High blood pressure is a major risk factor in the development of CHD (Kannel et al., 1978; Newman et al., 1986). Epidemiological studies have indicated that essential hypertension tends to be associated with other risk factors of CHD, such as hyperlipidemia, diabetes and obesity (Ferrannini et al., 1987). One goal of antihypertensive therapy should be to reduce CHD by lowering blood pressure; therefore, an antihypertensive drug that can reduce the above risk factors is preferred for the management of hypertension.

Recent clinical studies, however, have revealed that some antihypertensive drugs have unfavorable effects on plasma lipid profiles (Giles, 1992; Kasiske et al., 1995). For example, some beta adrenergic receptor blockers increase plasma TG levels and decrease HDL levels. Thiazide-type diuretics in general increase TG and LDL levels. Although most of the clinical and experimental data indicate that calcium antagonists generally do not influence plasma lipid profiles (Giles, 1992; Kasiske et al., 1995), several exceptions indicated that they can decrease plasma TG and increase HDL levels (Caule et al., 1991; Kazumi et al., 1990; Kihara, 1991; Morris et al., 1993). Because high TG and low HDL levels are often associated with hypertension and thought to accelerate atherosclerosis, it is important to evaluate the effect of calcium antagonists on these lipid profiles.

**ABSTRACT**

We evaluated the effect of AE0047, a dihydropyridine-type calcium antagonist, on the plasma lipid levels of obese Zucker rats. In rats treated orally with 3 to 10 mg/kg/day AE0047 for 7 days, plasma triglyceride (TG) and TG-rich lipoprotein levels dose-dependently decreased, whereas those of high-density lipoprotein cholesterol increased. Total cholesterol and low-density lipoprotein levels did not change. To elucidate the mechanism by which AE0047 decreases plasma TG levels, we examined the effect of AE0047 on the synthesis and secretion of TG-rich lipoproteins in human intestinal cell line Caco-2, as well as on the association and degradation of very low density lipoprotein (VLDL) in human hepatoblastoma cells HepG2.

When Caco-2 cells were grown on a membrane filter and $^{14}$C-oleic acid was added to the apical side, $10^{-6}$ and $10^{-6}$ M AE0047 inhibited basolateral secretion of $^{14}$C-TG. AE0047 also suppressed the basolateral secretion of apolipoprotein B. In HepG2 cells, AE0047 increased the cellular uptake of $^{125}$I-VLDL. These results suggested that AE0047 decreased plasma TG level by the inhibition of intestinal chylomicron secretion and the enhancement of hepatic uptake of VLDL. AE0047 may be beneficial for the treatment of hypertensive patients with hypertriglyceridemia to reduce the risk factors of coronary heart disease.
TG metabolism: (1) intestinal absorption of exogenous lipid and secretion of chylomicrons, (2) synthesis and secretion of VLDL by the liver, (3) catabolism of lipoproteins by lipases and (4) resorption of the lipoproteins into the liver. Because TG-rich lipoproteins (chylomicrons and VLDL) are exclusively generated by the liver and intestine, we used human hepatic (HepG2) and intestinal (Caco-2) cell lines in which the synthesis, secretion and clearance of lipoproteins have been widely studied (Javit, 1990; Levy et al., 1995). We identified two possible mechanisms from in vitro studies using these cell lines.

**Methods**

**Materials.** AE0047 was synthesized in our laboratory. Nilvadipine was obtained from Fujisawa Pharmaceutical Co. (Tokyo, Japan) and purified in our laboratory. LPL (derived from Pseudomonas sp.) and lipid assay kits for total cholesterol, TG and HDL cholesterol were purchased from Wako Chemicals (Osaka, Japan). BSA (essentially fatty acid free), nonessential amino acid solution and LPDS were obtained from Sigma Chemical Co. (St. Louis, MA). The protein assay kit was obtained from BioRad Laboratories (Hercules, CA), 14C-oleic acid (50–62 mCi/mmol) was from Amersham International (Bucks, UK), sodium [125I]iodide was from DuPont/NEN Research Products (Dreieich, Germany) and FCS was from ICN Biochemicals Japan (Osaka, Japan).

