Effects of Efonidipine Hydrochloride on Cholesterol Esterification Mediated by Beta-Very Low Density Lipoprotein in J774 Macrophages

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ABSTRACT—The effects of efonidipine hydrochloride (efonidipine), a dihydropyridine calcium antagonist, on the cholesterol ester metabolism induced by beta-migrating very low density lipoprotein (β-VLDL) in J774 macrophages were studied. The cholesteryl ester content in the macrophages was increased by incubation with β-VLDL, and the increase was inhibited by efonidipine. Oleic acid incorporation into cellular cholesteryl ester was increased by β-VLDL in J774 macrophages. The incorporation at an early phase of β-VLDL induction (0–3 hr) was inhibited by efonidipine. This inhibitory effect of efonidipine was greater at an early phase of β-VLDL induction (0–3 hr) than at a late phase of the induction (8–11 hr). Pretreatment of the cells with efonidipine enhanced the inhibitory effect. Efonidipine also inhibited β-VLDL degradation but not the binding and association in macrophages without pretreatment. β-VLDL binding and association to macrophages were decreased by pretreatment of the cells with efonidipine. β-VLDL metabolism was also decreased by dibutyryl cyclic AMP pretreatment. The decrease of β-VLDL metabolism by efonidipine was prevented by co-treatment with efonidipine and HA1004, a protein kinase A inhibitor. Furthermore, efonidipine increased the intracellular cyclic AMP content in J774 macrophages. These findings suggest that efonidipine suppresses cholesterol ester deposition in atherosclerotic foam cells by inhibiting the modified lipoprotein metabolism and cholesterol esterification mainly through elevation of the cellular cyclic AMP level.

Keywords: Efonidipine, Macrophage, Cholesteryl ester, β-Very low density lipoprotein, Cyclic AMP

Cholesterol ester deposition in atherosclerotic intimal thickening is mainly due to the accumulation of lipid laden foam cells derived from monocyte/macrophages and intimal smooth muscle cells (1, 2). Cholesterol ester accumulation in the foam cells is caused by incorporation of beta-migrating very low density lipoprotein (β-VLDL) or modified lipoproteins such as acetylation or oxidation of low density lipoprotein (LDL) (3–7) and due to subsequent induction of cholesterol esterification in the cells (4, 8–11). These β-VLDL or modified lipoproteins are recognized by a scavenger receptor or LDL receptor related receptor family on the cell surface and internalized into the cells (10, 11). On the contrary, high density lipoproteins (HDL) diminish the intracellular cholesterol ester content by stimulating cholesterol ester hydrolysis activity and cholesterol translocation to the cell membrane and by enhancing the efflux of cholesterol from the cell membrane (12, 13).

Calcium cation mobilization into cells and intracellular calcium cation contents affect the cholesterol ester metabolism in macrophages. There are several reports that calcium antagonists show antiatherogenic effects in animal models with inhibition of cholesterol ester accumulation (14–17). These calcium antagonists suppress the cholesterol ester accumulation in macrophages by inhibiting acyl-CoA cholesterol acyl-transferase (ACAT) and enhancing cholesterol ester hydrolysis (18, 19). ACAT activity was induced by modified lipoprotein receptors including the scavenger-receptor-mediated pathway. However, the mechanism of the inhibitory effects of calcium antagonists on the induction of ACAT activity mediated by modified lipoprotein remain unknown.

We have confirmed that efonidipine hydrochloride (efonidipine), a dihydropyridine calcium antagonist having a potent calcium antagonist action, a very slow onset and long-lasting antihypertensive action and diuretic and
natriuretic actions (20), suppressed aortic cholesterol ester accumulation and surface involvement in cholesterol fed atherosclerotic rabbits without affecting the plasma cholesterol level (21).

In this study, to clarify the mechanisms of the inhibitory effect of efonidipine on cholesteryl ester deposition, we examined the effect of efonidipine on cholesteryl ester formation mediated by β-VLDL and δ-VLDL metabolism in J774 macrophages.

MATERIALS AND METHODS

Materials

Efonidipine (Lot No. 110P8701) was synthesized in the Central Laboratories of Nissan Chemical Industries (Funabashi). Nicardipine was purchased from Mediolast S.p.A. (Milano, Italy). HA1004 and dibutyryl cyclic AMP (diBu-cAMP) were obtained from Seikagaku Kogyo Co., Ltd. (Tokyo) and Sigma (St. Louis, MO, USA), respectively. The cyclic AMP enzyme immunoassay system was obtained from Amersham International plc (Amersham, UK). All compounds were dissolved in dimethylsulfoxide (DMSO) and added to the medium at a final concentration of 0.2%. 14C-Oleic acid and 125I-Na were obtained from New England Nuclear (Boston, MA, USA).

