Enhanced expression of ATP-binding cassette transporter A1 in non-rafts decreases the sensitivity of vascular endothelial cells to Shiga toxin

Short Title: Stx sensitivity decreased by enhancement of ABCA1 expression

Authors: Nobutaka Higashi[1], Youko Matsumura[1], Fumiko Mizuno[1], Kazuki Kasahara[1], Shigeki Sugiura[2], Keiichi Mikasa[3], and Eiji Kita[1]

Affiliation: Department of Bacteriology[1], Medical Genetics Research Center[2], Center for Infectious Diseases[3], Nara Medical University, School of Medicine Kashihara, Nara 634-8521, Japan

Correspondence Author: Eiji Kita, M.D., Ph.D.
Department of Bacteriology,
Nara Medical University, School of Medicine
Kashihara, Nara 634-8521, Japan
Tel: + 81-744-29-8839,
Fax: + 81-744-29-7375
e-mail: eijikita@naramed-u.ac.jp

Abstract

Shiga toxin (Stx) binds to globotriaosyl ceramide (Gb3) receptors on the surface of vascular endothelial cells, which is followed by Gb3-dependent endocytosis, and initiates a cascade leading to cell damage. The Gb3 receptor is localized in lipid rafts, in which cholesterol is tightly packed primarily with sphingolipids in a liquid-ordered state. Recent studies have indicated that phosphodiesterase (PDE) type 4 inhibitors enhance the expression of ATP-binding cassette 1 (ABCA1) which promotes cholesterol efflux from non-rafts at the plasma membrane. Here we report that rolipram, a PDE4 inhibitor, reduced the sensitivity to Stx2 of human umbilical vascular endothelial cells in association with increased apolipoproteinA-I (apoA-I)-mediated cholesterol efflux, and shift of some Gb3 molecules from lipid rafts into non-rafts. Although rolipram treatment did not reduce Gb3 content at the plasma membrane and Stx binding to whole cells of HUVECs, it reduced Stx2 endocytosis. Knockdown of ABCA1 by transfection with siRNA ABCA1 in vascular endothelial cells abrogated the protective effect of rolipram on Stx2-exposed cells. Our present results suggest that the expression level of ABCA1 protein is one of critical determinants of Stx sensitivity levels in vascular endothelial cells.

Key Words: Shiga toxin, ABCA1, Gb3, cholesterol efflux, phosphodiesterase inhibitor, vascular endothelial cells

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157 infection is the leading cause of hemolytic uremic syndrome (HUS) in children [1]. The pathophysiology of HUS is not completely understood, although STEC strains have a variety of virulent factors including Shiga toxin (Stx) (Stx1 and Stx2) [2]. One established mechanism of STEC-associated HUS is that Stx bound to the specific Gb3 (globotriaosyl ceramide) receptor induces cytotoxicity in vascular endothelial cells through inhibition of protein synthesis, thereby initiating thrombotic microangiopathic damage. Many investigators have demonstrated that an overproduction of inflammatory cytokines/chemokines is also involved in STEC-associated HUS [3, 4]. Severity of HUS is related to serum levels of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8 [5-8], suggesting that inflammatory response is required for either initiating or developing the vascular damage following STEC infection. In connection with this, several studies [9, 10] have suggested that the cytokine milieu in the blood of individual patients determines whether endothelial cells survive or undergo apoptosis even if exposed to sub-inhibitory concentrations of Stx.

Plasma membrane Gb3 exists in lipid rafts or detergent-resistant microdomains (DRM) [11]. Kovbasnjuk et al. [12] have demonstrated that disruption of lipid rafts in intestinal epithelial cells by cholesterol depletion did not affect the amount of Gb3-bound Stx1 B-subunit, while Stx1 B-subunit internalization was significantly decreased. In addition, the A-subunit of Gb3-bound toxin in lipid rafts stimulates Gb3-dependent endocytosis of the toxin, and the cross-linking of Gb3 and lipid rafts is important for internalization of Stx [13]. However, it is unresolved whether enhanced cholesterol efflux at the plasma membrane alters the sensitivity to Stx of vascular

endothelial cells.

Our previous study [14] demonstrated that phosphodiesterase type 3 (PDE3) and PDE4 inhibitors prevented acute encephalopathy in STEC-infected mice. Both inhibitors cause dose-dependent suppression of TNF-α production from endotoxin-stimulated macrophages, mesangial cells, and microglia. This effect seems to protect vascular endothelial cells from Stx toxicity, because TNF-α enhances Gb3 expression in endothelial cells [15-19]. Additionally, PDE4 inhibitors are known to promote cholesterol efflux from vascular endothelial cells by enhancing the expression of ATP cassette binding protein 1 (ABCA1), which mediates the active release of cellular cholesterol and phospholipids to apolipoprotein A (apoA)-I [20]. Furthermore, destabilization of lipid rafts by cholesterol extraction potently inhibits Stx B-subunit transport from early endosomes to the trans-Golgi network in HeLa cells [21].

Thus, we investigated the role of ABCA1 expression in determining the sensitivity to Stx of vascular endothelial cells, using human umbilical vein endothelial cells (HUVECs) and rolipram (a PDE4 inhibitor). This study shows the first evidence that enhancement of ABCA1 expression decreased the sensitivity to Stx of HUVECs in association with an alteration in Gb3 behavior at the plasma membrane, and also with reduction of Stx endocytosis.

2. Results

2.1. Distribution of membrane molecules in TNF-α-stimulated HUVECs

Subconfluent monolayers of HUVECs were stimulated with or without 100 U/ml TNF-α for 24 h. The plasma membrane obtained from the post-nuclear supernatant of cell lysates was fractionated using the Triton X-based method followed by centrifugation in a discontinuous sucrose gradient, but the membrane was not enriched in this study. For the plasma membrane fractions of HUVECs cultured without TNF-α, Western blots identified caveolin-1, flotillin-1, GM1 and Gb3 in fractions 3-5 (density range: 1.055-1.115 g/ml), while TfR and ABCA1 were identified in fractions 9-11 (1.130-1.180 g/ml) (Fig. 1A). Stimulation for 24 h with 100 U/ml TNF-α did not change the location of these molecules in membrane fractions of HUVECs, compared to unstimulated cells (Fig. 1B), though the intensity of antibody-bound Gb3 bands seemingly increased. In the following experiments, HUVECs were used for assays after 24 h of stimulation with 100 U/ml TNF-α, unless otherwise indicated.

2.2. Effect of rolipram on cholesterol efflux and ABCA1 expression

We first examined the effect of rolipram on apoA-I-mediated cholesterol efflux from HUVECs which were labeled with 0.5 μCi/ml [1, 2-^{3}H] cholesterol. Rolipram (10 μg/ml) alone did not enhance [^{3}H] cholesterol efflux (Fig. 2A). However, this PDE4 inhibitor at doses of higher than 5 μg/ml significantly increased the efflux in the presence of 10 μg/ml apoA-I (5 and 7.5 μg/ml: $p < 0.05$, 10 μg/ml: $p < 0.01$ vs. apoA-I alone). This enhancement occurred only at non-rafts (TSF) (5 μg/ml: $p < 0.05$, 7.5 mg/ml; $p < 0.025$, 10 μg/ml: $p < 0.01$ vs. rolipram-untreated), but not at lipid rafts (DRM) (Fig. 2B).

Since rolipram enhanced apoA-I-mediated cholesterol efflux from TSF, we examined the effects of this inhibitor on ABCA1 mRNA expression and its protein levels in HUVECs. A 24 h treatment with 7.5 μg/ml rolipram alone enhanced the expression of ABCA1 mRNA, compared with 10 μg/ml apoA-I alone (Fig. 3A). Combination of rolipram and apoA-I most markedly enhanced its expression in HUVECs. Western blot assay using pooled TSF demonstrated that 10 μg/ml apoA-I alone did not enhance the expression of ABCA1 protein in HUVECs, while 7.5 μg/ml rolipram apparently increased it, independently of apoA-I (Fig. 3B). The latter finding was consistent with the increased amount of biotin-labeled ABCA1 proteins on the surface of HUVECs (Figure 3C); densitometry analysis of the labeled proteins showed that rolipram at doses of higher than 5 μg/ml significantly increased ABCA1 expression on the surface of HUVECs (5 and 7.5 μg/ml: $p < 0.05$, 10 μg/ml: $p < 0.01$ vs. apoA-I alone) (Fig. 3D). Since rolipram did not change the amount of biotin-labeled flotillin-1, this inhibitor seemed to selectively increase the expression of surface ABCA1. This enhancement was in parallel to the increase in concentrations of intracellular cAMP in HUVECs treated with rolipram; rolipram at doses of higher than 5 μg/ml significantly increased cAMP concentrations even in the absence of apoA-I (5 μg/ml; $p < 0.05$, 7.5 μg/ml; $p < 0.025$, 10 mg/ml; $p < 0.01$, vs. without rolipram treatment) (Fig. 3E). At a dose of 10 μg/ml, however, the presence of apoA-I augmented cAMP production to a significant extent, compared with rolipram alone ($p < 0.05$).

2.3. Effect of rolipram on Gb3 at the cell membrane of HUVECs

Next, we quantified the amount of Gb3 in neutral glycolipid separated from the plasma membrane of HUVECs receiving various treatments. Gb3 content was increased

only by stimulation with TNF-α, and was not further increased by additional treatments (Fig. 4A). Then, we determined whether treatment with combination treatment (7.5 μg/ml rolipram and 10 μg/ml apoA-I) altered the distribution of cell-surface molecules using membrane fractions separated by the discontinuous sucrose gradient centrifugation. The combination shifted a part of Gb3 molecules from DRM into TSF (Fig. 4B), while GM1 co-localized with Gb3 at DRM was not shifted into TSF. Apparently, the combination increased the expression of ABCA1 at TSF, but did not change the localization of this protein. The combination did not affect the distribution and expression of other molecules. When the same gradients were tested for the Stx2-binding ability by the overlay assay, the binding was found in fractions 10 and 11 of TSF. In contrast, Stx2 binding was apparently decreased at fractions 3 and 4, but increased at fraction 5 of DRM (Fig. 4C). In the Stx2 binding assay using HUVECs, however, the amount of the labeled toxin binding to the cells was slightly, but not significantly, reduced by increasing concentrations of rolipram (Fig. 4D).

