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Short Communication

Selective Blockade of the Endothelin Subtype A Receptor Decreases Early Atherosclerosis in Hamsters Fed Cholesterol

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Recent studies suggest that endothelin and its receptors may be involved in atherogenesis. To test this hypothesis, cholesterol-fed hamsters were treated with a selective endothelin subtype A (ET_A) receptor antagonist BMS-182874. Characterization of hamster atherosclerotic plaques indicated that they contained a fibrous cap of smooth muscle cells, large macrophage-foam cells, and epitopes of oxidized low density lipoprotein. Messenger RNA for both ET_A and ET_B receptors was detected in aortic endothelial cells, in medial smooth muscle cells, and in macrophage-foam cells and smooth muscle cells of the fibro-fatty plaques. BMS-182874 inhibited the endothelin-1-induced pressor response whereas the depressor effect was unaltered, suggesting that vascular ET_A receptors were selectively blocked in vivo. In hyperlipidemic hamsters, BMS-182874 decreased the area of the fatty streak by reducing the number and size of macrophage-foam cells. The results indicated that ET_A receptors and thus endothelin promoted the early inflammatory phase of atherosclerosis. (Am J Pathol 1995, 146:819–826)

Endothelin is a 21-amino-acid peptide with potent vasoactive properties.[1] Three isopeptides of endothelin (ET-1, -2, and -3) mediate their action by binding with two subtypes of endothelin receptors denoted as ET_A and ET_B.[2,3] The amino acid sequence of the two receptors have a 60% homology[2] yet they are functionally distinct. Activation of ET_A receptors on the surface of medial smooth muscle cells produces contraction, whereas ET_B receptors on the arterial endothelium induce relaxation of medial smooth muscle cells through the release of prostacyclin and nitric oxide.[4–6]

Besides controlling vascular tone, endothelin appears to have other biological functions. For example, there is evidence suggesting a role for endothelin and its receptors in atherogenesis. Patients with atherosclerosis have elevated plasma endothelin concentrations,[7] and messenger RNA for preproendothelin and for ET_A and ET_B receptors was detected in human atherosclerotic plaques.[8] *In vitro*, oxidized low density lipoprotein (LDL) stimulated endothelial cells[9] and macrophages[10] to release endothelin. This peptide was a chemoattractant for monocytes[11] and mitogenic for vascular smooth muscle cells.[12]

We used the hamster model[13] to investigate the role of endothelin receptors and endothelin in atherogenesis by treating hamsters with an orally active and selective ET_A receptor antagonist BMS-182874.[14,15] First the hamster atherosclerotic plaque in the aortic arch was partially characterized and then the location of messenger RNA for vascular endothelin receptors in the same arterial segment was determined. Next the effect of BMS-182874 on the ET-1-induced pressor response was tested to ascertain the extent of vascular ET_A receptor blockade *in vivo*. Finally the

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effect of BMS-182874 on early atherosclerosis in the lesion-prone aortic arch was studied in hyperlipidemic hamsters.

Materials and Methods

Fifty-seven male F_1B hamsters (Biobreeders, Fitchburg, MA) were used in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals prepared by ILAR (Publication No. NIH 85-23, 1985).

Characterizing Hamster Atherosclerosis

Three hamsters were fed a diet of chow (Purina 5001) supplemented with 0.05% cholesterol and 10% coconut oil for 3 weeks, which resulted in mild hyperlipidemia and in the accumulation of macrophage-foam cells in the aortic arch.[13] Another four hamsters were fed chow supplemented with 0.3% cholesterol and 10% coconut oil for 12 to 15 months. This diet produced severe hyperlipidemia that resulted in the formation of atherosclerotic plaques in the aortic arch. Aortic arches from both groups were used to identify the presence of arterial oxidized LDL, smooth muscle cells, and macrophage-foam cells as well as the location of messenger RNA for the vascular ET_A and ET_B receptors.