Preparation of lipoproteins

β-VLDL was prepared from diet induced hypercholesterolemic rabbits by ultracentrifugation at a density of 1.006 according to the methods of Morisaki et al. (22). Lipoprotein-deficient serum (LpDS) was prepared from fetal bovine serum (FBS) as the bottom fraction of the ultracentrifugation, having a density of 1.25. δ-VLDL and LpDS were dialyzed against phosphate-buffered saline (PBS). δ-VLDL was labeled with 125I-Na according to the method of Goldstein et al. using ICI (23).

Cholesterol ester accumulation by δ-VLDL in J774 macrophages

J774 macrophages were cultured in a 24-well plate with 10% FBS-DME medium. In the study on the early phase of β-VLDL induction, cells were pre-cultured in fresh 10% FBS-DME medium with or without drugs for 8 hr, and the cells were subsequently cultured in the 10% FBS-DME medium containing 100 µg cholesterol/ml δ-VLDL, 148 MBq/ml 14C-oleic acid (3.7 MBq/µl of 10% BSA, pH8) and the drugs for 3 hr. In the study on the late phase of δ-VLDL induction, the cells were pre-cultured in the δ-VLDL containing medium with or without drugs for 8 hr, and they were subsequently cultured in the medium containing δ-VLDL, 14C-oleic acid and the drugs, as above, for 3 hr. Then the cells were washed with PBS and briefly sonicated in PBS. The cells were extracted with chloroform : methanol (2 : 1), and the extracts were separated by TLC (1st development, isopropylether : acetic acid / 96 : 4; 2nd development, petroleum ether : diethyl ether : acetic acid / 90 : 10 : 1), and the 14C radioactivity in the cholesteryl ester fraction was counted. Cell protein was measured by a BCA kit (Pierce, Rockford, IL, USA).

Assay for cholesterol esterification in the cells

J774 macrophages were maintained at confluency with 10% FBS-DME medium. Cells were pre-cultured in 10% FBS-DME medium with or without the drugs for 8 hr; Then all media were changed to 5% LpDS containing DME medium, and the drugs were added to all cultures. The binding assay, cell association assay and degradation assay were performed according to the method of Goldstein et al. (3). For the binding assay, the cells were first placed on ice for 1 hr and then 40 µg/ml 125I-δ-VLDL was added to the medium. The cells were placed on ice for another 1 hr. The cells were washed with 0.2% BSA-containing PBS twice and PBS once. They were then digested with 0.1 N NaOH, and the radioactivity and protein content were measured. For the cell association assay, 40 µg/ml 125I-δ-VLDL was added to the medium, and the cells were cultured for 3 hr. The cells were washed and digested as described above, and the radioactivity and protein content were measured. For the degradation assay, 40 µg/ml 125I-δ-VLDL was added to the medium and the cells were cultured for 12 hr. An aliquot of the culture media was precipitated by 10% TCA, and the supernatant was extracted with chloroform containing KI and H2O2. The radioactivities in the aqueous phase was measured.

Intracellular cAMP contents in J774 macrophages

J774 macrophages maintained in T-75 flasks were seeded on 12-well culture plates at 2 x 10⁶ cells/well with 10%
FBS-DME medium and cultured until confluency. Then the cells were cultured with efonidipine or nicardipine containing 10% FBS-DME medium for 1, 3 or 8 hr, and the cellular cAMP was extracted with ice-cold 65% ethanol and dried under nitrogen. The residual precipitates were dissolved in 0.1 N NaOH, and the cellular proteins were measured. The extracts were dissolved in 150 μl assay buffer, and 100 μl of this solution was subjected to enzyme immunoassay for cAMP (Amersham).

**Statistical analyses**

All assays were carried out in triplicate sets of culture wells. Statistical analyses were performed by Student's t-test for unpaired data. Differences with a P value of <0.05 or <0.01 were considered to be significant.

**RESULTS**

**Effect of efonidipine on cholesteryl ester accumulation**

Figure 1 shows the effect of efonidipine on cholesteryl ester accumulation induced by β-VLDL in macrophages. The cellular cholesteryl ester content was drastically increased by incubation with β-VLDL. This increase was inhibited by efonidipine in a concentration-depend-
Fig. 3. Effects of efonidipine hydrochloride and nicardipine pretreatment on cholesterol esterification in J774 macrophages.