2.4. Effect of rolipram on the Stx2 sensitivity of HUVECs

In association with these changes, rolipram at doses of higher than 5 μg/ml significantly increased the percentage of surviving cells, as determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) assay (Fig. 5A), and also decreased the number of apoptotic cells as determined by TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay (Fig. 5B) after 24 h incubation with 10 nM Stx2 (in both assays; 5 and 7.5 μg/ml: $p < 0.05$, 10 μg/ml: $p < 0.01$ vs. without rolipram). Increased cell survival after incubation with 10 nM Stx2 was associated with the suppression of caspase-3 and -8 activities in rolipram-treated cells (5

and 7.5 μg/ml: $p < 0.05$, 10 μg/ml: $p < 0.025$, vs. rolipram-untreated cells) (Fig. 5C). In parallel to the decreased activities of caspase-3 and -8, 10 μg/ml rolipram reduced DNA fragmentation in HUVECs after 24 h incubation with 10 nM Stx2 (Fig. 5D); its reduction levels appeared to be almost comparable to those achieved by 20 μM Z-IETD-FMK (caspase-8-specific inhibitor).

2.5 Effect of rolipram on small interfering (si) RNA-transfected HUVECs

In order to clarify the relatedness between ABCA1 expression and Stx2 sensitivity, we determined whether knockdown of ABCA1 protein impaired the protective effect of rolipram on Stx2-exposed HUVECs. As shown in Figs. 6A and B, the level of ABCA1 protein expression apparently decreased at the plasma membrane of ABCA1 siRNA-transfected cells, while the expression was not changed in negative control siRNA-transfected cells. Expression of caveolin-1 appeared to be at similar levels between both transfected cells. Down-regulation of ABCA1 expression significantly reduced the level of cholesterol efflux in the presence of apoA-I, but not in the presence of BSA alone (Fig. 6C). Unlike untransfected HUVECs, Gb3 behavior was not altered in ABCA1 siRNA-transfected cells by treatment with the combination of rolipram and apoA-I (Fig, 6D). In negative control siRNA-transfected HUVECs, combination treatment induced the same alteration for Gb3 behavior as observed in untransfected cells (data not shown). Furthermore, down-regulation of ABCA1 significantly impaired rolipram-induced cell resistance to Stx2 in the presence of apoA-I as determined by MTT assay (5 and 7.5 μg/ml rolipram: $p < 0.05$, 10 μg/ml rolipram: $p < 0.01$ vs. control siRNA-transfected cells) (Fig. 6E).

Finally, we examined the effect of rolipram on the Stx2 endocytosis by HUVECs

using biotin-labeled Stx2. The combination treatment (7.5 μg/ml rolipram and 10 μg/ml apoA-I) significantly reduced the level of Stx2 endocytosis during a 60-min incubation period in both untransfected HUVECs (at 5 min: $p < 0.05$, at 10 min: $p < 0.025$, at 15 - 60 min: $p < 0.01$) and negative control siRNA-transfected cells (at 5 min: $p < 0.05$, at 10 - 60 min: $p < 0.025$), compared with untransfected cells receiving only apoA-I treatment (Fig. 7A). In contrast, it did not significantly decrease the level of Stx2 endocytosis in ABCA1 siRNA-transfected HUVECs at indicated time points. Fig. 7B shows comparison of Stx2 endocytosis levels, expressed as % of control (rolipram-untreated untransfected cells) at 20 min; treatment with 7.5 μg/ml rolipram reduced Stx2 endocytosis in untransfected cells by 89% and in control siRNA-transfected cells by 79%. In contrast, rolipram did not decrease Stx2 endocytosis in ABCA1 siRNA-transfected cells to a significant extent.

In order to examine the difference in intracellular location of Stx2 between rolipram-treated HUVECs and rolipram-untreated control cells, Oregon Green 488-labeled Stx2 was used instead of biotin-labeled Stx2 for endocytosis assay. After treatment with rolipram and apoA-I, HUVECs were incubated with 20 nM Oregon Green 488-labeled Stx2 on ice for 30 min. After incubation on ice, the cells were shifted to 37°C for 5 or 30 min, and then fixed at 37°C. In this assay, however, formation of tubules containing Stx2 was not clearly demonstrated in both cells as examined by ordinary fluorescence microscopy. At 10 min of incubation at 37°C, apparently higher degrees of fluorescence dye-labeled Stx2 were observed in untreated control cells than in rolipram-treated cells (Fig. 7C). After 30 min at 37°C, the majority of the fluorescence dye-labeled Stx2 were accumulated near to the nucleus in control cells, while the toxins were diffusely scattered in the cytoplasm of rolipram-treated cells.

Similar difference in intracellular location of the labeled Stx2 was observed between the rolipram-treated control siRNA-transfected cells and the rolipram-treated ABCA1 siRNA-transfected cells; the toxin was diffusely present in the cytoplasm of the former cells, while it was accumulated near to the nucleus in the latter cells (data not shown).

3. Discussion

This study demonstrates that rolipram at doses of higher than 5 μg/ml significantly reduced the sensitivity to Stx2 of TNF-α-stimulated HUVECs, and this effect may result from enhancement of ABCA1 expression. Rolipram was able to increase ABCA1 expression independently of apoA-I stimulation, although the presence of apoA-I augmented such effect of rolipram. Enhanced ABCA1 expression by rolipram led to the increase in apoA-I-mediated cholesterol efflux at TSF (non-rafts) of the treated cells. Although rolipram neither altered Gb3 levels in plasma membranes nor the Stx2 binding to the cells, it caused the shift of some Gb3 molecules into TSF without impairing their ability to bind to Stx2. Survival of rolipram-treated HUVECs after incubation with Stx2 was closely related to enhanced ABCA1 expression and also altered Gb3 behavior at plasma membranes; these changes are thought to account for the decrease in both Stx2 endocytosis and Stx2-induced apoptosis. Moreover, down-regulation of ABCA1 in HUVECs by transfection of ABCA1 siRNA prevented the rolipram-induced Gb3 shift in plasma membranes, and impaired the protective effect of rolipram on Stx2-exposed cells. These facts suggest that the expression level of ABCA1 may be one of critical determinants of the sensitivity to Stx2 in HUVECs.

A number of observations indicate that TNF-α is one of powerful stimulators for up-regulation of Gb3 in endothelial cells [15-19], and contributes to the pathologic process in HUS [4-6, 22], although serum levels of this cytokine in HUS patients are not always elevated. Actually, only TNF-α increased Gb3 content in plasma membranes. Rolipram did not affect Stx2 binding to TNF-α-stimulated HUVECs, while it decreased Stx2 endocytosis and/or intracellular trafficking. PDE inhibitors are known to increase intracellular cAMP concentrations, thereby suppressing the productions of inflammatory

cytokines [14, 23]. Furthermore, cAMP up-regulates ABCA1 expression [24], and exogenous apoA-I binding to cellular ABCA1 increases intracellular cAMP levels [25, 26]. Especially, the combination of rolipram (higher than 5 μg/ml) and 10 μg/ml apoA-I was found to up-regulate ABCA1 expression to a significant extent in accordance with elevation of intracellular cAMP levels. Since apoA-I alone did not enhance ABCA1 expression to a significant extent, the effect of the combination treatment on ABCA1 expression is possibly due to the increase in the amount of exogenous apoA-I binding to cellular ABCA1, the expression of which is up-regulated by rolipram. The increased binding may further enhance the synthesis of cAMP, thereby leading to a greater extent of ABCA1 expression. In parallel to the increase in ABCA1 expression, the sensitivity to Stx2 of rolipram-treated cells was significantly decreased as demonstrated by MTT assay, TUNEL analysis, caspase activity, and DNA fragmentation. Up-regulation of ABCA1 expression is therefore considered to decrease Stx2 cytotoxicity in HUVECs.

Rolipram shifted some Gb3 molecules from DRM into TSF without affecting their Stx2-binding ability. ABCA1 is shown to cause a change in overall lipid packing of the plasma membrane, including a marked redistribution of cholesterol and sphingomyelin from lipid rafts to non-rafts [27]. Such ability of ABCA1 may account for the shift of some Gb3 molecules induced by rolipram. Because different types of Gb3 pools exist in plasma membranes of HUVECs [21, 28], we hypothesize that rolipram-induced Gb3 shift occurs in different Gb3 species; some of Gb3 pools are sensitive to rolipram treatment while others are not. Interestingly, GM1 was not shifted into TSF by rolipram, despite the co-localization of GM1 and Gb3 in DRM. Because recent studies suggest a fundamental difference between Gb3- and GM1-containing rafts [29, 30], it can be also hypothesized that rolipram-induced alteration in Gb3 receptors may possibly occur in

different lipid rafts [30, 31].

It is shown that cholesterol depletion affects Stx internalization without reducing the level of Stx binding to the cells [12]. While MβCD binds directly to cholesterol, rolipram promoted only apoA-I-mediated cholesterol efflux from TSF where apoA-I preferentially acquired cholesterol [32, 33]. Rolipram did not affect Stx2 binding to treated cells; we thought that the capacity for Stx2 binding of Gb3 receptors shifted into TSF compensated for the decreased Stx2 binding in DRM. Nevertheless, rolipram significantly reduced Stx2 endocytosis in HUVECs, compared with untreated cells. In ABCA1 siRNA-transfected HUVECs, rolipram neither induced the shift of Gb3 receptors into TSF nor reduced Stx2 endocytosis; accordingly, the cells were sensitive to the toxin as much as rolipram-untreated untransfected cells. We therefore hypothesize that Stx2 bound to Gb3 receptors at TSF of rolipram-treated HUVECs is less efficiently endocytosed, leading to reduced levels of Stx2 cytotoxicity. In addition, fluorescence dye-labeled Stx2 was accumulated at the site adjacent to the nucleus (probably at the endoplasmic reticulum) in the cells sensitive to Stx2, implying that the toxin bound to Gb3 at DRM is effectively internalized, followed by the retrograde transport through the Golgi apparatus to the endoplasmic reticulum [21]. In contrast, rolipram enhanced the resistance to Stx2 of both untransfected HUVECs and control siRNA-transfected cells in association with reduced levels of Stx2 endocytosis. Different from intracellular location of Stx2 in Stx2-sensitive cells, the toxin diffusely existed in the cytoplasm of both rolipram-treated untransfected cells and rolipram-treated control siRNA-transfected cells. These facts strongly support our hypothesis that Gb3 receptors existing in non-rafts, capable of binding to Stx2, are involved in reduction of Stx2 endocytosis. In addition, transfection experiments suggest that such reduced levels of

the endocytosis and/or internalization of Stx2 by rolipram are due to the shift of Gb3 receptors in plasma membranes by enhancement of ABCA1 expression. In connection with this, ABCA1 has been shown to alter functions of the plasma membrane, such as endocytosis and phagocytosis [34-36]. Therefore, the different intracellular localization of Stx2 between Stx2-sensitive and Stx2-resistant cells suggests that ABCA1 expression must be required to reduce the endocytosis and/or intracellular trafficking of Gb3-bound Stx2 in vascular endothelial cells.