To identify smooth muscle cells, hamster aortic arches were perfused with 10% neutral buffered formalin at 100 mm Hg, imbedded in paraffin, and sectioned. Endogenous peroxidase activity in the paraffin sections was quenched by 3% H_2O_2. After blocking with 10% horse serum, sections were incubated with an α-actin antibody (BioGenix, San Ramon, CA). A secondary biotinylated antibody was applied followed by streptavidin conjugated to peroxidase and the chromogen was diaminobenzidene (Vector Laboratories, Burlingame, CA). Nonimmune immunoglobulin G was substituted for the primary antibody and served as a negative control. To identify epitopes of oxidized LDL, an antibody to malondialdehyde-conjugated LDL[16] was used (MDA2, kindly donated by Dr. Michael Rosenfeld). The chromogen was alkaline phosphatase (Vector), which avoided the need to block endogenous peroxidase activity and prevented further oxidation of arterial proteins. To detect nonspecific esterase activity of arterial macrophages, frozen sections of hamster plaque were fixed in 10% neutral buffered formalin and immersed in α-naphthylbutyrate.

Detecting Messenger RNA for Vascular-Endothelin Receptors

Human ET_A and ET_B cDNA clones[17,18] were random primer labeled with digoxigenin-coupled dUTP according to standard procedures.[19] *In situ* hybridization was performed on frozen sections (8 μ) of hamster aortic arch that were mounted on glass slides and fixed with ethanol-acetic acid. The slides were treated with HCl and proteinase to permeabilize the cells and then with Denhardt's solution and formamide-dextran. A heat-denatured cDNA probe (salmon sperm) was applied, the slides were washed, and the cDNA probes for the endothelin receptors were hybridized for 1 hour. After a second wash, an antidigoxigenin antibody (coupled to alkaline phosphatase) was added. After a wash, the nitroblue tetrazolium substrate was pipetted onto the slides and the blue/purple reaction product indicated the presence of the messenger RNA. No counterstain was used. For negative controls, the endothelin receptor cDNAs were excluded. The specificity of the probes was demonstrated in Chinese hamster ovary cells that were stably transfected with the human ET_A or ET_B cDNA. *In situ* hybridization with the ET_A probe labeled only the chinese hamster ovary/ET_A cells. Conversely, the ET_B probe stained only the Chinese hamster ovary/ET_B cells (data not shown).

Evaluating ET_A Receptor Blockade with BMS-182874

This study elucidated the effect of the orally active and selective ET_A receptor antagonist BMS-182874 (5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-napthalenesulfonamide[14,15] on the ET-1-induced depressor/pressor response in hamsters fed chow. This determined whether BMS-182874 selectively blocked ET_A receptors *in vivo*. The carotid artery and jugular vein of anesthetized hamsters were catheterized, and mean arterial pressure and heart rate were monitored 3 hours after surgery in conscious unrestrained animals as described previously.[20] Hamsters (n = 4) received four bolus intravenous (i.v.) doses of human ET-1 (1 nmol/kg body weight; Peptides International, Louisville, KY) at 45-minute intervals to determine whether multiple injections of ET-1 induced tachyphylaxis of the depressor/pressor response. $NaHCO_3$ vehicle was given 15 minutes before the third ET-1 dose. In a separate study, hamsters received three bolus i.v. injections of ET-1 (45 minutes apart), and BMS-182874 was gavaged orally 15 minutes before the third ET-1 dose. Blood pressure and

heart rate were monitored on a polygraph (Grass Instruments, Quincy, MA). Four hamsters were orally dosed with 75 μmol/kg BMS-182874 and another four received 150 μmol/kg.

The Effect of BMS-182874 on Plasma Lipids, Blood Pressure, and Atherosclerosis

In the atherosclerosis study, three groups of hamsters were fed chow plus 0.05% cholesterol and 10% coconut oil; control hamsters received vehicle (5% $NaHCO_3$), and two other groups were dosed orally with either 150 or 75 μmol/kg/day BMS-182874 for 21 days. The atherogenic diet and drug treatment were started simultaneously. After 18 days, the hamsters were fasted overnight and then anesthetized with 50% CO_2/50% O_2 and bled once to measure plasma lipids. Plasma cholesterol and triglyceride were measured enzymatically with commercial kits on a Cobas Mira automated analyzer. High density lipoprotein cholesterol was assayed after phosphotungstate precipitation of lipoproteins containing apolipoprotein B.