A: In the early phase, the cell culture medium was changed to 10% FBS-DME medium containing the indicated concentration of drugs, and the cells were cultured for 8 hr. β-VLDL (100 µg cholesterol/ml) and 14C-oleic acid (37 MBq/well) were added, and the culture was continued for 3 hr. B: In the late phase, the cell culture medium was changed to the 10% FBS-DME medium containing the indicated concentration of drugs and β-VLDL (100 µg cholesterol/ml), and the cells were cultured for 8 hr. 14C-oleic acid (37 MBq/well) was added to the medium, and the culture was continued for 3 hr. ●: efonidipine, ○: nicardipine.

Each value is a mean ± S.D. of triplicate assays; * and ** indicate significant differences from the control at P < 0.05 and P < 0.01, respectively.

ent manner from 10⁻⁹ M to 10⁻⁷ M, and its IC₅₀ was 10.81 nM in the 24 hr incubation (Fig. 1). At 10⁻⁸ M, the inhibitory effect of efonidipine was stronger than that of nicardipine.

Effects of efonidipine on cholesterol esterification

Figures 2 and 3 show the effects of efonidipine and nicardipine on the 14C-oleic acid incorporation into the cellular cholesteryl ester fraction in J774 macrophages. There was marked 14C-oleic acid incorporation into the macrophages in the presence of β-VLDL both at the early and late phases of the incubation.

Efonidipine inhibited the β-VLDL-induced 14C-oleic acid incorporation as shown in Figs. 2 and 3. This inhibitory activity was almost the same as that of nicardipine with respect to the potency and concentration-dependency. The inhibition at the late phase of induction was greater after an 8-hr pretreatment of the cells with efonidipine (IC₅₀: 0.583 nM) than without pretreatment (IC₅₀: 6933.3 nM) as shown in Figs. 3B and 2B. The same effect was observed in the early phase of induction. When the cells were pretreated with efonidipine for 8 hr, the inhibitory effect at the early phase of induction (IC₅₀: 0.258 nM) was greater than that at the late phase of induction (IC₅₀: 0.583 nM) as shown in Fig. 3. Also, when the cells were pretreated with efonidipine, the inhibitory effect at the early phase of induction (IC₅₀: 63.81 nM) was greater than that at the late phase of induction (IC₅₀: 6933.3 nM) as shown in Fig. 2. The highest concentration of efonidipine (10⁻⁵ M) also inhibited the basal 14C-oleic acid incorporation at the early phase of induction.

Effects of efonidipine on 125I-β-VLDL binding, cell association and degradation in J774 macrophages

To clarify the mechanism of the inhibitory effect of efonidipine on the cholesterol esterification induction mediated by β-VLDL, the effects of efonidipine on 125I-β-VLDL binding, cell association and degradation in J774 macrophages were studied. In the cells not pretreated with drugs, the 125I-β-VLDL degradation in the cells was decreased by efonidipine, but the binding and cell association to the cells were not affected by it (Fig. 4A). This inhibitory effect was significant but the concentration dependency was not clear. In the cells pretreated with drugs for 8 hr, 125I-β-VLDL binding and cell association were significantly decreased by efonidipine pretreatment: by 35.4% at 10⁻⁶ M and by 40.8% at 10⁻⁶ M, and the degradation was also significantly decreased by efonidi-
pine pretreatment: by 46.1% at $10^{-6}$ M and its IC$_{50}$ was 1788 nM (Fig. 4B). This inhibitory activity of efonidipine was the same as that of nicardipine.

Next, the effect of HA1004, a cyclic AMP (cAMP)-dependent protein kinase inhibitor, on the inhibition by efonidipine and the effect of dibutyril cyclic AMP (diBu-cAMP) on $^{125}$I-$\beta$-VLDL binding, cell association and degradation in J774 macrophages were studied. Cells were pretreated for 8 hr with efonidipine and HA1004 before the assay. In the binding and cell association, the inhibitory effect of efonidipine was completely prevented by the pretreatment with 30 $\mu$M HA1004, and the inhibitory effect of efonidipine on the degradation was partially prevented by the pretreatment with 30 $\mu$M HA1004 (Fig. 5). The binding, cell association and degradation were significantly decreased in the cells pretreated with diBu-cAMP by 32.2%, 61.2% and 77.4%, respectively (Fig. 5).