Cell culture insert (0.4-μm pores, 4.9 cm²) and six-well tissue culture plates were purchased from Becton Dickinson (Lincoln Park, NJ). Silica gel G TLC plates were obtained from E. Merck (Darmstat, Germany), and collagen-coated tissue culture dishes were from Iwaki Glass (Chiba, Japan).

**Animals.** Female obese Zucker rats (10 or 11 weeks old) were obtained from Charles River Inc. (Wilmington, MA) and housed for 1 week before study. Animals were maintained on normal laboratory chow (CE-2) and water ad libitum.

**Fig. 1.** Chemical formula of AE0047.

**Fig. 2.** Effect of AE0047 and nilvadipine on the plasma lipid profiles of Zucker rats. Rats were administered the indicated amounts of drugs for 7 days. Animals were deprived of food overnight before the blood sampling; 2 hr after the final administration, blood was drawn. There were 11 study animals receiving AE0047 (3 mg/kg), 10 receiving AE0047 (10 mg/kg) and vehicle and 8 receiving nilvadipine. All values represent mean ± S.E. Significantly different from vehicle: **, P < .01; *, P < .05 (Dunnett's method).
Drug administration and plasma lipid determination. AE0047 and nilvadipine (40 mg each) were dissolved in 0.4 ml of 99.5% ethanol and 0.1 ml of Tween 80 and then diluted with water to the desired concentrations. These solutions were prepared each day immediately before use. Then, 10 ml/kg concentrations of the drug solutions, corresponding to 3 or 10 mg/kg AE0047 and 10 mg/kg nilvadipine, were orally administered once per day for 7 days to Zucker rats (n = 8–11). Animals were deprived of food overnight before blood sampling but were allowed free access to tap water. Two hours after the final administration on day 7, the animals were anesthetized with CO2, blood was withdrawn from the heart and plasma was prepared by centrifugation. Levels of plasma total cholesterol, TG and HDL cholesterol were measured using commercial assay kits. Chyomicrons, VLDL and LDL were analyzed by heparin-calcium precipitation (Matzno et al., 1994).

Determination of lipase activities in postheparin plasma. AE0047 and nilvadipine were orally administered for 7 days to Zucker rats as described above (n = 7 or 8). Two hours after the last administration, 1000 IU/kg heparin was injected intravenously. Postheparin plasma was prepared, and lipase activities were measured as reported (Matzno et al., 1994).

Synthesis and secretion of TG and apo B in Caco-2 cells. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in medium A (Eagle’s minimum essential medium (DMEM) supplemented with 1% (v/v) nonessential amino acids, 100 IU/ml penicillin G, 0.1 mg/ml streptomycin sulfate and 20% heat-inactivated FCS) in a humidified atmosphere of 95% air/5% CO2 at 37°C. The cells were subcultured in 75-ml flasks. Monolayers were studied at passages 23 to 27.

To study TG and apo B, Caco-2 cells (5 × 10^5 cells/1.5 ml of medium A) were seeded onto filters (cell culture insert) that were placed in a well of six-well plates containing 2 ml/well of medium A. Cells reached confluence within 2 or 3 days. Media of the upper (apical) and lower (basolateral) sides were exchanged with fresh media every 2 or 3 days until the transepithelial electrical resistance reached ~300 Ωcm². The resistance was measured using a Millicell-ERS (Millipore, Bedford, MA).

At 7 or 8 days after seeding, media of the both sides of chamber were changed to medium B (medium A supplemented with 1.5% BSA) containing AE0047 or nilvadipine. The drugs were dissolved in dimethylsulfoxide, and the final concentration in the medium was ≤0.1%. The cells were incubated with the drugs for the indicated periods; then, 1 μCi of 14C-oleic acid (400 μM) was added to the apical side. After a 16-hr incubation, 14C-TG was extracted from the basolateral medium and cell lysate according to the method of Bligh and Dyer (1959). TG was separated by TLC using petroleum ether/ethyl ether/acetone acid (80:20:1, v/v) as the solvent system. Radioactivity in the TG fraction was measured using a liquid scintillation counter.