In connection with Stx endocytosis, Römer et al. [37] have shown that the Gb3-binding nontoxic Stx B induces endocytic plasma membrane invaginations, and tubule occurrence increases on energy depletion and inhibition of dynamin or actin functions. Scission of the tubular invaginations is preceded by cholesterol-dependent membrane reorganization, though Stx B subunits are able to induce the invagination after plasma membrane cholesterol is extracted [37, 38]. Furthermore, dynamin and cholesterol are shown to contribute to the scission of Stx B subunit-induced membrane tubules; either inhibition of dynamin by dynasore or cholesterol extraction by MßCD partially protects cells from Stx-induced cytotoxicity [38]. According to these facts, enhanced efflux of cholesterol by treatment with rolipram is supposed to increase the accumulation of Stx2 in membrane connected tubules, which is thought to contribute to cell protection from Stx cytotoxicity. However, we were unable to observe the apparent tubules containing Stx2 in HUVECs, which were treated either with a combination of 7.5 μg/ml rolipram and 10 μg/ml apoA-I or with apoA-I alone, at 10 and 30 min after being shifted to 37°C. Though we do not explain the reason for the failure to observe the tubules containing Stx2, we may have several speculations for this result. One possible explanation is that since tubules containing Stx2 can not be observed in

rolipram-untreated cells, tubules containing Stx2 may not be clearly identified by an ordinary fluorescence microscope. In addition, assuming that rolipram treatment does not induce accumulation of Stx2 in membrane connected tubules, levels of rolipram-promoted ABCA1-mediated cholesterol efflux might not be sufficient to inhibit the scission of Stx-induced tubular invaginations, unlike MßCD capable of inducing much higher levels of cholesterol extraction. Alternatively, since rolipram induces Gb3 shift from rafts into non-rafts, Stx-binding Gb3 molecules in non-rafts might be incapable of inducing tubular invaginations; this assumption is consistent with the idea that tubular B subunits of Stx are in a raft-like lipid environment [37]. Finally, it is in the least speculated that Stx-induced tubular invaginations may take place in endothelial cells less than in epithelial cells. At present, we can not give a well-grounded evidence for each of these speculations. Despite of the failure to demonstrate the tubules containing Stx2, we do not claim that rolipram decreases Stx2 cytotoxicity by the mechanism(s) different from inhibition of the scission of Stx2-induced tubular membrane invaginations. We must explore all the possibilities of these speculations in further study in order to determine whether or not rolipram-induced enhancement of ABCA1 expression inhibits the scission of Stx2-induced tubules.

Our data do not allow us to draw conclusions about whether the severity of clinical disease in STEC-infected patients correlates with their levels of ABCA1 expression and/or function. However, present results suggest that the expression level of ABCA1 protein might be one of critical determinants of Stx sensitivity levels in vascular endothelial cells. Further investigation using ABCA1-deficient mice is necessary to determine the actual involvement of ABCA1 in HUS development of STEC-infected

patients.

4. Conclusion

In this study, we show that up-regulation of ABCA1 expression by rolipram, a PDE 4 inhibitor, decreased the sensitivity to Stx2 of HUVECs by reducing the endocytosis and/or intracellular trafficking of Gb3-bound Stx2. The protective effect of rolipram was dependent on enhancement of ABCA1 expression, which causes an alteration of Gb3 behavior at the plasma membrane. Knockdown of ABCA1 abrogated the protective effect of rolipram on Stx2-exposed HUVECs; rolipram did not induce the alteration for Gb3 behavior in plasma membranes and decrease Stx2 endocytosis. Our present results therefore suggest that the expression level of ABCA1 protein must be one of critical determinants of Stx sensitivity levels in vascular endothelial cells.

5. Materials and Methods

5.1. Cells, Shiga toxin (Stx), and reagents

HUVECs were purchased from Sankou Junyaku Co., Ltd. (Tokyo, Japan). Endothelial basal medium (EBM) (Medium 131) and microvascular growth supplement (MVGS) were obtained from Cascade Biologics, Inc. (Portland, OR). Rolipram (racemate of 4-[3-cyclopentyloxy-4-methoxyphenyl-2-pyrrolidone]) (PDE4 inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO), and fresh solution of the drug was prepared by use of dimethyl sulfoxide (DMSO) diluted 1:1 000 with culture medium [14]. Methyl-ß-cyclodextrin (MßCD), recombinant human TNF-α, apoA-I, cholesterol, **mercaptoethanesulfonic acid (MESNa), iodoacetamide,** and Hanks' balanced salts solution (HBSS) were all from Sigma-Aldrich. Recombinant Stx2 was prepared as described before [18]: its cytotoxic potency was 4×10^6 50% cytotoxic doses (CD50)/μg of protein for 24 h as tested in Vero cells. EZ-Link Sulfo-NHS-SS-biotin (sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate) and Halt™ Phosphatase Inhibitor Cocktail and Halt™ Protease Inhibitor Cocktail were purchased from Pierce Biotechnology (Rockford, IL). Oregon Green 488 and FluoReporter protein labeling kit (F-6153) were obtained from Molecular Probes (Eugene, OR). Lipoprotein-deficient bovine serum (LP-BS) and BSA were obtained from Biomedical Technologies, Inc., (Stoughton, MA). Culture plates and dishes were obtained from BD Falcon™ (San Jose, CA), and Lab-Tek chamber slides from Nunc Inc., (Naperville, Ill). ECL (enhanced chemiluminescence) Western Blotting Detection Reagents, Hybond-P (PVDF membranes), streptavidin-Sepharose™ (average particle size: 34 μm, binding capacity: > 300 nmol biotin/ml medium), [1, 2-^{3}H] cholesterol (48 Ci/mmol), Na^{125}I (100 mCi/ml) and cAMP assay kit (RPA 509) were all from GE Healthcare UK Ltd.

(Buckinghamshire, England). BioMax XAR Film for radioautography and RC-DC Protein Assay Kit II were obtained from Eastman Kodak (Rochester, NY), and Bio-Rad Laboratories (Hercules, CA), respectively. Purified Gb3 was purchased from Accurate Chemicals and Scientific Corp. (Westbury, NY), and pre-coated high-performance thin-layer chromatography (HPTLC)-silica gel $60F_{254}$ plates (100 × 200 mm, Art. 13728) was from Merck KGaA (Darmstadt, Germany). ProNectin® F was from Sanyo Chemical Industries (Kyoto, Japan). Horseradish peroxidase (HRP)-conjugated streptavidin was from Cell Sciences, Inc. (Canton, MA). In Situ Cell Death Detection kit and apoptotic DNA ladder kit were from Roche Applied Science (Tokyo). Colorimetric cell proliferation assay kit I for MTT was from Boehringer Mannheim GmbH (Ingelheim, Germany). Other chemical reagents were purchased from Wako Pure Chemicals (Kyoto), unless otherwise stated.

5.2. Antibodies

Antibodies for immunoblotting were purchased from the indicated commercial sources as follows. Anti-caveolin monoclonal antibody (MAb) (clone 2234, Mouse IgG2a, used at 1:5 000), anti-flotillin-1 MAb (clone 18, Mouse IgG1, used at 1:500), and anti-transferrin receptor (TfR) MAb (clone 2/Transferrin, mouse IgG1, used at 1:2 000) were obtained from BD Transduction Laboratories (San Diego, CA), anti-CD77/Gb3 MAb (clone 38-13, rat IgM, used at 1:4) from Coulter-Immunotech (Marseille, France), rabbit polyclonal antibody against ABCA1 (rabbit IgG, used at 1:500) from Novus Biological Inc. (Littleton, CO), mouse polyclonal antibody against β-actin (used at 1:10,000) from R & D Systems (Minneapolis, MN), anti-Stx 2 MAb (11E10, mouse IgG1, used at 1:10) from HyCult Biotechnology (Uden, The

Netherlands), HRP-conjugated cholera toxin subunit B (CTxB-HRP) (1:500) from List Biological Lab. Inc. (Campbell, CA), HRP-conjugated goat anti-mouse IgG (used at 1:2 500) and goat anti-rat IgM antibodies (used at 1:5 000) from AbD Serotec (Raleigh, NC), and HRP-conjugated goat anti-rabbit IgG (used at 15 000) antibody from Bethyl Laboratories Inc. (Montgomery, TX).

5.3. Culture of HUVECs

HUVECs were grown in complete medium (CM) (EBM containing MVGS) at 37°C in humidified 5% CO_2 as described before [18]. All culture plates and dishes were coated with ProNectin® F before use. Cells were used at passage three when > 97% of cells were positive for the endothelial-specific marker, von Willebrand factor, as determined by an indirect immunofluorescence staining procedure. For each assay, cells were seeded in wells of six-well (5×10^4 in 2 ml CM/well), 12-well (2.5×10^3 in 1.5 ml CM/well), 24-well (1.5×10^3 in 1 ml CM/well) or 96-well (10^3 in 100 µl CM/well) culture plates. When cells were grown to an 80% confluent monolayer, medium was replaced by EBM containing 10% LP-BS (EBM/LP-BS) to prevent further growth. HBSS supplemented with 10 mM HEPES (pH 7.4) was used for washing of cell monolayers, either with or without 0.25% (wt/vol) BSA (HBSS/BSA), before subsequent treatment. For cell treatment, EBM/LP-BS was used to dilute treating agents.

HUVECs were stimulated with 100 U/ml TNF-α in EBM/LP-BS for 24 h before treatment with rolipram. Rolipram solution was added to culture of HUVECs at a 5% volume of medium (final concentration range: 1-10 µg/ml), and control cells were treated with the same volume of DMSO diluted 1:1 000 with EBM/LP-BS.

