigate the effects of BHT on expression of P-selectin (PADGEM, GMP-140), intercellular adhesion molecule-1 (ICAM-1) and class II MHC (Ia) antigen, we proposed an experiment on rats. Male rats (n=18 per group) were fed either a normal cholesterol control diet, a normal cholesterol diet containing 0.5% BHT (BD), a high cholesterol diet containing 1.5% cholesterol and 0.1% sodium cholate (CD), or the CD diet containing 0.5% BHT (BCD). Rats were sacrificed after 3 days, and after 1, 2, 4, 10, and 17 weeks of dietary treatment. Although there was no gross or light microscopic atherosclerotic lesions, scanning electron microscopy revealed monocytic adhesion to aortic endothelium and mild endothelial injuries in CD and BCD groups. Immunohistochemically, the addition of BHT to a high cholesterol diet inhibited P-selectin expression but not in ICAM-1 and Ia antigen. These findings suggest that in rats, high cholesterol diets induce expression of ICAM-1, P-selectin and Ia antigen. In addition, the antiatherogenic effect of BHT may play a role in the inhibition of P-selectin.

Key Words : *Antioxidants; Hypercholesterolemia; Diet; Immunohistochemistry; Electron scanning microscopy; Rats*

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INTRODUCTION

Clinical, epidemiologic and experimental evidence linking hyperlipidemia to atherogenesis is well-established. Many mechanisms have been postulated regarding the role of lipids in lesion formation. Most of these link plasma low density lipoprotein cholesterol (LDL) and atherogenesis. LDL has been known to be damaging or toxic to cells in vitro, and cytotoxicity induced by LDL can result from lipoprotein oxidation (1, 2). Oxidized LDL is chemotactic for leukocytes to inhibit macrophage motility, to be cytotoxic, to stimulate endothelial cells to release chemotactic factors, and to modulate gene expression (3-5).

Treatment of human umbilical vein endothelial cells with minimally modified LDL (MM-LDL) for four and 24 hr has been shown to increase P-selectin (GMP-140) mRNA expression in endothelial cells (6). Other studies have shown that oxidants alone will stimulate expression of P-selectin in endothelial cells (7).

P-selectin (PADGEM, GMP140) is a membrane-associated glycoprotein that can be translocated from its intracellular storage pool (Weibel-Palade bodies) to the surface of endothelial cells where it acts as a ligand for leukocyte adhesion (8-10). P-selectin is not detected in normal arterial endothelium but is expressed along with ICAM-1 in endothelium overlying atherosclerotic plaques, suggesting a role in the recruitment of monocytes in atherogenesis (11).

Intercellular adhesion molecule-1 (ICAM-1) is a molecule of the immunoglobulin superfamily involved in cell recognition and adhesion (12). Expression of ICAM-1 is increased by certain cytokines (13, 14), lysophosphadicylcholine (15), and shear stress (16), and can be decreased by certain anti-inflammatory drugs (8). Luscinskas et al. (14) have observed that ICAM-1 is essential for leukocytic transmigration.

Endothelial cells, smooth muscle cells, and macrophages do not normally express class II MHC (Ia) antigens. However, expression of Ia has been observed in atherosclerotic lesions in rats (18). Ia antigen expression provides a sensitive indicator for cell activation or immunologic reactions in the vessel.

Antioxidants such as butylated hydroxytoluene (19) and probucol (20, 21) have been shown to inhibit atherogenesis in animal models, perhaps through the inhibition of oxidative modification of LDL (5, 20, 21). Although expression of these adhesion molecules is important to early atherogen-

esis, few study has studied the relationship of antioxidant with the molecules. We, therefore, investigated the effect of an antioxidant on aortic expression of P-selectin, ICAM-1, and class II MHC (Ia) antigen in rats fed a hypercholesterolemic diet. Butylated hydroxytoluene (BHT) was used as the antioxidant, because it is used as a food preservative and thus is applicable to human diets.