Apo B secretion was examined under the same conditions as described above. After an 18-hr incubation, the apo B contents of the basolateral medium and cell lysate were measured by enzyme-linked immunosorbent assay. Briefly, medium or cell suspension was placed into 96-well plates and incubated for 17 hr at 4°C. After blocking nonspecific binding with 5% skim milk in PBS, plates were washed three times with TTBS (10 mM Tris-buffered saline containing 0.01% Tween 80, pH 7.4), and then 50 μl of anti-apo B antibody (diluted 1:5000 with PBS containing 0.1% BSA) was added and the plate was incubated for 2 hr. After two washes with TTBS, peroxidase-conjugated anti-goat IgG (diluted 1:5000 with PBS containing 0.1% BSA) was added and incubated for 2 hr. After washing with TTBS, peroxidase was detected with o-phenylenediamine, and the amount of antigen was determined as absorbance at 490 nm.

Synthesis of TG and FFA in sliced liver from Zucker rats. Zucker rats (n = 3–5) were administered AE0047 and nilvadipine for 7 days as described above. At 2 hr after the last administration, rats were bled from the heart, and the livers were perfused with saline. The livers were removed and sliced. TG and FFA synthesis was examined according to Yagasaki et al. (1984). Briefly, liver slices weighing 200 mg were placed in a screwcap tube (10 ml) containing 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, and 0.8 mCi of 14C-acetate. The tube was gassed with 100% O2 and incubated at 37°C for 3 hr in a metabolic shaker at 125 strokes/min. Thereafter, the tube was placed on ice to stop the reaction, and the liver was homogenized. TG and FFA labeled with 14C were extracted from the homogenate with chloroform/methanol (2:1, v/v) and separated on TLC, and radioactivity was measured as described above.

Preparation and 125I-labeling of lipoproteins. Fresh blood collected from healthy human volunteers was immediately mixed with 1 mg/ml EDTA and centrifuged (1500 × g, 10 min), and the plasma was collected. VLDL (d < 1.006) and LDL (1.006 < d ≤ 1.067) were separated by ultracentrifugation at 110,000 × g for 20 hr and stored at 4°C in the presence of 5 mM EDTA.

125I-LDL and 125I-VLDL were prepared as previously described (Yamauchi et al., 1996). 125I-LDL was prepared from 125I-VLDL by lipase digestion. Briefly, 30 μl of 125I-VLDL (0.1 mg of protein/ml) was mixed with 30 μl of digestion buffer (0.15 M NaCl, 4% BSA in 0.2 M Tris · HCl, pH 8.6) and LPL (1 IU/ml) and incubated at 37°C for 1 hr.

Incorporation of lipoproteins by HepG2 cells. HepG2 cells were subcultured in 100-cm² collagen-coated tissue culture dishes containing Williams’ E medium supplemented with penicillin G (100 IU/ml), 0.1 mg/ml streptomycin sulfate (medium C) and 10% heat-inactivated FCS.

For the following experiment, 2.5 × 10⁵ cells/well/ml of HepG2 cells were seeded onto 12-well plates. On the following day, cells were incubated for 48 hr with medium C containing the drugs and 5% LPDS. The media were exchanged with 1 ml of medium C containing labeled lipoproteins (1 μg/ml, drugs and 0.1% BSA). After incubation for the indicated periods, 200 μl of the medium was removed, mixed with 100 μl of 20% trichloroacetic acid, stored at 4°C for 30 min and centrifuged (10,000 × g 5 min). Trichloroacetic acid-soluble radioactivity was measured. The remaining cells were washed with ice-cold PBS and scrapped off the dishes with a rubber policeman. The radioactivity levels were measured, and the data represent total lipoproteins incorporated by HepG2 (i.e., sum of association and degradation).