5.4. Isolation of plasma membranes

Plasma membranes were isolated and fractionated by equilibrium density-gradient centrifugation according to the method of Ilangumaran *et al.* [37] with slight modifications. Briefly, HUVECs were washed in cold HBSS twice and once in TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA). The cell pellet was resuspended at the ratio of 5×10^7 cells /ml in TKM buffer containing 73% (wt/vol) sucrose, 1:100 dilutions of Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail, and 0.05% (wt/vol) sodium azide. The cell suspension was homogenized using a Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a handy-sonicator (three 20-s bursts; Tomyseiko, Tokyo). The nuclei and the cell debris were removed by centrifugation for 20 min at 3,200 × g. A portion of the **post-nuclear** supernatant (used as a plasma membrane fraction in some experiments), equivalent to 2.5×10^7 cells, were incubated with 1% (vol/vol) Triton X-100 for 30 min on ice. Sucrose concentration was adjusted to 40% (wt/vol) in the final volume of 1.0 ml and the homogenates were placed at the bottom of SW41 tubes (Beckman Instruments, Nyon, Switzerland). They were overlaid with 6.0 ml of 36% (wt/vol) sucrose and 3.0 ml of 5% (wt/vol) sucrose in TKM buffer, and centrifuged at 250 000 × g for 18 h at 4°C in an L-70 Ultracentrifuge (Beckman Instruments). One-ml fractions were collected from the top, numbered 1-12, and stored at -20°C. After centrifugation, the densities of all sucrose fractions were measured using a refractometer (Master-α, ATAGO Inc., Tokyo). In this study, DRM with lower buoyant densities (1.055-1.115 g/cm^3) was collected from the 5-36% sucrose interface (fractions 3 - 5) where an apparently visible band was formed. Fractions 9 - 11 were collected and used as Triton X-soluble fraction (TSF). **In this study, the plasma membrane was not enriched, and the**

pellet at the bottom of the gradient was not re-extracted. The amount of protein per fraction was determined using RC-DC Protein Assay Kit II.

5.5. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot

Various cell surface molecules in the density gradient fractions separated by SDS-PAGE were evaluated by Western blotting as previously described [38]. Briefly, 30 μl of the gradient fractions were directly solubilized in 3 × reducing sample buffer [150 mM Tris-HCl (pH 6.8), 6% (wt/vol) SDS, 30% (vol/vol) glycerol, 0.01% (wt/vol) bromophenol blue, 15% (vol/vol) β-mercaptoethanol]; this mixture was boiled for 5 min, and quenched on ice for 1 min. Proteins were was loaded on to each lane of SDS-12% polyacrylamide gel and separated using a minigel apparatus (Bio-Rad). Fractionated proteins were transferred to Hybond-P membranes by using a semidry transfer apparatus (ATTO Cooperation, Tokyo). The membranes were blocked in 3% (wt/vol) skim milk/0.25% (wt/vol) BSA in 20 mM Tris-HCl-150 mM NaCl-5 mM $MgCl_2$-0.15 mM $CaCl_2$ (pH 7.4) (blocking solution), incubated overnight with primary antibodies at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 45 min at room temperature. To detect ganglioside GM1, blots were incubated with CTxB-HRP. Specific interactions were revealed using ECL system following the supplier's instructions. Densitometry analysis was performed using Gel analyst system (ICONIX, Paris, France) with NIH Image software (version 1.62). All bands shown migrated at the expected size, as determined by comparison with molecular weight standards (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blot assay of the plasma membrane isolated from transfected HUVECs, total protein (50 μg) was loaded on the gel after solubilized in 3 × reducing sample buffer. The expression of ABCA1 and caveolin-1

was evaluated using specific antibodies, and compared with the expression of β-actin.

5.6. Determination of ABCA1 expression

5.6.1. Detection of ABCA1 mRNA by RT-PCR

Total RNA was extracted using the TRIzol reagent from HUVECs. One μg of total RNA was mixed with human ABCA1-specific or GAPDH-specific primers; one-step RT-PCR was performed using SuperScript III One-Step RT-PCR as described before [38]. Sequences of the specific primers for ABCA1 were 5'-GACATCCTGAAGCCA ATCCTG-3' (forward), and 5'-CCTTGTGGCTGGAGTGTCAGG T-3' (reverse) [20]. Those for GAPDH were 5'-GAAGGTGAAGGTCGGATC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse) [39]. Amplification was performed by initially denaturing DNA at 95°C for 3 min. Thereafter, denaturing was at 95°C for 75 s, annealing at 54.6°C for 75 s, and extension at 72°C for 55 s for a total of 31 cycles with a final extension period of five min. PCR products were resolved by electrophoresis on 2.0% agarose gels and visualized by ethidium bromide staining.

5.6.2. Cell-surface ABCA1 biotinylation

Subconfluent monolayers were surface-biotinylated with 1 mg/ml EZ-Link Sulfo-NHS-SS-biotin for 30 min on ice [40]. The labeled cells were washed with 50 mM Tris-HCl, pH 7.4, containing 100 mM glycine for 10 min on ice, then lysed with 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% SDS, 1:100 dilutions of protease/phosphatase inhibitors, and centrifuged at 14,000 × g for 10 min at room temperature. The resulting supernatant (100 μl) was precipitated overnight at 4 °C with 70 μl of

streptavidin-Sepharose. The gel was pelleted, and treated at 37°C for 30 min in 50 μl of 3 × reducing Laemmli sample buffer. The volume of 30 μl was run on an 8% SDS-PAGE. Fractionated proteins were identified by Western blotting using anti-ABCA1 antibody or anti-flotillin-1 MAb, and developed with ECL system for measurement of intensity of each band. Total proteins were stained with Coomassie Brilliant Blue.

5.7. Small interfering RNA (siRNA) transfection

To modulate ABCA1 expression in HUVECs, the cells were transfected with siRNA targeting to ABCA1 (SI03025190, Qiagen) or negative control siRNA (SI03650318, Qiagen) according to the protocol provided by the manufacturer. Briefly, 5 x 10^4 cells were seeded in a six well tissue culture plate, and grown to 80% confluence in CM. Both of ABCA1 and negative control siRNA (50 nM) were diluted in 25 μl of serum-free EBM, respectively. The siRNA for ABCA1 (GGGACTTAGTGGGACGAAATCTCTT) and negative control siRNA were mixed with RNAiFect (Qiagen), respectively, to allow formation of a transfection complex. Cells were washed with HBSS/BSA, and incubated with respective siRNA complexes (1 nM of siRNA) in EBM/LP-BS. After incubation at 37°C for 6 h, the medium was replaced by CM containing 30 μg/ml cholesterol, and grown for an additional 36 h before TNF-α stimulation. The efficiency of the silencing was monitored by RT-PCR and Western blotting. Reduction in target protein expression after siRNA incubations averaged 85.3%.

5.8. Cholesterol efflux assay

HUVEC monolayers in 24-well plates were labeled with 0.5 μCi/ml [1, 2-^{3}H] cholesterol in EBM/LP-BS as described previously [41]. After 24 h, labeled cells were washed twice with HBSS/BSA. Cholesterol-enrichment was performed by 24 h-incubation with 30 μg/ml cholesterol in EBM/LP-BS. After washing with HBSS/BSA, rolipram (5-10 μg/ml) in 1 ml of EBM/LP-BS with or without 10 μg/ml apoA-I was added for 24 h. As controls, labeled cells were incubated with 10 μg/ml apoA-I alone for 24 h or with 7.5 mM MßCD alone for 30 min after cholesterol enrichment. For transfected cells, 10% (wt/vol) BSA was used as a control for apoA-I. After incubation, the medium was aspirated and centrifuged to remove any dissociated cells, and cells in wells were lyzed in 1N NaOH. Aliquots of medium and cell lysates were assayed by liquid scintillation counting. Results represent radioactivity in the medium as a percentage of the total radioactivity (medium plus cell lysates) [32]. Each assay was performed in triplicate. The same experiments were repeated three times.

For determination of the level of [^{3}H] cholesterol in the plasma membrane, HUVEC monolayers were cultured in 4-well plates before labeling with 0.5 μCi/ml [1, 2-^{3}H] cholesterol and subsequent cholesterol enrichment. After stimulation with 10 μg/ml apoA-I for 24 h, DRM (pooled fractions 3 - 5) and TSF (pooled fractions 9 - 11) were prepared from the plasma membrane as already described. Quantity of [^{3}H] cholesterol in these two preparations was determined by liquid-scintillation counting. Cholesterol levels were expressed as cam per well. Each assay was performed in triplicate. The same experiments were repeated twice.

5.9. Gb3 content and Stx2 binding assay

Neutral glycolipids were isolated from the plasma membrane (equivalent to 5 x 10^6

HUVECs) as previously described [18]. Briefly, neutral glycolipids separated from gangliosides by DEAE-Sephadex A-25 (GE Healthcare) chromatography were dried, dissolved in chloroform-methanol (2:1, vol/vol), and 25 μg dry weight of each sample was separated on TLC plates with chloroform/methanol/water (65:25:4, vol/vol/vol). Different concentrations of purified Gb3 were also separated on the same plate. For comparison of Gb3 content, TLC immunoblot was performed as described previously [18]. Briefly, TLC plates were immersed in 0.5% (vol/vol) polyisobutylmethacrylate in acetone, air dried, sprayed with 20 mM Tris-HCl, pH 7.4, supplemented with 150 mM NaCl, 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Hybond-P membranes were placed on the TLC plate and then pressed at 0.07 Pa at 180°C in TLC thermal blotter (ATTO). After drying, the membranes were immersed in methanol for 1 min, and then soaked in the blocking solution for 30 min at room temperature. Membranes were subjected to Western blot analysis using an anti-Gb3 MAb and ECL system [18]. The amount of Gb3 in a test sample was calculated based on the standard curve of the intensity made by using different concentrations of standard Gb3, and values were expressed as nmol/10^6 cells. The assay was repeated twice; each assay was performed in triplicate.

For the Stx 2 overlay assay with sucrose gradient fractions, a volume of 900 μl (per each fraction) from pooled sucrose gradient fractions was dot-blotted on Hybond-P membranes, and the membranes were blocked in the blocking solution overnight at 37°C. After three washes in 20 mM Tris-buffered saline (pH 7.4), the membranes were incubated for 1 h with 5 nM $Na^{125}I$–labeled Stx2 at room temperature with shaking. Binding of the toxin was revealed by autoradiography using BioMax imaging films [18, 44].