Fig. 4. Effects of efonidipine hydrochloride and nicardipine $\beta$-VLDL binding, cell association and degradation in J774 macrophages. Cells were maintained on a 48-well culture plate. A: The cells were not pretreated with drugs before the $\beta$-VLDL receptor assays. B: the cells were pretreated with drugs for 8 hr before the assay. ●: efonidipine, ▲: nicardipine. Each value is a mean±S.D. of triplicate assays; * and ** indicate significant differences from the control at P<0.05 and P<0.01, respectively.
Effect of efonidipine on intracellular cAMP contents in J774 macrophages

Figure 6 shows the effect of efonidipine on intracellular cAMP contents in J774 macrophages. Efonidipine significantly increased the intracellular cAMP content by 60.3% (18.3 ± 1.4 to 29.3 ± 1.6 fmole/μg protein) at 10⁻⁷ M in the 3-hr pretreatment. In 8-hr pretreatment, efonidipine more potently increased the intracellular cAMP content in a concentration-dependent manner and increased it by 128.5% (18.3 ± 1.4 to 41.8 ± 0.6 fmole/μg protein) at 10⁻⁷ M. Nicardipine also increased the cAMP level to the same degree as efonidipine by the 3-hr pretreatment of the cells.

DISCUSSION

Deposition of lipid-laden foam cells derived from monocytes/macrophages and smooth muscle cells in the intimal lesion is a key event in the atheromatous intimal thickening and causes the cholesterol ester accumulation in the intima. Monocyte-derived macrophages deposit cholesterol ester through a modified lipoprotein scavenging pathway. In the present study, we examined the effect of efonidipine hydrochloride (efonidipine), a dihydropyridine calcium antagonist, on the cholesteryl ester metabolism in J774 macrophages.

Efonidipine inhibited cholesteryl ester accumulation in the J774 macrophages induced by β-VLDL (Fig. 1). This
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inhibition was observed in a concentration-dependent manner at $10^{-9}$ M to $10^{-7}$ M, and higher inhibition was observed at $10^{-5}$ M. Nifedipine has been reported to inhibit cholesteryl ester accumulation in macrophages (19). Recently, we have reported that efonidipine suppressed cholesteryl ester accumulation in the arterial wall by 64% ($P < 0.05$) in the aortic arch at 100 mg/kg dosage in cholesterol-fed rabbits, and the maximal plasma efonidipine concentration reached 82.1 ± 17.0 ng/ml (115 nM) under this condition (21). Our data indicate that efonidipine has a greater inhibitory effect on cholesteryl ester accumulation induced by modified lipoprotein than nicardipine, suggesting that efonidipine has a potent inhibitory effect on cholesteryl ester deposition and foam cell formation in the atheromatous lesion.

To evaluate the inhibitory effect of efonidipine on cholesteryl ester accumulation, the effect on cholesteryl esterification induced by $\beta$-VLDL was examined. Efondipine inhibited $\beta$-VLDL inducible cholesteryl ester formation in J774 macrophages (Figs. 2 and 3). Intracellular cholesteryl ester accumulation is proposed to be related to the activation or induction of acyl-CoA cholesteryl acyltransferase (ACAT) and suppression of the neutral cholesteryl ester hydrolase activity via a scavenger pathway (8-10). Verapamil has been reported to inhibit cholesteryl esterification in whole macrophages but not in cell homogenate (24). The inhibitory effect of efonidipine on cholesteryl ester formation induced by $\beta$-VLDL was greater in efonidipine-pretreated cells than in non-pretreated cells in the both the early and late phases of the induction. Furthermore, this inhibitory effect was greater at the early phase of the induction (0 to 3 hr after $\beta$-VLDL addition) than at the late phase (8 to 11 hr after $\beta$-VLDL addition). The effect in the early phase is thought to reflect the effect on the induction of ACAT activity, and the effect in the late phase is thought to reflect the effect on the induced ACAT activity rather than its induction. The discrepancy of the inhibitory effect of efondipine in the early phase and the late phase suggests that efonidipine may have inhibitory effects on the intracellular induction of the ACAT activity through metabolizing modified lipoproteins via the scavenger pathway. These inhibitory effects of efonidipine by pretreatment, as shown in Figs. 2A and 3A, were almost the same as those of nicardipine with respect to the potency and dose-dependent manner and similar to its calcium antagonistic effect, decreasing $\text{Ca}^{2+}$ uptake by 67% at $10^{-7}$ M (25). At the early phase of induction, $10^{-5}$ M efonidipine inhibited the basal level of cholesteryl esterification activity (Fig. 2). This suggests that these calcium antagonists may also inhibit the ACAT activity expressed in the basal level. Nifedipine has been reported to inhibit cholesteryl ester formation in cultured arterial smooth muscle cells (18). In our study, efonidipine also inhibited cholesteryl esterification in rabbit intimal smooth muscle cells to the same degree as in the J774 macrophages (data was not shown).