At 21 days, hamsters were lightly anesthetized with methoxyflurane and a catheter was inserted into the carotid artery. Three hours after surgery and receiving the last dose of drug, blood pressure and heart rate were monitored in conscious, unrestrained hamsters. The hamsters were re-anesthetized with methoxyflurane and perfused at 100 mm Hg with 10% neutral buffered formalin, and the aortic arch was dissected and stained with oil red O. Atherosclerosis consistently occurs along the inner curvature of the aortic arch. The arches (from the aortic valves to the start of the descending thoracic aorta) were mounted *en face* and macrophage-foam cell number, foam cell size, and fatty streak area were quantified by computer-assisted image analysis (Automatix, Billerica, MA) as described previously.[21]

For the depressor/pressor studies, a paired *t*-test was used to compare the depressor/pressor effect of BMS-182874 when given between the second and third ET-1 challenge. In the atherosclerosis experiment, an analysis of variance followed by a Tukey's test was used to determine differences between blood pressure, plasma lipids, and atherosclerosis parameters of control and treated hamsters. Data were transformed when necessary to comply with Bartlett's homogeneity of group variances. Groups of data that failed the Bartlett's test were analyzed with a nonparametric Mann-Whitney U test.

Results

F_1B hamsters fed 0.3% cholesterol and 10% coconut oil were severely hyperlipidemic (total cholesterols ranged from 700 to 2600 mg/dl), and macrophage-foam cell accumulation along the inner curvature of the aortic arch was accelerated, leading to the formation of fibro-fatty plaques in the same region by 12 months (Figure 1a). The plaques contained a dense connective tissue matrix and a necrotic core of cholesterol crystals. The fibrous cap was composed of smooth muscle cells that were labeled with an antibody to α-actin (Figure 1b). Within the plaque were large round foam cells that had nonspecific esterase activity, indicating that they were probably macrophages (Figure 3a, b). Immunostaining with the MDA2 antibody revealed malondialdehyde-lysine residues that were primarily localized within smooth muscle cells of the fibrous cap and in the large macrophage-foam cells (Figure 1c).

In the aortic arch of hamsters fed 0.05% cholesterol and 10% coconut oil for 3 weeks, the human cDNA

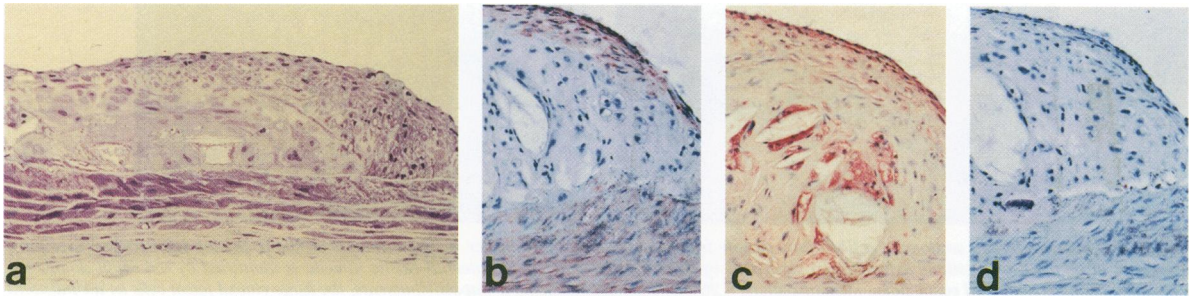

Figure 1. **a:** *Low magnification of a fibro-fatty plaque in the aortic arch from a hamster that was hypercholesterolemic for 12 months. Methacrylate section stained with hematoxylin; magnification, ×115.* **b:** *Semiserial paraffin sections of a hamster atherosclerotic plaque. An antibody against α-actin labeled smooth muscle cells of the fibrous cap and media with a brown peroxidase reaction product. Hematoxylin counter stain; magnification, ×90.* **c:** *An antibody (MDA2) against malondialdehyde detects epitopes of oxidized LDL in smooth muscle cells of the fibrous cap and in large macrophage-foam cells of the plaque. Hematoxylin counter stain; magnification, ×90.* **d:** *There was no staining when the primary antibody was substituted with nonimmune immunoglobulin G. Hematoxylin stain; magnification, ×90.*