Statistical analysis. Statistical analysis was made with an unpaired t test for two groups. For more than three groups, we used one-way analysis of variance and then Dunnett’s multiple-compari-
Results

**In vivo effects of AE0047 on plasma lipid profiles of obese Zucker rats.** Plasma lipid profiles of hypertriglyceridemic Zucker rats given AE0047 or nilvadipine for 7 days are shown in figure 2. AE0047 (3 and 10 mg/kg/day) dose-dependently decreased plasma TG level without altering total cholesterol and LDL levels. AE0047 (10 mg/kg) also decreased plasma TG-rich lipoproteins (chylomicrons and VLDL), whereas HDL cholesterol significantly increased. The administration of 10 mg/kg nilvadipine, a typical DHP-type calcium antagonist, tended to decrease plasma TG and chylomicron levels.

**Effect of AE0047 on plasma LPL activity in Zucker rats.** To assess the mechanism by which AE0047 decreased the plasma TG level of Zucker rats, we administered AE0047 or nilvadipine for 7 days and measured the lipase activity in postheparin plasma, which represents the sum of the LPL and H-TGL activities. The results showed that neither drug affected the lipase activity (fig. 3).

**Inhibition of the secretion of TG and apo B by AE0047 in Caco-2 cells.** Dietary TG is adsorbed and secreted as chylomicrons from the intestine. To check this process, we used the human intestinal cell line (Caco-2). Approximately 80% of the secreted 14C-TG appeared in the basolateral medium when 14C-oleic acid was added to the apical side of Caco-2 cells and incubated for 18 hr in the presence of the drug. 14C-TG contents in the cellular lysate (synthesis) and basolateral medium (secretion) were measured. All values represent mean ± S.E. Significantly different from vehicle: **, P < .01 (unpaired t test).
pressed in a dose-dependent manner between $10^{-7}$ and $10^{-5}$ M AE0047 (fig. 5). On the contrary, $10^{-5}$ M nilvadipine affected neither cellular TG synthesis nor its secretion (fig. 5).

We next examined the effect of AE0047 on the secretion of apo B, a major apolipoprotein constituent of TG-rich lipoproteins (VLDL and chylomicrons). As shown in figure 6, $10^{-5}$ M AE0047 inhibited the basolateral secretion of apo B without affecting its cellular level.

**Effect of AE0047 on TG and FFA synthesis in hepatocytes.** We evaluated the effect of AE0047 and nilvadipine on the hepatic synthesis of FFA and TG by using liver slices from Zucker rats that had been treated for 7 days with the drugs. Figure 7 shows that neither AE0047 nor nilvadipine influenced the hepatic synthesis of FFA and TG.

**Enhancement of VLDL incorporation by AE0047 in HepG2 cells.** Sequential reactions including binding, incorporation and degradation by the liver constitute the major pathway of lipoprotein clearance from the circulation. We evaluated the effect of AE0047 or nilvadipine on this step using a human hepatic cell line (HepG2). Figure 8 shows the total incorporation (association and degradation) of $^{125}$I-labeled LDL, IDL and VLDL. The total incorporation efficiency of VLDL was only 45.9% and 70.3% of that of LDL and IDL, respectively, after a 6-hr incubation. We next studied the incorporation of lipoproteins in HepG2 cells exposed to AE0047 (fig. 9). Cells were incubated for 48 hr with AE0047 before the addition of lipoproteins. AE0047 (0.1–10 μM) did not affect the incorporation of labeled LDL (fig. 9A) or IDL (fig. 9B). In contrast, VLDL incorporation was significantly increased by 10 μM AE0047 (fig. 9C).

We also studied the effect of various concentrations of nilvadipine on the incorporation of VLDL by HepG2 cells (fig. 10). In contrast to AE0047, this calcium antagonist did not affect the VLDL incorporation, even at a concentration of 10 μM.