MATERIALS AND METHODS

Experimental design

Seventy-two male Sprague-Dawley rats (weigh>200 g, purchased from The Korean Chemical Institute, Taejon, Korea) were randomly assigned to one of four dietary treatments (n=18 per group): 1) control group, 2) BHT diet (BD) group, 3) cholesterol diet (CD) group, or 4) CD+ BHT (BCD) group. The control group was fed a commercial rat pellet diet (Dae-Jong, Korea). The cholesterol diet contained 1.5% (w/w) cholesterol (Sigma) and 0.1% (w/w) sodium cholate (Sigma), and the BHT diet contained 0.5% (w/w) butylated hydroxytoluene (Sigma). We used 0.5% BHT in this experiment based upon previous studies (19, 22, 23) while showed no reduction of atherosclerosis by 0.1% BHT but reductions at the 1.0% level. Rats were housed alone or in pairs in stainless steel cages and given food and water ad libitum.

Three days and one, two, four, ten, and 17 weeks following initiation of the experimental diets, under ether anesthesia, three animals from each diet group were euthanized. The heart and aorta were excised after perfusion of heparin-saline and 0.5% paraformaldehyde solution. Blood was collected at necropsy by cardiac puncture from the animals necropsied at each time, and sera were analyzed for cholesterol content using a Hitachi 736-20 automatic chemical analyzer. A proximal portion of the aorta (>0.5 cm in length) was excised and processed for electron microscopy, and the subsequent distal section (>1 cm long) of the aorta was processed for immunohistochemistry. The remaining aorta was used for routine light microscopic study. These tissue specimens were embedded in Paraplast (Oxford, U.S.A.). Four μ m-thick microsections were cut and stained with hematoxylin and eosin.

Scanning electron microscopy

Tissue specimens for scanning electron microscopy were rinsed in Millonig's phosphate buffer (pH 7.2), fixed in 2.5% glutaraldehyde for 2.5 hr at 4℃ and postfixed with 1% osmium tetroxide solution (24). Specimens were dehydrated with alcohol, replaced with isoamyl acetate, and dried with a Ladd 2800 critical point dryer. The sections were examined using a Hitachi S-2500 scanning electron microscope at 25 kV after gold coating by an Eiko IB-3 ion coater.

Immunohistochemistry

ICAM-1 (anti-rat CD54, monoclonal) and P-selectin (anti-rat, polyclonal) antibodies were purchased from Pharmingen (San Diego, CA, U.S.A.), and class II major histocompatibility complex (anti-rat Rt1.B, monoclonal) antibody was purchased from Cedarlane (Hornby, Canada).

The immunohistochemical study results were examined by three pathologists (C-S L, D-H P and D-Y K). Three sections from each animal were used for the study. The immunoreactivity score was arrived when two or more sections showed the same reactivity. All fields of the arterial section were examined. When inter-observer differences occurred, slides were reviewed by the group and a collective decision was made. The final reactivities were expressed as negative (0), weakly positive (1+) and strong positive (2+).

Six μm-thick frozen sections from rat aortas were incubated with 3% hydrogen peroxide for five minutes to block endogenous peroxidase, washed, and then incubated with primary antibodies to ICAM-1 (1:100), P-selectin (1:100), and anti-rat Rt1.B (1:100) overnight at 4℃, respectively. After washing, slides were then incubated with species-appropriate biotinylated secondary antibodies for 30 min (Dako LSAB+kit, Japan), washed, and then incubated with streptavidin-conjugated horseradish peroxidase (HRP) for 20 min. HRP visualization was carried out using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as substrate. Sections were counterstained with Meyer's hematoxylin solution (Sigma).

Positive controls for endothelial adhesion molecule expression were prepared as follow (25). Three age-matched rats on a control diet were injected intravenously with bacterial lipopolysaccharide (LPS) (a phenol extract from *E. coli* type 055:B5, 200 μg/kg body weights in sterile 0.9 g/L NaCl: Sigma) 1½ hr before sacrifice. Slides in which primary antibodies were omitted served as negative controls for each antibody used in this study.