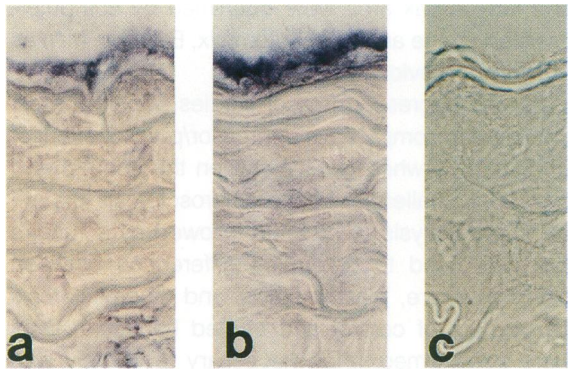

Figure 2. *Frozen sections of the aortic arch from a hamster fed 0.05% cholesterol and 10% coconut oil for 3 weeks (total cholesterol, 200 mg/dl).* **a:** *After* in situ *hybridization with the cDNA probe for the ET_A receptor, blue/purple nitroblue tetrazolium reaction product was localized in the endothelial cells, and less was present in the medial smooth muscle cells. No counterstain; magnification, ×390.* **b:** *Significant levels of messenger RNA for the ET_B receptor were in the endothelium, and less was localized in medial smooth muscle cells. Magnification, ×390.* **c:** *No staining occurred without the endothelin receptor cDNA probe. Magnification, ×390.*

probes for ET_A and ET_B receptors hybridized to endothelial cells and to a lesser extent on medial smooth muscle cells, whereas there was no staining when the endothelin receptor probes were omitted (Figure 2a, b, c). *In situ* hybridization with the same endothelin receptor cDNAs was performed on fibro-fatty atherosclerotic plaques. As mentioned previously, the lesions contained large round foam cells that had nonspecific esterase activity, indicating that they were probably macrophages (Figure 3a, b). Infiltrating between the macrophage-foam cells were smooth muscle cells identified with an anti-α-actin antibody (data not shown). The ET_A receptor cDNA hybridized to the sections, suggesting that macrophage-foam cells and smooth muscle cells in the lesion, as well as medial smooth muscle cells, contained messenger RNA for the ET_A receptor (Figure 3c). There was also messenger RNA for the ET_B receptor in macrophage-foam cells and smooth muscle cells of the plaque, whereas less hybridization occurred in smooth muscle cells of the media (Figure 3d).

In the endothelin depressor/pressor study, the first bolus i.v. dose of ET-1 (1 nmol/kg) immediately decreased blood pressure for 2 minutes (probably mediated by the release of nitric oxide via ET-1 stimulation of endothelial ET_B receptors), then it produced a submaximal increase of blood pressure (likely mediated in part by ET_A receptors on smooth muscle