**Discussion**

**AE0047 decreases plasma TG levels in Zucker rats.** We evaluated the effect of AE0047 on TG metabolism in the hypertriglyceridemic obese Zucker rat. This animal model is characterized by primary hypertriglyceridemia (Bray, 1977) and is widely used to evaluate the hypolipidemic action of drugs (Kasin et al., 1992). We demonstrated that AE0047 significantly decreased plasma TG and TG-rich lipoprotein levels and increased HDL levels in obese Zucker rats (fig. 2).

It is controversial whether hypertriglyceridemia is an independent risk factor for CHD. However, recent epidemiolog-
ical studies have confirmed that it is indeed a risk factor in certain populations (Cambien et al., 1986; Castelli, 1986; Fontbonne et al., 1989). The association of hypertriglyceridemia with a low plasma HDL level (Gotto, 1992), high procoagulant activities (Hoffman et al., 1992; Simpson et al., 1983) and high plasminogen activator inhibitor activities (Raccah et al., 1993) may facilitate atherosclerosis. Therefore, hypertriglyceridemia must be treated to prevent CHD, especially in patients with multiple risk factors for CHD, such as hypertension, obesity and diabetes. The hypotriglyceridemic action of AE0047, as well as its potent antihypertensive activity, may help reduce the incidence of CHD. We previously showed that AE0047 has antiatherogenic activity in rabbits fed with cholesterol (Yamanaga et al., 1993).

**Hypothesis of TG-lowering and HDL-increasing actions.** We first evaluated the effect of AE0047 on the lipase activities of Zucker rats because LPL and H-TGL play important roles in TG metabolism by degrading TG of VLDL, IDL and chylomicrons. However, AE0047 administration did not change the plasma lipase activities (fig. 3). Moreover, AE0047 did not affect the hepatic synthesis of FFA and TG in the Zucker rats (fig. 7). In contrast, AE0047 inhibited TG secretion in Caco-2 cells in a dose- and time-dependent fashion without affecting cellular TG synthesis (figs. 4 and 5). AE0047 also decreased the secretion of apo B (fig. 6), a major apolipoprotein in VLDL and chylomicrons. These results suggested that AE0047 inhibits the secretion of TG-rich lipoprotein particles into the medium in Caco-2 cells. In vitro data also suggested that one of the TG-lowering mechanisms of AE0047 is the reduction in chylomicron secretion from intestine.

The above notion that AE0047 may reduce intestinal chylomicron secretion is supported by the following. Hughes et al. (1988) demonstrated that calcium ionophores increase the synthesis and secretion of apo B in Caco-2 cells. Strauss and Jacob (1981) showed that calcium stimulates the secretion of TG in the isolated jejunum of the hamster. On the contrary, a benzothiazepine-type calcium antagonist (TA-3090) inhibits TG secretion in jejunal explants (Levy et al., 1992).

The studies on HepG2 cells indicated another possibility. AE0047 increased only VLDL incorporation in HepG2 cells (fig. 9), suggesting that AE0047 facilitates the hepatic clearance of VLDL in Zucker rats. Then, to explain the selectivity of hepatic VLDL incorporation, we noticed the role of hepatic LRP. In fact, VLDL and IDL include both apo B and apo E in their particles. However, the portion of these apolipoproteins in the cellular binding is different. Previous studies (Connelly and Kukis, 1981; Granot et al., 1994) showed that large and TG-rich lipid particles caused more rapid binding to apo E-specific sites (i.e., LRP). On the other hand, Krul et al. (1985) reported that apo B-specific binding in VLDL increased with the reduction of diameter and that virtually all of LDL binding is mediated by apo B. Taken together, these results suggested that VLDL incorporation is mainly medi-
ated by apo E (through LRP), whereas IDL is mainly mediated by apo B (through LDL receptor). Thus, LRP enhancement by AE0047 in HepG2 cells might increase only the VLDL incorporation.