Data analysis

Serum cholesterol data were analyzed using one-way analysis of variance with a significance level of $p<0.05$.

RESULTS

General

The rats remained healthy and grew well throughout the experimental period. At necropsy, no significant atheroscle-

rotic lesions were observed in the heart or aorta. The livers of all the rats in the cholesterol-treated groups revealed variable fatty changes without cirrhosis, whereas in the control and BD group, livers were grossly normal.

Total cholesterol

Significant increases in serum cholesterol level were found in the CD and BCD groups ($p<0.001$) (Fig. 1). The addition of BHT gave mildly increased or similar cholesterol levels in the BD and BCD groups, respectively ($p>0.05$).

Light and scanning electron microscopy

On light microscopic examination, all thoracic and abdominal aortas from the animals appeared normal, with no detectable atherosclerotic lesions. On scanning electron microscopic examinations, luminal surfaces in the control animals revealed a regular, parallel arrangement of endothelial cells. No monocytic or platelet adhesions were observed (Fig. 2A). Animals fed the control diet plus BHT showed no endothelial damage or cellular adhesion at any point in the study (Fig. 2B). In contrast, monocytic adhesion to the endothelium was observed from one week of the experiment in animals fed with the hypercholesterolemic diet.

The extent of monocytic adhesion was small, but increased with time. From the 10 week to the end of the study, mild endothelial injury, and adhesion of monocytes and platelets were observed (Fig. 2C). BHT addition to the cholesterol treatment did not change these pathologic findings significantly (Fig. 2D).

Immunohistochemistry

The aortas of LPS-injected rats (positive controls) revealed diffuse staining for all three antigens (ICAM-1, P-selectin and Ia) along the endothelial layer (figures not shown). Slides from negative controls showed no staining of the arterial wall. Table 1 shows data regarding the expression of adhesion molecules and Ia antigen.

DISCUSSION

In animals with diet-induced hyperlipidemia, the early pathologic changes in arteries include adherence of mononuclear leukocytes to the endothelium and accumulation of macrophages in the intima (26, 27). Although rats are generally known to be resistant to experimental atherosclerosis (28, 29), these studies suggest that rats may provide a useful model for studying of oxidation and endothelial leukocyte interaction. In addition, the presence of a pro-adhesive

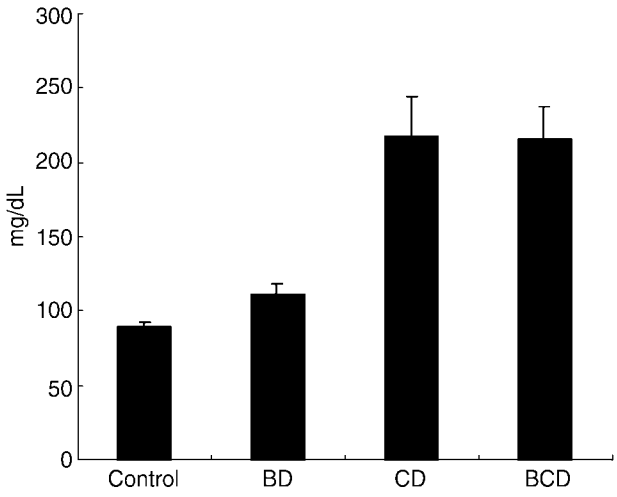

Fig. 1. Mean serum cholesterol levels of rats (±SEM). Serum cholesterol increased significantly in CD and BCD groups ($p<0.001$). The addition of BHT did not cause any significant difference in the levels ($p>0.05$).

response of the rat endothelium to a cholesterol diet suggests that endothelium-leukocyte interaction alone is not sufficient to promote development of an atherosclerotic lesion. The main purpose of the present experiment was not to observe the atherosclerotic lesion but to see the effect of BHT on expression of intercellular adhesion molecules.