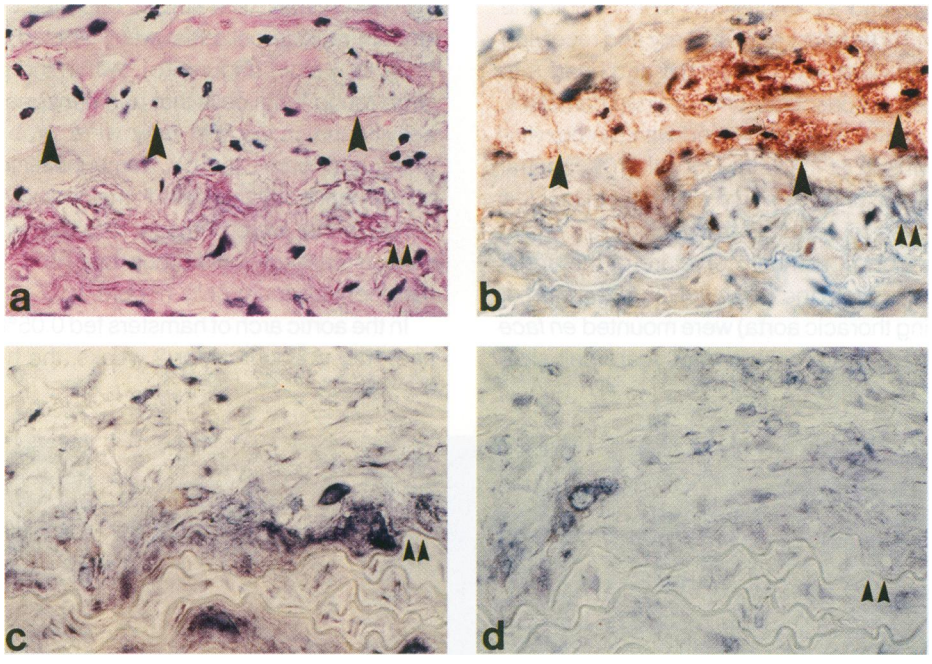

Figure 3. *Semiserial frozen sections of a fibro-fatty plaque from a cholesterol-fed hamster.* **a:** *The lumen is visible in the top left corner. There were clusters of large foam cells (single arrowheads) separated by thin elongated cells. The internal elastic lamella is identified by the small double arrowheads. Hematoxylin and eosin stain; magnification, ×260.* **b:** *With the substrate α-naphthylbutyrate, the large round foam cells in an adjacent section had nonspecific esterase activity (ie, brown reaction product), indicating that they were macrophage-foam cells (single arrowheads). Methyl green counterstain; magnification, ×260. Between the macrophage-foam cells were thin smooth muscle cells that were labeled with the anti-α-actin antibody (data not shown).* **c:** In situ *hybridization of an adjacent section showing significant messenger RNA for ET_A receptors in the macrophage-foam cells and smooth muscle cells of the fibro-fatty lesion. The foam cells were lightly labeled as most of the cell body contained droplets of lipid and the labeled cytoplasm formed thin strands around the clear droplets. Smooth muscle cells in the media were also labeled. Magnification, ×260.* **d:** *Messenger RNA for ET_B receptors was also present in macrophage-foam cells and in smooth muscle cells of the fibro-fatty plaque with lesser amounts in the media. Magnification, ×260. There was no staining of the plaque when the endothelin receptor cDNAs were excluded (data not shown but similar to Figure 2c).*

cells).[4-6] Blood pressure returned to normal levels by 30 minutes. Subsequent ET-1 challenges (45 minutes apart) caused maximal depressor and pressor responses without evidence of tachyphylaxis, and there was no effect of the $NaHCO_3$ vehicle (Figure 4a). In separate animals, BMS-182874 was gavaged orally between the second and third i.v. ET-1 challenge. BMS-182874 (75 and 150 μmol/kg) failed to alter the ET-1 depressor response, but it reduced the ET-1 pressor effect by 41 and 64%, respectively. This suggested that BMS-182874 antagonized ET_A receptors (Figure 4b, c).