The assay of Stx 2-binding to HUVECs was performed according to a previously

described method [14]. Briefly, subconfluent cell monolayers in 100 μl of EBM/LP-BS in 96-well plates were treated for 24 h with rolipram (0 - 10 μg/ml) and 10 μg/ml apoA-I. After washing with cold HBSS/BSA, monolayers were incubated with 5 nM $Na^{125}I$–labeled Stx2 in 100 μl of EBM/LP-BS for 1 h at 4°C. After incubation, monolayers were washed three times with cold HBSS/BSA to remove unbound Stx2, and solubilized in 100 μl/well of 1 N NaOH. Radioactivity in cell lysates was measured by γ-counting. The level of specific binding was determined by subtracting counts obtained in the presence of 100-fold excess unlabeled toxin from those obtained in the presence of ^{125}I–labeled -toxin alone.

5.10. Assay for internalized toxin

Endocytosis was performed as described previously [37, 38] using biotin-labeled Stx 2. Biotinylation of Stx 2 was performed according to the supplier's instructions using EZ-Link Sulfo-NHS-SS-Biotin. Approximately 86.3 ± 5.4% of the label was incorporated into Stx 2. Subconfluent monolayers of HUVECs in wells of 24-well culture plates, which were stimulated with 100 U/ml TNF-α for 24 h, were treated for 24 h with 7.5 μg/ml rolipram and 10 μg/ml apoA-I or with 10 μg/ml apoA-I alone in 1 ml of EBM/LP-BS per well. After 24 h of incubation, medium was removed and monolayers were washed twice with cold HBSS/BSA. Immediately, 10 nM biotin-labeled Stx2 in 1 ml of cold EBM/LP-BS was added to wells and incubated on ice for 30 min. The cells were shifted to 37 °C, and incubated for 30 min. At indicated time points, the cells were washed twice with cold washing buffer (0.14 M NaCl, 2 mM $CaCl_2$, and 20 mM HEPES, pH 8.6) to remove unbound toxin. To remove the SS-linked biotin on cell surface-bound protein, half of the cells were treated with 100 mM MESNa

in the same buffer containing 2 mg/ml BSA for 20 min at 0°C. After washing, excess MESNa was quenched with 150 mM iodoacetamide for 20 min. The other half was incubated with BSA-containing buffer alone and similarly treated with iodoacetamide. Quntification of Stx2 was performed as described before [31]. Both cells were washed twice with washing buffer, and lysed in 150 μl of blocking buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl, 0.2% BSA, 0.1% SDS, and 1% Triton X-100). After this lysate was aspirated, each well was washed with 50 μl of the blocking buffer. The first lysates and the second washing solution were combined, and 100 μl of the combined solution was transferred into 96-well plates pre-coated with anti-Stx2 MAb. Biotinylated Stx2 was visualized with HRP-conjugated streptavidin and *o*-phenyldiamine dihydrochloride (OPD). Addition of 3 M HCl stopped the reaction, and optical density (OD) was measured at 492 nm. OD from cells treated with MESNa represents the amount of internalized toxin, whereas OD from untreated cells represents the total amount of toxin associated with the cells (bound plus internalized). Endocytosis of Stx2 was reported as internalized toxin in percentage of total cell-associated toxin.

For microscopic observation of intracellular Stx2, Stx2 was labeled with Oregon Green 488, using a FluoReporter protein labeling kit as previously described [23]. HUVECs (5×10^4) in chambers of Lab-Tek chamber slides (4-chamber type) in a volume of 400 μl of EBM/LP-BS were treated with 7.5 μg/ml rolipram and 10 μg/ml apoA-I or 10 μg/ml apoA-I alone for 24 h. After washing with HBSS/BSA, cells were incubated with 20 nM Oregon Green-labeled Stx2 in 400 μl of EBM/LP-BS on ice for 30 min. After washing with cold HBSS/BSA, the cells were shifted to 37°C. After 10 or 30 min of incubation at 37°C, the cells were fixed at 37°C with 3% (vol/vol)

paraformaldehyde containing 2% (wt/vol) sucrose for 30 min, and then mounted. Slides were analyzed by fluorescence microscopy (Olympus BX 50, Olympus, Tokyo).

5.11. Intracellular cAMP measurement

HUVECs were lysed in 500 µl of 50 mM sodium acetate, and clear supernatants were obtained by centrifugation. Concentrations of cAMP in each clear supernatant were determined using a cAMP assay kit following the manufacturer's instructions. Results were expressed in fmol per µg cell protein as determined by comparison with a standard curve. Each assay was performed in quadruplicate. The same experiments were repeated twice.

5.12. Other assays

For cytotoxic assays, subconfluent monolayers of HUVECs in 96-well culture plates (for cytotoxic assay) or in chambers of Lab-Tek chamber slides (for apoptosis assay) were treated with rolipram (1-10 µg/ml) for 24 h. After washing with HBSS/BSA, cells were incubated for 24 h with 10 nM Stx2 in 100 µl (96-well culture plates) or 500 µl (Lab-Tek chamber slides) of EBM/LP-BS containing 10 µg/ml apoA-I. Cell viability was determined by MTT assay using a Colorimetric Cell Proliferation Assay Kit I as previously reported [45]. Absorbance at 540 nm was measured in an ELISA plate reader (Bio-Rad). Apoptosis of HUVECs was assessed by TUNEL assay using the In Situ Cell Death Detection kit according to the manufacturer's instructions. Consecutive oil immersion (100 × objective) fields were counted (a minimum of 500 cells), and the apoptotic index was calculated as the percentage of stained cells.

For DNA fragmentation assay, DNA was extracted from subconfluent monolayers of

HUVECs in 12-well culture plates after indicated treatments. Extracted DNA was treated with DNase-free RNase for 30 min, and DNA concentrations were measured. Equal amounts of DNA (2 μg) were loaded on 1.8% agarose gels. After electrophoresis, gels were visualized by ethidium bromide staining.

Activities of caspase-3 and -8 were determined using Caspase Colorimetric Protease Assay kits according to the manufacturer's instructions. In brief, HUVECs in 12-well culture plates were treated with rolipram (0-10 μg/ml) for 24 h. After washing with HBSS/BSA, cells were incubated for 24 h with 10 nM Stx2 in 1 ml of EBM/LP-BS containing 10 μg/ml apoA-I. After 3 washes with HBSS, cells were extracted with Cell Lysis Buffer. Concurrently, samples for a negative control were extracted from the cells without exposure to Stx2. A standard curve using the absorbance of *p*-nitroanilide standards was constructed, and then the specific activities in each sample were calculated according to the manufacturer's protocol.

5.13. Statistics

Data were obtained from two or three independent experiments with triplicate or quadruplicate samples in each experiment. Statistical analyses were performed using a two-tailed Student's *t*-test or single-factor ANOVA. A P value of < 0.05 was considered to be significant. A Bonferroni correction was utilized to determine statistical significance when multiple comparisons were made.

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We declare that no conflict of interest exists.

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Figure legends

Figure 1. Distribution of cell-surface molecules of TNF-α-stimulated HUVECs

Subconfluent monolayers of HUVECs in 6-well culture plates were stimulated with or without 100 U/ml TNF-α in EBM/LP-BS for 24 h. Cell extracts were fractionated using the Triton X-based method followed by centrifugation in a discontinuous sucrose gradient as described in the Materials and Methods. Equal volumes of the fractions collected from the gradient were separated by SDS-PAGE, and analyzed by Western blot using antibodies specific for the indicated proteins. Bound antibodies were visualized by ECL system. GM1 was detected by incubation with CTxB-HRP before visualization with ECL system. Representative results from three separate experiments with similar results are shown. A) before TNF-α stimulation, B) after TNF-α stimulation.

Figure 2. Effect of rolipram on apoA-I-mediated cholesterol efflux

Subconfluent monolayers in 24-well or 4-well culture plates were labeled with 0.5 μCi/mL [^{3}H] cholesterol for 24 h, and enriched with 30 μg/mL cholesterol. A) Determination of [^{3}H] cholesterol efflux from HUVECs. Labeled monolayers in 24-well culture plates were treated with rolipram (0 -10 μg/ml) for 24 h, either in the presence or absence of 10 μg/ml apoA-I. For controls, labeled monolayers were treated with 7.5 mM MβCD alone for 30 min or 10 μg/ml apoA-I alone after cholesterol enrichment. The amount of [^{3}H] cholesterol in medium and cell lysates was quantified by liquid-scintillation counting. Cholesterol detected in the medium is expressed as % of total [^{3}H] cholesterol incorporated in the cell. Results are obtained from three independent experiments, and each bar represents means ± SD of nine wells. * $p < 0.05$,

** $p < 0.01$, vs. apoA-I alone. *NS*, not significant. B) Determination of [^{3}H] cholesterol levels in DRM and TSF. DRM and TSF were prepared from the plasma membrane of [^{3}H] cholesterol-labeled HUVECs in 4-well culture plates after treatment with rolipram (0 -10 μg/ml) and 10 μg/ml apoA-I. Levels of [^{3}H] cholesterol in both fractions were measured by liquid-scintillation counting, and cholesterol levels in each fraction were expressed as cpm per well. Results are obtained from two independent experiments, and each bar represents means ± SD of six wells. * $p < 0.05$, ** $p < 0.025$, *** $p < 0.01$ vs. TSF prepared from rolipram-untreated cells.