Adhesion of monocytes on the endothelial surface is a early event in human and experimental atherosclerosis (26). Cybulsky et al. (30) first identified an inducible endothelial adhesion molecule that was selective for mononuclear cells. The localized nature of this leukocytic-endothelial interaction may be a consequence of local changes in the endothelial surface expression of adhesion molecules. Several families of adhesion receptors, e.g., the integrins, the adhesion molecules of the immunoglobulin superfamilies, the cadherins, the Lec-CAMs (leukocyte-cellular adhesion molecules) and homing receptors mediate cell-substrate and cell-cell adhesion (31).

Sugama et al. (32) divided endothelial adhesiveness into early- and late-phase responses and found that the initial response was dependent on P-selectin expression, while the late response was ICAM-1-dependent. P-selectin is a membrane-associated glycoprotein, and is structurally related to ELAM-1 (33) and the MEL-14 lymphocyte homing receptor involved in leukocyte adhesion to vascular endothelium (34). P-selectin can also be induced by MM-LDL (6) or other oxygen radicals (7) in endothelial cell culture. ICAM-1 is a cell surface glycoprotein expressed on vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, fibroblasts, tissue macrophages and mitogen-stimulated T lymphocytes (35) . ICAM-1 expression is greatest on endothelium (36). Under normal (nonstress) conditions, ICAM-1 is expressed in fetal but not adult mesenchymal