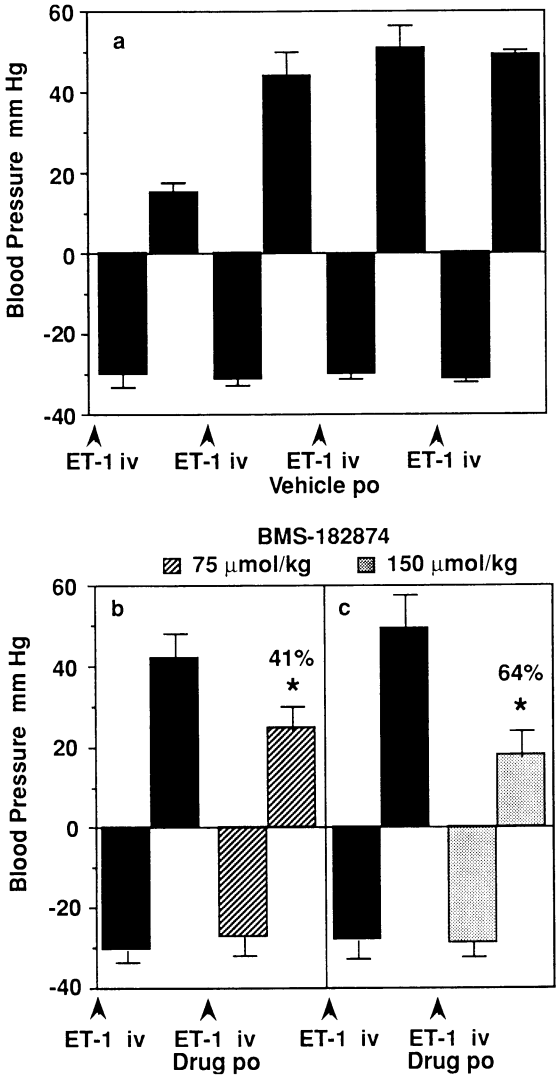

Figure 4. a: *The effect of ET-1 (1 nmol/kg) on blood pressure (mean ± SEM) in male F_1B hamsters fed chow. Sequential i.v. doses of ET-1 (45 minutes apart) resulted in maximal depressor and pressor responses after the second ET-1 challenge. There was no tachyphylaxis and the vehicle (po) had no effect.* b: *The effect of BMS-182874 on the ET-1-induced depressor and pressor response when the compound was given (po) between the second and third ET-1 challenge. BMS-182874 (75 μmol/kg) inhibited the pressor response by 41% ($P < 0.05$), whereas the depressor response was unaffected.* c: *At 150 μmol/kg, BMS-182874 decreased the pressor effect by 64% ($P < 0.05$).*

In the atherosclerosis study, 150 μmol/kg BMS-182874 decreased plasma total cholesterol, LDL plus very low density lipoprotein cholesterol and total triglycerides by 20, 29, and 40%, respectively, compared with controls (Table 1). With the 75 μmol/kg dose of BMS-182874, plasma lipids were unchanged. Neither dose of BMS-182874 altered mean arterial pressure as compared with controls, although heart rate was slightly elevated (Table 1). Compared with controls, 150 μmol/kg BMS-182874 decreased the number of macrophage-foam cells/mm^2, average foam cell size (μ^2) and fatty streak area (μ^2) by 81, 36, and 84%, respectively (Table 1). BMS-182874 at 75 μmol/kg reduced these parameters by 44, 29, and 52% (Table 1 and Figure 5).

Discussion

Golden Syrian hamsters (F_1B hybrid strain) fed a mild atherogenic diet of chow supplemented with 0.05% cholesterol and 10% coconut oil became hyperlipidemic, and most of the plasma cholesterol was in the LDL fraction.[13] Along the inner curvature of the aortic arch, macrophage-foam cells rapidly settled in the intima eventually forming a fatty streak.[13] Increasing the dietary cholesterol concentration to 0.3% produced severe hyperlipidemia and accelerated macrophage-foam cell accumulation that resulted in the formation of fibro-fatty plaques along the inner curvature of the aortic arch by 12 months. Therefore, in the aortic arch of hamsters, the fatty streak progressed into an atherosclerotic plaque. The hamster plaque shared some features found in advanced human atherosclerosis, such as a fibrous cap of smooth muscle cells, large macrophage-foam cells, epitopes of oxidized LDL, a dense connective tissue matrix, and a necrotic core of cholesterol crystals.

In situ hybridization demonstrated messenger RNA for ET_A and ET_B receptors in endothelial cells and in medial smooth muscle cells of the aortic arch. Messenger RNA for both receptor subtypes was present in macrophage-foam cells and in smooth muscle cells of atherosclerotic plaques. Messenger RNA for both endothelin receptors was also detected in human lesions.[8] Although elevated messenger RNA levels do not always lead to increased protein synthesis, the results were consistent with the concept that ET_A and ET_B receptors were present in the aortic arch and in advanced atherosclerotic lesions of hyperlipidemic hamsters. Interestingly, peritoneal macrophages appear to have ET_B receptors,[22] indicating a diversity of endothelin receptor expression in macrophages that may depend on their functional state and/or tissue location.