Figure 3. Effect of rolipram on ABCA1 expression and intracellular cAMP concentration

Subconfluent monolayers of HUVECs in 24-well (for A, C and E) or 12-well (for B and D) culture plates were incubated for 24 h with 7.5 μg/ml rolipram alone, 10 μg/ml apoA-I alone or the combination of rolipram and apoA-I. A) RT-PCR assay. Total RNA was extracted from HUVECs after respective treatments, and 1 μg of RNA was mixed with human ABCA1-specific or GAPDH-specific primers. One-step RT-PCR was performed using SuperScript III One-Step RT-PCR. PCR products were resolved by electrophoresis on 2.0% agarose gels and visualized by ethidium bromide staining. B) Western blot assay. TSF was separated from the plasma membrane of HUVECs after respective treatments. Thirty μg of TSF protein (pooled fractions 9-11) was analyzed by SDS-PAGE and identified by Western blot with anti-ABCA1 antibody. Blots were developed with ECL system. C) Comparison of biotin-labeled surface ABCA1 protein. Surface proteins of HUVECs were labeled with Sulfo-NHS-SS-Biotin after respective treatments. Biotin-labeled ABCA1 and flotillin-1 were precipitated from cell lysates

with streptavidin-Sepharose. These two proteins in the precipitates were fractionated by SDS-PAGE and identified by Western blot with specific antibodies. Blots were developed with ECL system, and total proteins were stained with Coomassie Brilliant Blue. In *A*, *B* and *C*, representative results from three separate experiments with similar results are shown. D) Quantification of biotin-labeled ABCA1. Surface proteins of HUVECs were labeled with Sulfo-NHS-SS-Biotin after 24 h-treatment with rolipram (0 - 10 μg/ml) in the presence of 10 μg/ml apoA-I. The biotin-labeled ABCA1 proteins were precipitated from the lysates of HUVECs, and were analyzed by SDS-PAGE and Western blot using anti-ABCA1 antibody. Density of bound antibodies visualized by ECL system was measured. A densitometry unit of the band obtained from the apoA-I-treated but rolipram-untreated HUVECs (control cells) was arbitrary defined as 1.0. * $p < 0.05$, ** $p < 0.01$ vs. control cells. E) Measurement of intracellular cAMP concentrations. HUVECs were incubated with rolipram (0-10 μg/ml) for 24 h in the presence (□) or absence (’) of 10 μg/ml apoA-I. Cells were lyzed in 500 μl of 50 mM sodium acetate. Clear supernatants of cell lysates were obtained by centrifugation, and cAMP levels in each sample were determined using a cAMP assay kit. * $p < 0.05$, ** $p < 0.025$, *** $p < 0.01$ vs. rolipram-untreated cells. At 10 μg/ml of rolipram, the presence of apoA-I induced higher levels of cAMP, compared with the absence of apoA-I ($p < 0.05$). In *D* and *E*, results are obtained from three independent experiments, and each bar represents means ± SD of nine wells.

Figure 4. Induction of the cleavage of Gb3 and the redistribution of Gb3 at the plasma membrane by rolipram

A) Determination of Gb3 content in the membrane. Subconfluent monolayers of

HUVECs in 6-well culture plates were treated for 24 h with 7.5 μg/ml rolipram alone, 10 μg/ml apoA-I alone, or combination (rolipram and apoA-I). HUVECs receiving no treatments and those receiving only TNF-α stimulation were included in this assay. Total neutral glycolipids were extracted from HUVECs and separated from gangliosides by DEAE-chromatography. Separated samples (25 μg dry weight/50 μl) were fractionated on TLC with different concentrations of Gb3 standards. Gb3 was identified by TLC-immunoblot assay using an anti-Gb3 MAb. Bound antibodies were revealed by ECL system, and quantified by densitometry analysis. The amount of Gb3 in test samples was calculated based on the standard curves made by using different concentrations of standard Gb3, and expressed as nmol/10^6 cells. Results are obtained from two independent experiments, and each bar represents means ± SD of four cell samples. * p <0.0075 vs. untreated cells. *NS*, not significant between cell groups of different treatment. B) Western blot assay for membrane molecules. Subconfluent monolayers of HUVECs in 6-well culture plates were incubated for 24 h with 7.5 μg/ml rolipram and 10 μg/ml apoA-I. Cells were fractionated using the Triton X-based method followed by centrifugation in a discontinuous sucrose gradient. Membrane fractions were analyzed by Western blot as described in the legend for Fig. 1. C) Stx2-overlay assay of separated membrane fractions. Membrane fractions (900 μl/fraction) were dot-blotted on Hybond-P membranes. After blocking, the membranes were overlaid with 5 nM ^{125}I-labeled Stx2 for 1 h, and the binding of Stx2 was revealed by autoradiography. In *B* and *C*, representative results from three separate experiments with similar results are shown. D) Stx2 binding to HUVECs. Subconfluent monolayers of HUVEC in 96-well culture plates were treated for 24 h with rolipram (0-10 μg/ml) in the presence of 10 μg/ml apoA-I. After incubation, monolayers were washed with cold

HBSS/BSA, and then incubated with 5 nM $Na^{125}I$-labeled Stx2 in 100 μl of EBM/LP-BS for 1 h at 4°C. After washing three times with cold HBSS/BSA to remove unbound Stx2, monolayers were solubilized in 100 μl/well of 1 N NaOH. Radioactivity in cell lysates was measured by γ-counting. Results were obtained from three independent experiments. The plotted values are the mean ± SD of 9 wells. *NS*, not significant between groups of cells treated with different doses of rolipram.

Figure 5. Decrease in Stx2 sensitivity of HUVECs by rolipram

Subconfluent monolayers of HUVECs in 96-well culture plates (for MTT assay), in chambers of Lab-Tek chamber slides (for apoptosis assay) or in 12-well culture plates (for caspase activity assay and DNA extraction) were treated with rolipram (0-10 μg/ml) for 24 h. After washing with HBSS, monolayers were incubated for 24 h with 10 nM Stx2 in EBM/LP-BS containing 10 μg/ml apoA-I. A) Cell viability was measured by the MTT assay using the Colorimetric Cell Proliferation Assay Kit I, and expressed as absorbance at 540 nm. Values of absorbance at 540 nm after 24 h-incubation with medium plus 10 μg/ml apoA-I ranged from 1.14 to 1.28 (↕). B) Apoptotic cells were determined using the In Situ Cell Death Detection Kit. Results are expressed as (the number of TUNEL-positive cells / total cells examined) × 100 (%). C) The activity of caspase-3 (□) and -8 (’) was determined using a Caspase Colorimetric Protease Assay Kit. The specific activities of each sample were calculated according to the manufacturer's protocol. In *A*, *B*, and *C*, results are obtained from three separate experiments, and values represent the mean ± SD of 12 wells (in *A*), 6 chambers (in *B*) or 9 wells (in *C*). * $p < 0.05$, ** $p < 0.01$ vs. rolipram-untreated cells. D) Fragmentation patterns of DNA. Rolipram (5 or 10 μg/ml) was added to culture of HUVECs in 12-well

culture plates for 24 h prior to incubation with 10 nM Stx2, while 20 μM Z-IETD-FMK was added to the culture throughout incubation with Stx2. Subconfluent monolayers incubated with TNF-α alone, Stx2 alone and a combination of these two agents were also included in this assay. Ten μg/ml apoA-I was added to all cultures during 24 h incubation. Extracted DNA (2 μg) was fractionated by 1.8% agarose gel electrophoresis, and visualized by ethidium bromide staining. Representative results from three separate experiments with similar results are shown.

Figure 6. The effect of ABCA1 knockdown on Stx2 sensitivity of HUVECs

Untransfected HUVECs and transfected cells in 6-well (for preparation of the plasma membrane), 24-well (for cholesterol efflux assay) or 96-well (for MTT assay) culture plates were grown to subconfluence in CM containing 30 μg/ml cholesterol for 36 h before stimulation with 100 U/ml TNF-α. A) Western blot analysis for ABCA1 and caveolin-1. The plasma membrane was prepared from cell lysates of HUVECs after 24 h stimulation with TNF-α, and separated by SDS-PAGE and Western blot using MAbs to ABCA1, caveolin-1 and polyclonal antibodies to β-actin. Representative results from three separate experiments with similar results are shown. B) Expression levels of ABCA1 and caveolin-1 proteins. Protein expression levels on Western blots were determined by densitometry assay; density of bound antibodies was measured after visualization by ECL system. The expression level of each protein in ABCA1 siRNA -transfected cells (□) is expressed as % of expression levels in control siRNA -transfected cells (’). * $p < 0.005$ vs. control siRNA-transfected cells. C) Determination of [^{3}H] cholesterol efflux from whole cells. After 24 h of stimulation with TNF-α, HUVECs were labeled with 0.5 μCi/mL [^{3}H] cholesterol for 24 h, and enriched with 30

μg/ml cholesterol as described in the legend for Fig. 2. Labeled cells were treated for 24 h with 7.5 μg/ml rolipram in the presence of 10 μg/ml apoA-I or 10% (wt/vol) BSA. The amount of [^{3}H] cholesterol in medium and cell extract was quantified by liquid-scintillation counting. Cholesterol detected in the medium is expressed as % of total [^{3}H] cholesterol incorporated in ABCA1 siRNA-transfected cells (□) and control siRNA-transfected cells (’). * $p < 0.05$ vs. control siRNA-transfected cells. D) Western blot assay of membrane molecules of ABCA1 siRNA-transfected cells. The plasma membrane of TNF-α-stimulated cells was fractionated using the Triton X-based method followed by centrifugation in a discontinuous sucrose gradient, and analyzed by Western blot as described in the legend for Fig. 1. Representative results from three separate experiments with similar results are shown. E) Cell viability of transfected cells exposed to Stx2. After 24 h of stimulation with TNF-α, HUVECs transfected with ABCA1 siRNA (□) and control siRNA (■) were treated for 24 h with rolipram (0 - 10 μg/ml) before exposure to Stx2. Cell viability was measured by the MTT assay after 24 h of incubation with 10 nM Stx2 in the presence of 10 μg/ml apoA-I. Results are expressed as absorbance at 540 nm. Values of absorbance at 540 nm after 24 h-incubation with medium plus 10 μg/ml apoA-I (↕) ranged from 1.02 to 1.22 for control siRNA-transfected cells (■) and from 1.05 to 1.20 for ABCA1 siRNA -transfected cells (□), respectively. * $p < 0.05$, ** $p < 0.01$ vs. rolipram-untreated cells.

In *B*, *C*, and *E*, results were obtained from three individual experiments. Values represent the mean ± SD of triplicate cell samples (in *B*), six wells (in *C*) or nine wells (in *E*).

Figure 7. Decreased endocytosis of Stx2 by rolipram

A) Subconfluent monolayers of HUVECs in 24-well culture plates, which were stimulated with 100 U/ml TNF-α for 24 h, were treated for 24 h with 7.5 μg/ml rolipram and 10 μg/ml apoA-I or with 10 μg/ml apoA-I alone. After washing with cold HBSS/BSA, biotin-labeled Stx2 (10 nM) was bound to HUVECs on ice for 30 min. The cells were then shifted to 37°C for various lengths of time (5 - 60 min), and at the end of each incubation period, cells were washed with cold washing buffer. Half of the cells were treated with 0.1 M MESNa in the cold washing buffer containing 2 mg/ml BSA for 20 min at 0°C. After washing, excess MESNa was quenched with 150 mM iodoacetamide for 20 min. The other half was incubated with the same buffer devoid of MESNa. After quenching with 150 mM iodoacetamide for 20 min, both cells were washed twice with washing buffer, and lysed in 150 μl of blocking buffer. After this lysate was aspirated, each well was washed with 50 μl of the blocking buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl, 0.2% BSA, 0.1% SDS, and 1% Triton X-100). The lysate and the washing solution were combined, and 100 μl of the combined solution was transferred into 96-well plates pre-coated with anti-Stx2 MAb. Captured Stx2 was visualized with HRP-conjugated streptavidin and OPD. After the reaction was stopped by addition of 3 M HCl, and optical density (OD) was measured at 492 nm. Endocytosis of Stx2 was reported as internalized toxin (OD from cells treated with MESNa) in percentage of total cell-associated toxin (OD from MESNa-untreated cells). Results were obtained from three separate experiments; each done in triplicate. Each bar represents the mean ± SD of 9 wells. * p <0.05, ** p < 0.025, *** p < 0.01 vs. rolipram-untreated untransfected cells. At each time point, difference in endocytosis levels between rolipram-untreated untransfected cells and rolipram-treated ABCA1 siRNA-transfected cells was not significant. (□) untransfected cells without rolipram,

(') untransfected cells with rolipram, (○) control siRNA-transfected cells with rolipram, (●) ABCA1 siRNA-transfected cells with rolipram.