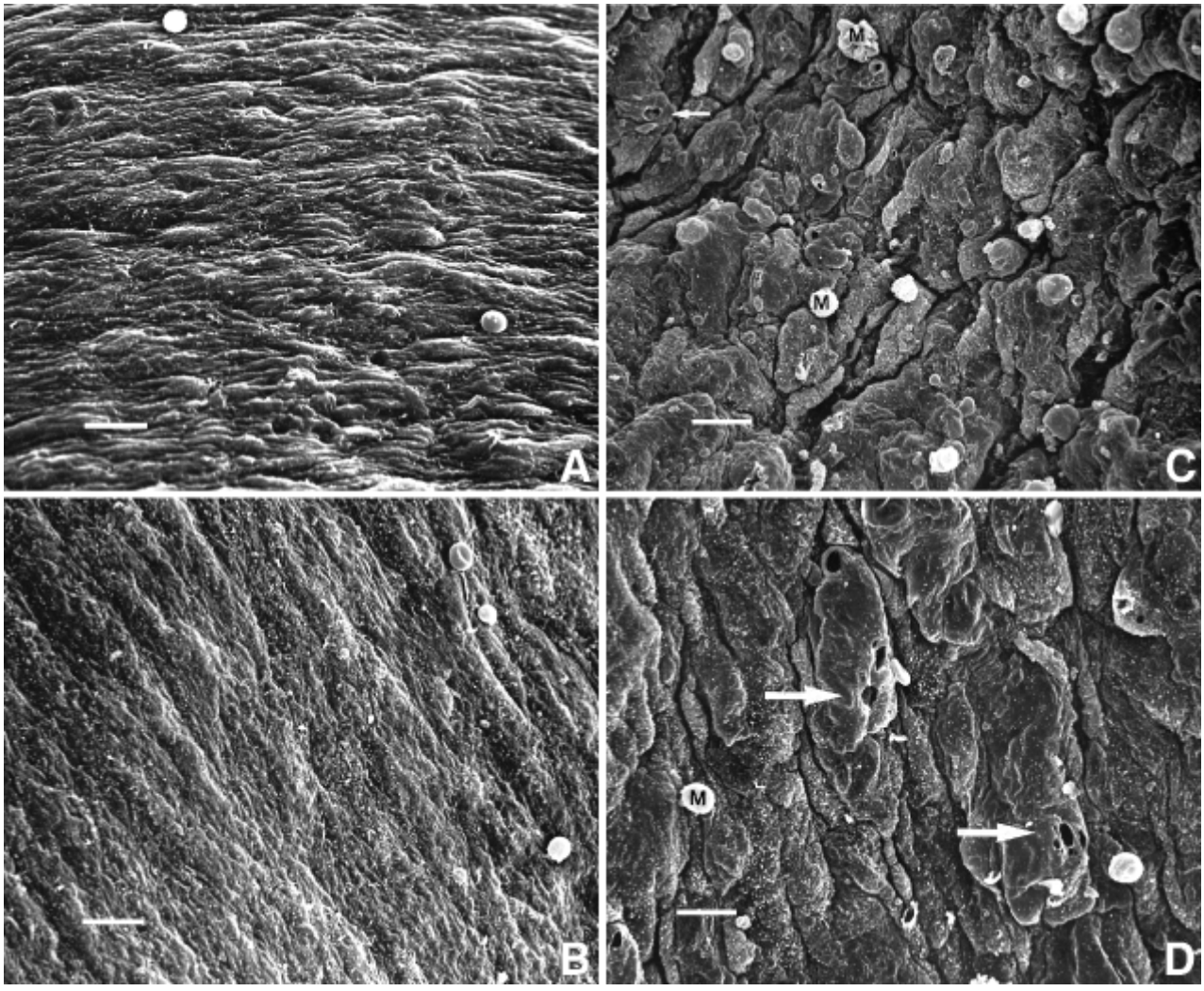

Fig. 2. Scanning electron micrographs of aortas of male Sprague-Dawley rats. A: control group. Well-arranged endothelial cells are present. 10 weeks. B: BD group. No specific abnormality could be noted. 10 weeks. C: CD group. Mild to moderate swelling of the endothelial cells with adhesion of monocytes (M). These are accompanied by infrequent endothelial injuries (arrow). 17 weeks. D: BCD group. The cytoplasmic membranes of the endothelium are dilated and perforated (arrows). There is some monocytic (M) adhesions. 17 weeks. A scale bar=10 μm.

*Control=commercial rat pellet diet; BD=control+0.5% butylated hydroxytoluene (BHT); CD=control+1.5% cholesterol+0.1% sodium cholate; BCD=control+CD+0.5% BHT.

cells, but can be reexpressed in adult cells during pathologic processes such as atherosclerosis (37). Increased endothelial ICAM-1 expression has been demonstrated in human atherosclerotic lesions (38), further supporting the role of ICAM-1 in atherogenesis. ICAM-1 plays a role in both intercellular adhesion and transendothelial migration (18, 39) along with other chemical factors. ICAM-1 may act synergistically with P-selectin for monocytic recruitment, since codistribution of ICAM-1 and P-selectin has been detected in areas of active monocytic infiltration, but not in inactive fibrous plaques (11). Cooperation between adhesion molecules may contribute to the initiation and development of macrophage-rich atherosclerotic lesions. Vascular smooth muscle cells do not normally express Ia antigens but in experimental (28, 51) and human (12) atherosclerosis.

In our immunohistochemical study, we observed expression of ICAM-1 and P-selectin after three days on the CD diet. P-selectin expression, but not ICAM-1 and Ia antigen, was decreased in the BHT-treated animals. We think the decreased expression of P-selectin is at least partly due to BHT treatment, because the difference of serum cholesterol between CD and BCD groups was not significant. Ia antigen has been expressed lately in both the CD (at 17 weeks)

Table 1. *Immunoreactivity of the rat aortic endothelium for ICAM-1, P-selectin and Ia antigen**