BMS-182874 inhibited the ET-1-induced pressor response, confirming that it was orally active and

Table 1. *Effect of BMS-182874 on Plasma Lipids, Body Weight, Blood Pressure, Heart Rate and Atherosclerosis of Hamsters Fed Cholesterol*

	Total cholesterol (mg/dl)	LDL + VLDL cholesterol (mg/dl)	HDL cholesterol (mg/dl)	Total triglyceride (mg/dl)	Body weight (g)	Mean blood pressure (mm Hg)	Heart rate (beats/min)	Fatty streak area ($\mu^2 \times 10^3$)	Macrophage-foam cells/mm^2	Foam cell size (μ^2)	n
Control	188 ± 7	131 ± 7	58 ± 2	254 ± 20	130 ± 3	105 ± 3	317 ± 6	63.7 ± 9.7	54 ± 15	42 ± 3	13
BMS-182874, 150 μmol/kg	151 ± 4*	93 ± 4*	58 ± 1	152 ± 17*	129 ± 2	104 ± 3	354 ± 8*	10.4 ± 2.9†	10 ± 3*	27 ± 2*	12
BMS-182874, 75 μmol/kg	184 ± 5	125 ± 5	59 ± 2	181 ± 26	129 ± 3	103 ± 4	361 ± 9*	30.7 ± 5.3*	30 ± 9*	30 ± 2*	13

Data are presented as mean ± SEM. VLDL, very low density lipoprotein.
* $P < 0.008$ compared with the control group (Tukey's test).
† $P < 0.001$ compared with the control group (Mann-Whitney U test).

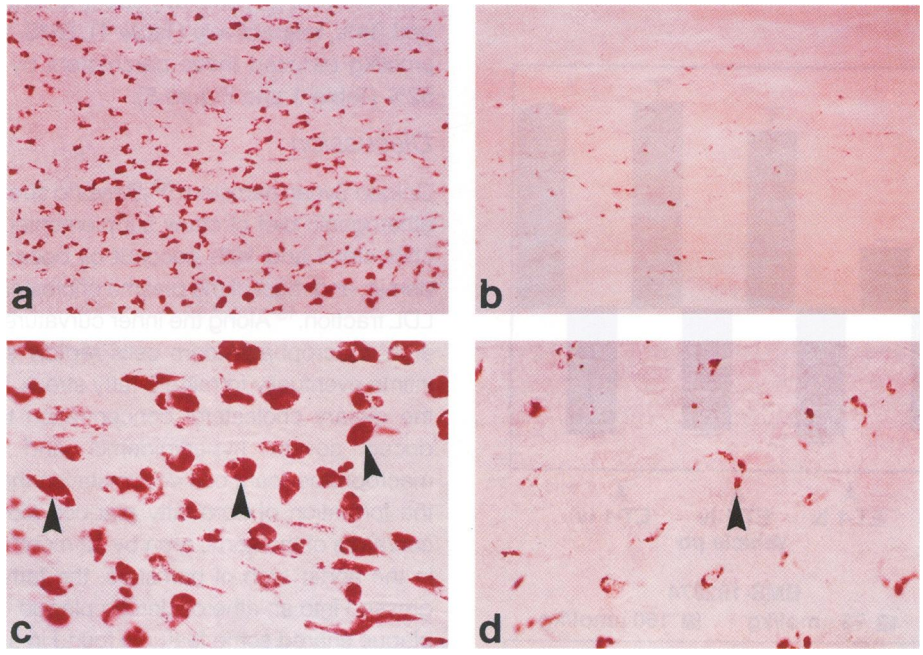

Figure 5. (a) *Photomicrograph of the aortic arch viewed* en face *from a hyperlipidemic control hamster (3 weeks). Blood flow was from left to right. There were many lipid inclusions located along the inner curvature. Oil red O stain; magnification, ×90.* b: *After 3 weeks of treatment with BMS-182874 (75 μmol/kg/day), the amount of neutral lipid was less than in the control specimen. Oil red O; magnification, ×90.* c: *At higher magnification, the intimal lipid particles of the control specimen corresponded to many subendothelial macrophage-foam cells engorged with neutral lipid (arrowheads); magnification, ×260.* d: *Increasing the magnification demonstrates that the number and size of the macrophage-foam cells (arrowhead) was reduced by BMS-182874 (75 μmol/kg/day) as compared with the control specimen in Figure 5c. Magnification, ×260.*