In addition, it is likely that hepatic LRP enhancement also increases lipolysis of lipoproteins. The present study showed that AE0047 treatment did not affect the plasma lipase activity (fig. 3). Then, we considered the possibility that AE0047 enhanced the lipase-VLDL association rate. We suggested that AE0047 stimulated hepatic LRP manifestation as mentioned above. LRP also recognizes the lipase/lipoprotein complex as well as apo E (Krieger and Herz, 1994); therefore, this multifunctional receptor might help the lipase/lipoprotein interaction, lipolysis and resulting HDL formation. Further investigations remain to clarify these hypotheses.

Although the above mechanisms can explain the in vivo action of AE0047 against the plasma TG level, other mechanisms that were not tested in the present study might be involved. For example, AE0047 in vivo might alter the composition of apolipoproteins because enrichment of apo E or depletion of apo C in TG-rich lipoproteins accelerates their uptake by the liver (Kowal et al., 1990; Takahashi et al., 1995). Moreover, AE0047 might inhibit VLDL secretion, as reported for a benzothiazepine-type calcium antagonist (Nossen et al., 1987). An indirect mechanism such as an enhanced hepatic blood flow might also be involved.
Plasma TG level may be reduced by AE0047 through its calcium channel-blocking activity. There are discrepancies regarding the effect of calcium antagonist on the TG metabolism; therefore, it is difficult to determine whether the plasma TG level is reduced through its calcium channel-blocking activity. Clinical data suggest that calcium antagonists generally do not affect plasma lipid profiles (Giles, 1992; Kasiski et al., 1995), but results have differed, presumably due to clinical settings. Nicardipine is a typical example that may lower (de Cesaris et al., 1991; Kihara, 1991), have no effect on (Soro et al., 1990; Wang et al., 1993) or increase (Naukkarinen, 1988) plasma TG levels. Similarly, many calcium antagonists, such as amlopidine (Canale et al., 1991), nifedipine (Kazumi et al., 1990; Maldonado et al., 1992), isradipine (Morris et al., 1993) and nilvadipine (Berger and Albert, 1992), sometimes decrease plasma TG levels.

In the present study, AE0047 significantly decreased plasma TG and chylomicron levels of Zucker rats. In contrast, nilvadipine tended to decrease them, but the effects were not statistically significant (fig. 2). The discrepancy between AE0047 and nilvadipine on the in vivo efficacy can be explained by the following. First, we showed here that AE0047, but not nilvadipine, inhibited the intestinal chylomicron secretion and enhanced the hepatic uptake of VLDL. Because intestine and liver are the major organs responsible for the lipid metabolism, these findings strongly suggest that AE0047 decreased plasma TG level mainly by the above mechanisms, which were not shared by nilvadipine. In addition, the discrepancy may be partially due to the poor bioavailability of nilvadipine (~5% in rats; Tokuma et al., 1987) compared with that of AE0047 (~20%; Ohkubo et al., in press) if the calcium channel-blocking activity of AE0047 caused the reduction of plasma TG in Zucker rats. The observation that the hypotensive activity of AE0047 in normotensive rats was twice than that of nilvadipine1 might reflect the difference in bioavailability. As mentioned above, the relationship between the calcium channel-blocking activity and TG metabolism remains obscure, however, so more detailed and precise studies are needed to address the above issue.

In conclusion, AE0047 is a calcium antagonist that decreases plasma TG and increases plasma HDL levels in Zucker rats. Experiments in vitro suggest that the plasma TG is reduced through the suppression of chylomicron secretion from the intestine, as well as by enhancement of VLDL uptake by the liver. Treating hypertensive patients who have hypertriglyceridemia with AE0047 might reduce the risk factors of CHD.

References


Send reprint requests to: Takeshi Yamauchi, Ph.D., Pharmacology Laboratories, Central Research Laboratories, The Green Cross Corporation, 2-25-1, Shodai-Ohtani, Hirakata, Osaka 573, Japan.