Verapamil has been reported to inhibit lysosomal degradation of LDL, thereby increasing the intralysosomal pH (26). In our study, efonidipine inhibited $\beta$-VLDL degradation in J774 macrophages but not cell association and binding to the cells in the cells not pretreated with the drug (Fig. 4). However, the inhibitory effect of efonidipine on $\beta$-VLDL binding and cell association appeared in the cells pretreated with efonidipine. These inhibitory effects of efonidipine on $\beta$-VLDL binding, cell association and degradation were completely or partially prevented by co-treatment with HA1004 and efonidipine (Fig. 5), and pretreatment with dibutyl cyclic AMP suppressed $\beta$-VLDL binding, cell association and degradation (Fig. 5). Furthermore, efonidipine increased the intracellular cAMP level in J774 macrophages by 3 or 8 hour treatment (Fig. 6). The present findings suggest that efonidipine may suppress $\beta$-VLDL receptor expression or inactivate the intracellular machinery in $\beta$-VLDL translocation through elevation of the intracellular cAMP level via calcium antagonism. The decrease of the degradation without pretreatment with efonidipine suggests that the intracellular effect of efonidipine occurred during this long incubation period (12 hr). Cyclic AMP has been shown to enhance cholesteryl ester hydrolysis (27-29). Nicardipine has been shown to enhance the intracellular cAMP level through inhibiting phosphodiesterase in arterial smooth muscle cells (30, 31). Efondipine also increases the cAMP level in the arterial wall (32). Elevation of cAMP by efonidipine or exogenous addition of cAMP may activate cholesteryl ester hydrolysis activity and increase the cellular free cholesterol pool. The increase of free cholesterol in the cells down-regulates LDL receptor activity in the LDL pathway but not modified lipoprotein metabolism in the scavenger pathway (1, 33). Furthermore, activation of neutral cholesteryl ester hydrolysis by G-CSF did not cause down-regulation of $\beta$-VLDL metabolism (34). These findings suggest that cAMP may regulate the scavenger pathway in a cholesteryl ester hydrolysis in an independent manner through down-regulating $\beta$-VLDL receptor activity, and the biphasic inhibitory effects of efonidipine in cellular cholesteryl ester content (Fig. 1) and cholesteryl esterification (Figs. 2 and 3) suggest that efonidipine may inhibit this scavenger pathway through a cAMP elevating effect at lower concentration and also directly inhibit ACAT activity at higher concentration. This effect of efonidipine may generally be observed in the calcium channel blockers with cellular cAMP elevation activity. The efficacy of efonidipine on cholesterol esterification and $\beta$-VLDL...
metabolism was similar to those of nicardpine, whereas the efficacy of efonidipine on the cholesteryl ester accumulation was more potent than nicardpine. As shown in Fig. 6, since the intracellular cAMP elevating effect of efonidipine was similar to that of nicardpine, the cholesteryl ester hydrolysis enhancing effect through elevating the cAMP level of efonidipine may be similar to that of nicardpine. This discrepancy suggests that efonidipine may have some additional effects on the cholesteryl ester accumulation such as intracellular cholesteryl transportation and cholesterol synthesis. Sterol carrier protein 2 has been reported to relate to cholesterol eflux from the plasma membrane by HDL when it is poor in cholesterol in the early phase of effluxing.

Efonidipine suppresses atheroma formation with inhibition on cholesterol ester accumulation (21). In the present study, efonidipine inhibited cholesteryl ester deposition in the macrophages more potently than nicardpine by inhibiting cholesteryl esterification induced by 3-VLDL through suppression of the scavenger receptor function. These findings suggest that efonidipine has greater anti-atherogenic activity via suppression of cholesteryl ester deposition in the artery. The details of these effects on the scavenger receptor function and the effect on intracellular cholesterol transportation, cholesteryl synthesis and cholesteryl ester hydrolysis remain to be studied.

REFERENCES

4 Goldstein JL, Ho YK, Basu SK and Brown MS: Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 76, 333–337 (1979)
5 Henriksen T, Mahoney EM and Steinberg D: Enhanced macro-

phase degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. Proc Natl Acad Sci USA 78, 6499–6503 (1981)
12 Slotte JP, Oram JF and Bierman EL: Binding of high density lipoprotein to cell receptors promotes translocation of cholesterol from intracellular membrane to cell surface. J Biol Chem 262, 12904–12907 (1987)


31 Sakamoto N, Terai M, Takenaka T and Maeno H: Inhibition of cyclic AMP phosphodiesterase by 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(n-benzyl-n-methylamino) ethyl ester 5-methyl ester hydrochloride (YC-93), a potent vasodilator. Biochem Pharmacol 27, 1269–1274 (1978)