blocked vascular ET_A receptors in hamsters. A similar result was obtained in rats with BMS-182874 (J. E. Bird, unpublished data) and with the peptidic ET_A receptor antagonist BQ 123.[23] In the atherosclerosis study, blockade of vascular ET_A receptors with BMS-182874 failed to reduce blood pressure of normotensive hamsters, although heart rate was slightly increased (other studies indicated that BMS-182874 did not alter heart rate). Surprisingly the 150 μmol/kg dose of BMS-182874 decreased plasma cholesterols and triglycerides. It was not due to a reduction in food consumption as there were parallel increases in average body weights of the three groups; however, the mechanism behind the hypolipidemic effect was unknown. Another study has confirmed that 150 μmol/kg BMS-182874 lowered total cholesterol and triglycerides, whereas the 75 μmol/kg dose did not significantly alter plasma lipids (data not shown).

In hamsters treated with BMS-182874, there was a decrease in the number and size of the macrophage-foam cells that reduced the area of the fatty streak. The 150 μmol/kg dose of BMS-182874 decreased LDL cholesterol concentrations, which probably contributed to the reduction of the fatty streak area, as cholestyramine (which decreased plasma LDL cholesterol) also inhibited fatty streak formation in this model.[13] Nevertheless, it was unlikely that the 29% decrease in LDL plus very low density lipoprotein cholesterol with BMS-182874 accounted for the entire 81% reduction in foam cell number, as a low cholesterol diet or cholestyramine reduced this lipid fraction by 75 and 56%, respectively, which resulted in a pro-

portional decline of foam cells/mm^2 of 94 and 63%, respectively.[13] In addition, 75 μmol/kg BMS-182874 failed to alter blood pressure and plasma lipids, yet macrophage-foam cell number and size was significantly reduced, suggesting that blockade of the ET_A receptor with BMS-182874 directly impeded the progression of atherosclerosis.

ET_A receptors on endothelial cells, macrophage-foam cells, and smooth muscle cells offer the potential for a selective ET_A receptor antagonist to modulate cell function. The evidence so far suggested that, during atherogenesis, oxidized LDL stimulated endothelin production by endothelial cells[9] and/or by macrophages,[10] which attracted monocytes to the artery wall.[11] BMS-182874 may have impeded endothelin production or monocyte chemotaxis to arterial endothelin, which would account for the decreased number of arterial foam cells. It was also feasible that arterial endothelin stimulated the production of monocyte chemotactic protein,[24] which in turn recruited monocytes to the artery wall. Perhaps BMS-182874 interfered with this process. By slowing the recruitment of leukocytes to the aorta, the residence time for arterial macrophages to scavenge lipoproteins was probably decreased, thus accounting for the small size of macrophage-foam cells. Additionally, BMS-182874 may have impeded the formation of foam cells by inhibiting the uptake of oxidized LDL, decreasing cholesterol ester synthesis, or increasing cholesterol ester hydrolysis.

In conclusion, arterial endothelial cells, smooth muscle cells, and macrophage-foam cells of cholesterol-fed hamsters had significant levels of messenger RNA for ET_A and ET_B receptors. Selective inhibition of vascular ET_A receptors with 75 μmol/kg BMS-182874 reduced the accumulation and the formation of macrophage-foam cells independently of other cardiovascular risk factors. The higher dose of BMS-182874 probably further suppressed atherogenesis by decreasing plasma LDL cholesterol. The results suggested that ET_A receptors and its ligand endothelin participated in the progression of early atherosclerosis.

Acknowledgments

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