		Experimental groups											
Molecule	*Timepoint*	*D*			*BD*			*CD*			*BCD*		
P-selectin	*3 d*	*0*	*0*	*0*	*0*	*0*	*0*	*2+*	*2+*	*2+*	*2+*	*1+*	*1+*
	1 w	*0*	*0*	*0*	*0*	*0*	*0*	*2+*	*2+*	*1+*	*0*	*0*	*0*
	2 w	*0*	*0*	*0*	*1+*	*1+*	*0*	*2+*	*2+*	*2+*	*1+*	*0*	*0*
	4 w	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	10 w	*1+*	*0*	*0*	*0*	*0*	*0*	*2+*	*2+*	*2+*	*0*	*0*	*0*
	17 w	*0*	*0*	*0*	*1+*	*1+*	*0*	*2+*	*2+*	*2+*	*2+*	*2+*	*2+*
ICAM-1	*3 d*	*0*	*0*	*0*	*0*	*0*	*0*	*2+*	*1+*	*0*	*2+*	*1+*	*1+*
	1 w	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2+*	*2+*	*2+*
	2 w	*0*	*0*	*0*	*0*	*0*	*0*	*1+*	*1+*	*1+*	*2+*	*2+*	*2+*
	4 w	*1+*	*0*	*0*	*1+*	*0*	*0*	*2+*	*2+*	*1+*	*2+*	*2+*	*2+*
	10 w	*0*	*0*	*0*	*1+*	*0*	*0*	*2+*	*2+*	*2+*	*2+*	*2+*	*2+*
	17 w	*1+*	*0*	*0*	*1+*	*0*	*0*	*2+*	*2+*	*2+*	*2+*	*2+*	*2+*
Class II MHC antigen	*3 d*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	1 w	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	2 w	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	4 w	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
	10 w	*0*	*0*	*0*	*0*	*0*	*0*	*1+*	*0*	*0*	*0*	*0*	*0*
	17 w	*0*	*0*	*0*	*1+*	*0*	*0*	*1+*	*0*	*0*	*1+*	*0*	*0*

**Details of numerical scoring of immunoreactivity described in Methods section. Each number represents result from an individual animal. D=commercial rat pellet diet ; BD=D+butylated hydroxytoluene (BHT); CD=D+1.5% Cholesterol+0.1% sodium cholate ; BCD=D+CD+0.5% BHT.*

and in the BCD group (at 10 and 17 weeks). It suggests that an immunologic reaction took place late in the experiment. However, BHT did not seem to effect Ia antigen expression.

The mechanism underlying the differences in BHT effects on expression among ICAM-1, P-selectin and Ia antigen is uncertain, but one possibility is that it is related to differences in vascular sensitivity to oxidized lipids or oxidation overall. This experiment suggests that lipid oxidation may affect the expression of P-selectin more than ICAM-1 or Ia antigen.

A previous study (19) has shown that BHT increased the plasma cholesterol concentration in rabbits. Our result showed similar but much smaller changes in the no-cholesterol treatment groups. However, the effect was not found in cholesterol-treated groups. It is not clear why the effects were different in these groups. BHT is structurally related to probucol but it does not have the cholesterol-lowering effects of it (40). Maybe it is due to species difference, but another important point is that the biological properties of BHT has not completely been determined yet. The activity of BHT on cholesterol level was mild in this experiment.

The scanning electron microscopic examination revealed scattered areas of monocytic adhesion on the endothelium with mild endothelial injury in CD and BCD groups, perhaps due to oxidized LDL (41). These findings are compatible with a very early change in atherosclerosis.

Although we expected that BHT inhibits pathologic change, it did not show any difference. One possible explanation is that the change was too early to see the effect of BHT. If the aorta had more advanced lesions, it might have been possible. The other assumption is that the concentration of BHT is below the threshold level to inhibit the lesion, since 1.0% is the lowest concentration ever reported to inhibit atherosclerosis (22, 23). While there were definite ultrastructural findings, it did not show any gross or light microscopic lesions. This probably relates to the relative resistance of the rat model to atherosclerotic lesion development (42). However, Humphreys (43) reported that spontaneous or diet-induced atherosclerotic lesions do occur in rats based upon a large number of observations. It may be possible that a longer experimental period would have resulted in lesions visible at the light microscopic level.

In conclusion, the present study shows that BHT selectively inhibited the expression of P-selectin but not ICAM-1 or Ia antigen in hypercholesterolemic rat aortas. BHT did not show any inhibitory effect in an early atherosclerotic lesion on the aortic intima. Our results suggest that the antiatherogenic effect of BHT may partly be due to inhibition of P-selectin.

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