B) Comparison of the degree of Stx2 endocytosis between untransfected and transfected cells receiving treatment with 7.5 μg/ml rolipram after a 20-min incubation period. The graph was made based on data from A. Quantification of the degree of endocytosis was expressed as percentage of rolipram-untreated untransfected cells (control). The error bars show the deviation between three independent experiments, each done in triplicate. * $p < 0.025$ vs. ABCA1 siRNA-transfected cells with rolipram.

C) For microscopic observation of intracellular Stx2, Stx2 was labeled with Oregon Green 488. HUVECs (5×10^4) in Lab-Tek chamber slides (4-chamber type) in a 400 μl volume of EBM/LP-BS were treated with 7.5 μg/ml rolipram and 10 μg/ml apoA-I for 24 h. After washing with HBSS/BSA, cells were incubated with 20 nM Oregon Green-labeled Stx2 in 400 μl EBM/LP-BS on ice for 30 min. After washing with cold HBSS/BSA, the cells were shifted to 37°C. After 10 or 30 min of incubation at 37°C, the cells were fixed at 37°C with 3% (vol/vol) paraformaldehyde containing 2% (wt/vol) sucrose for 30 min, and then mounted. Slides were analyzed by fluorescence microscopy.

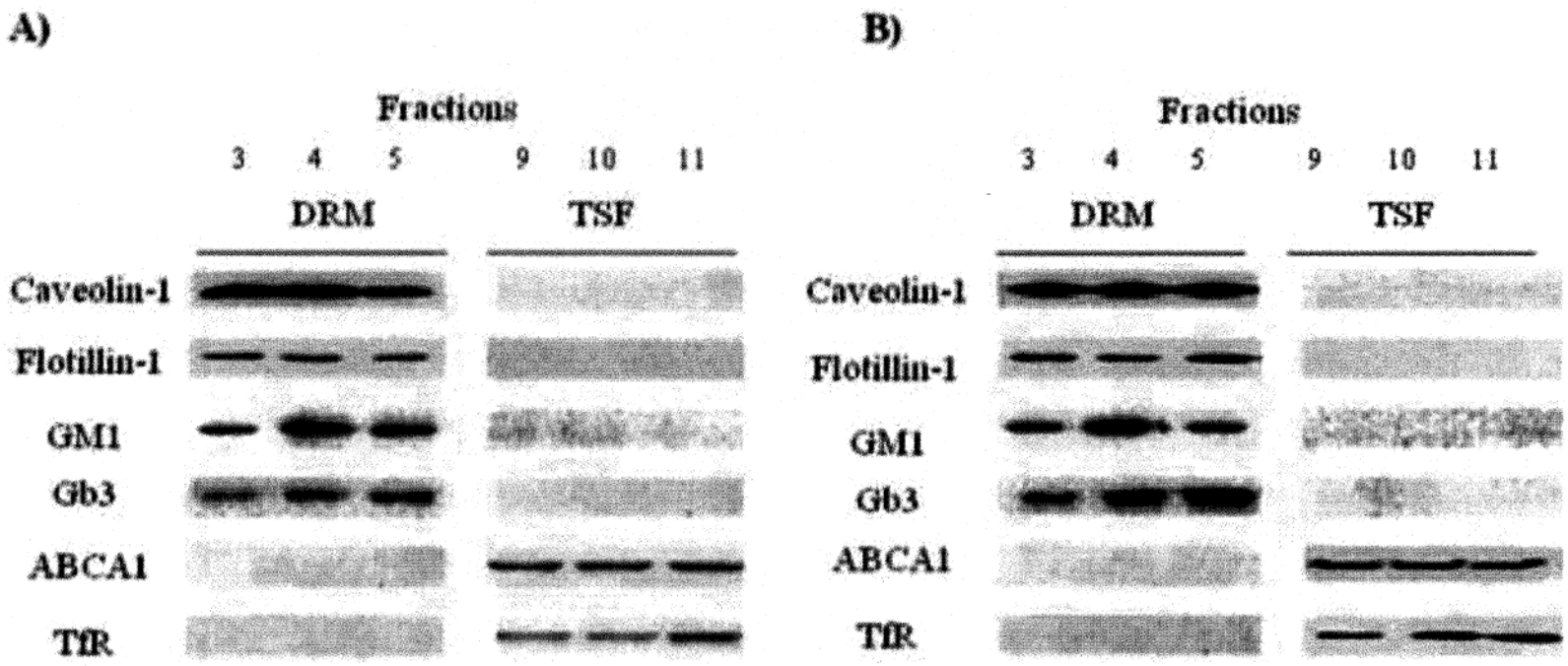

Fig. 1

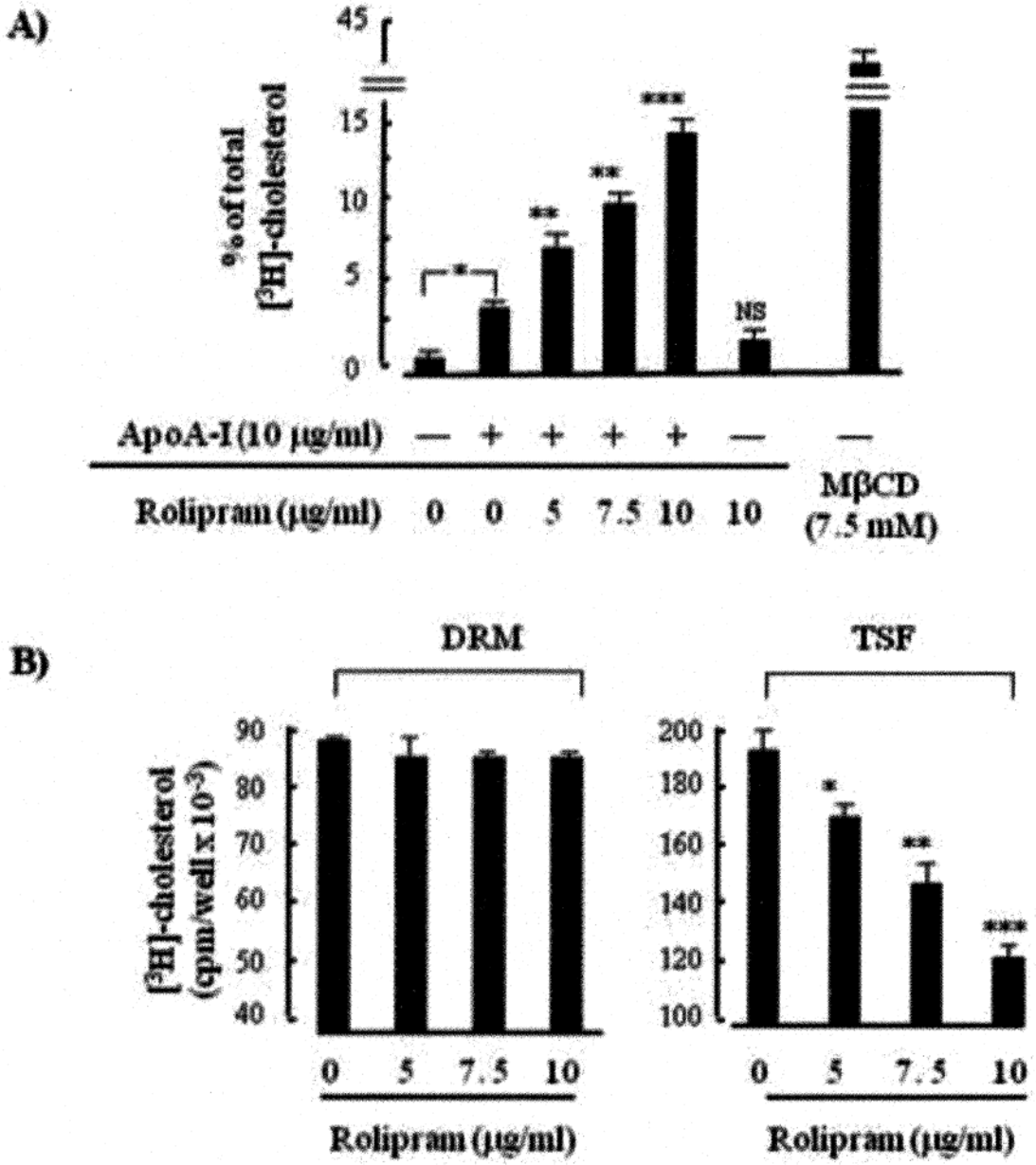

Fig. 2

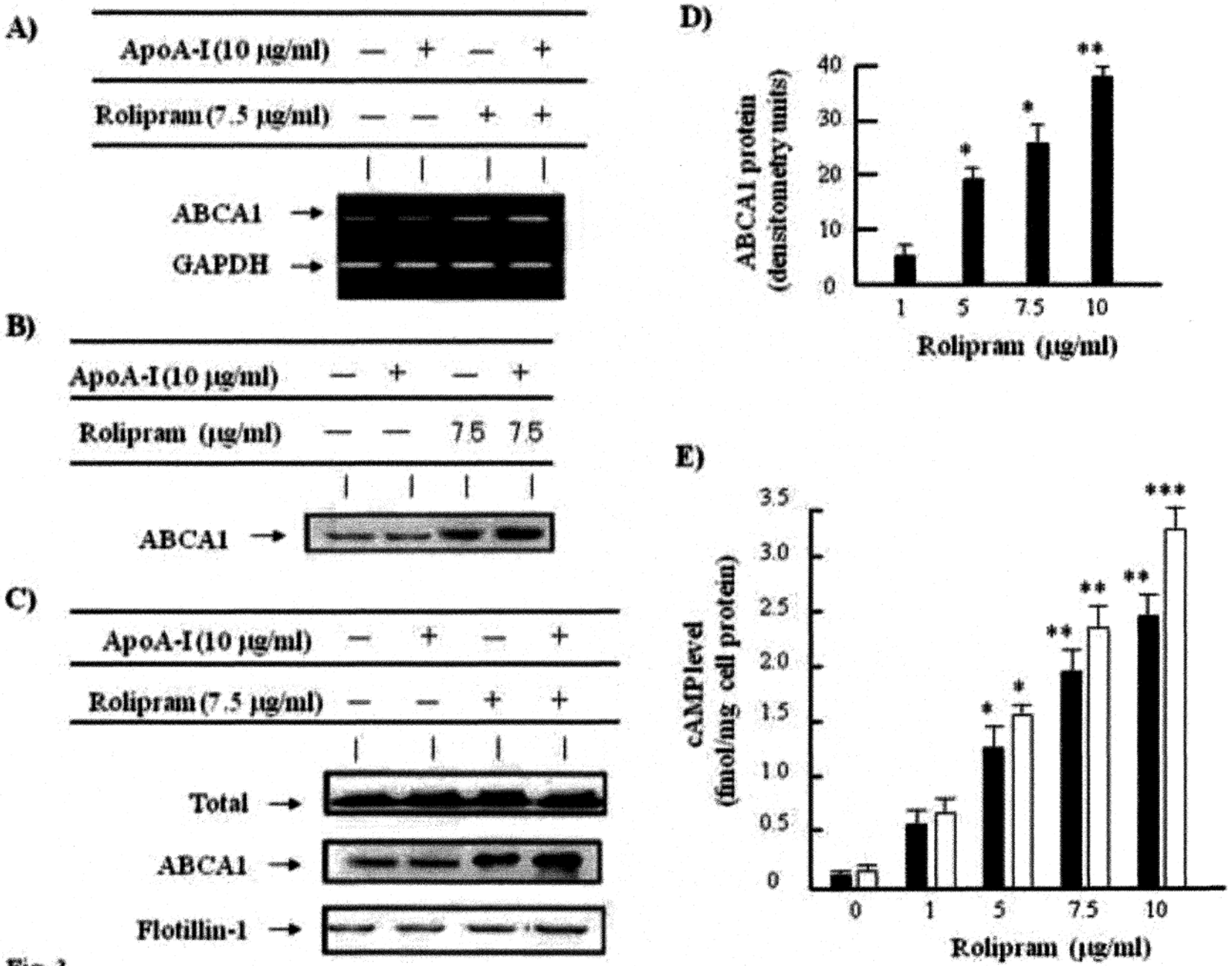

Fig. 3

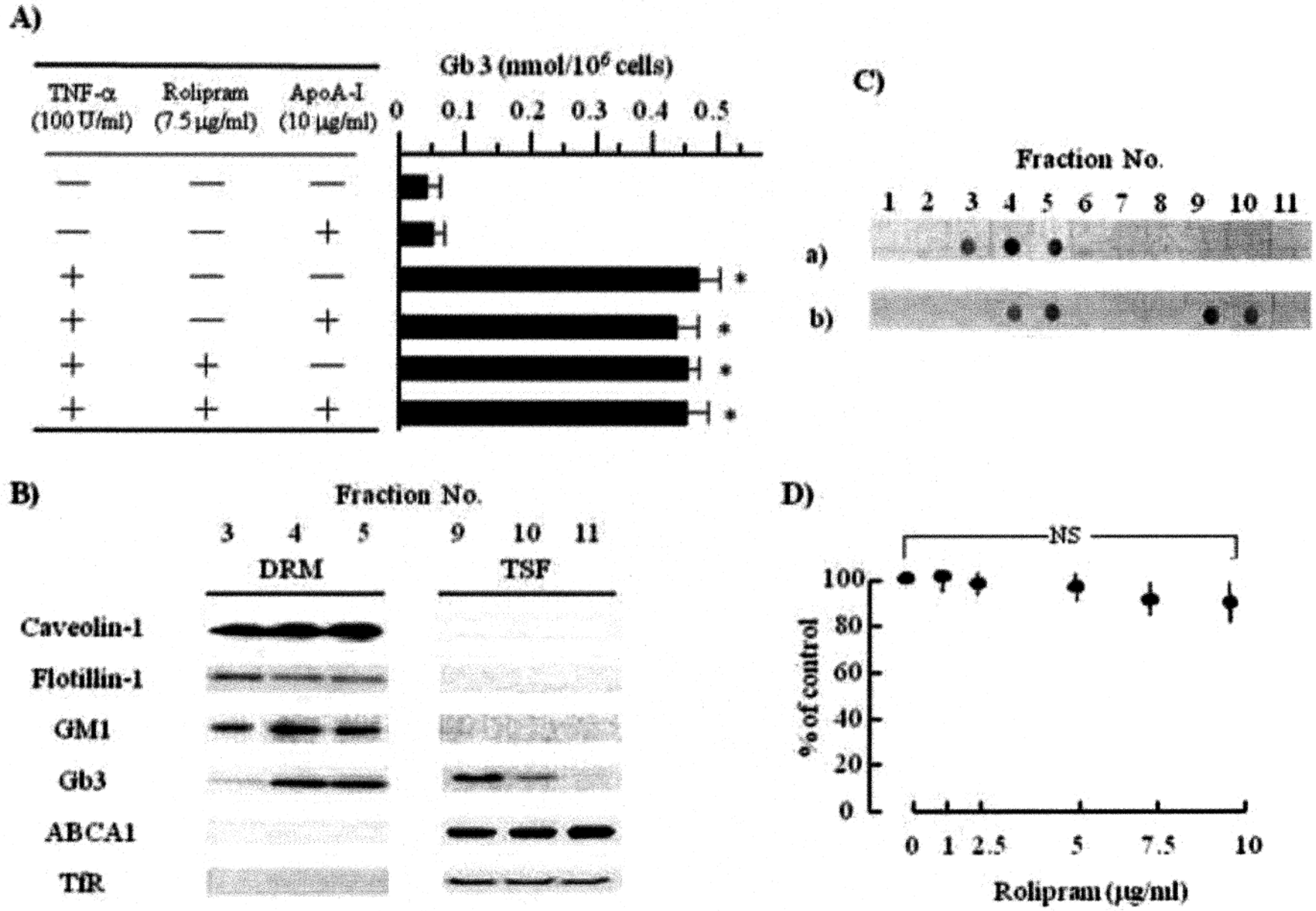

Fig. 4

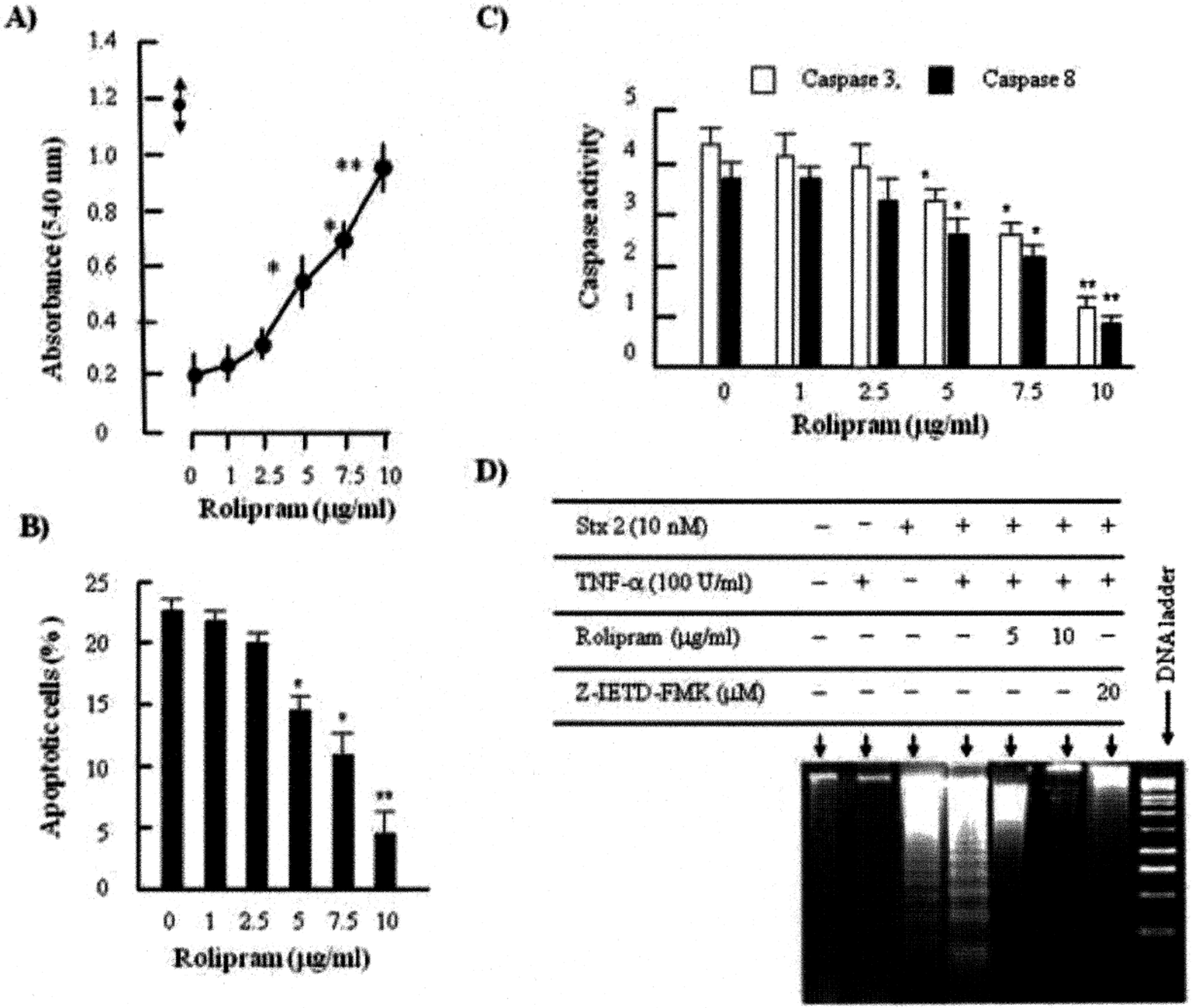

Fig. 5

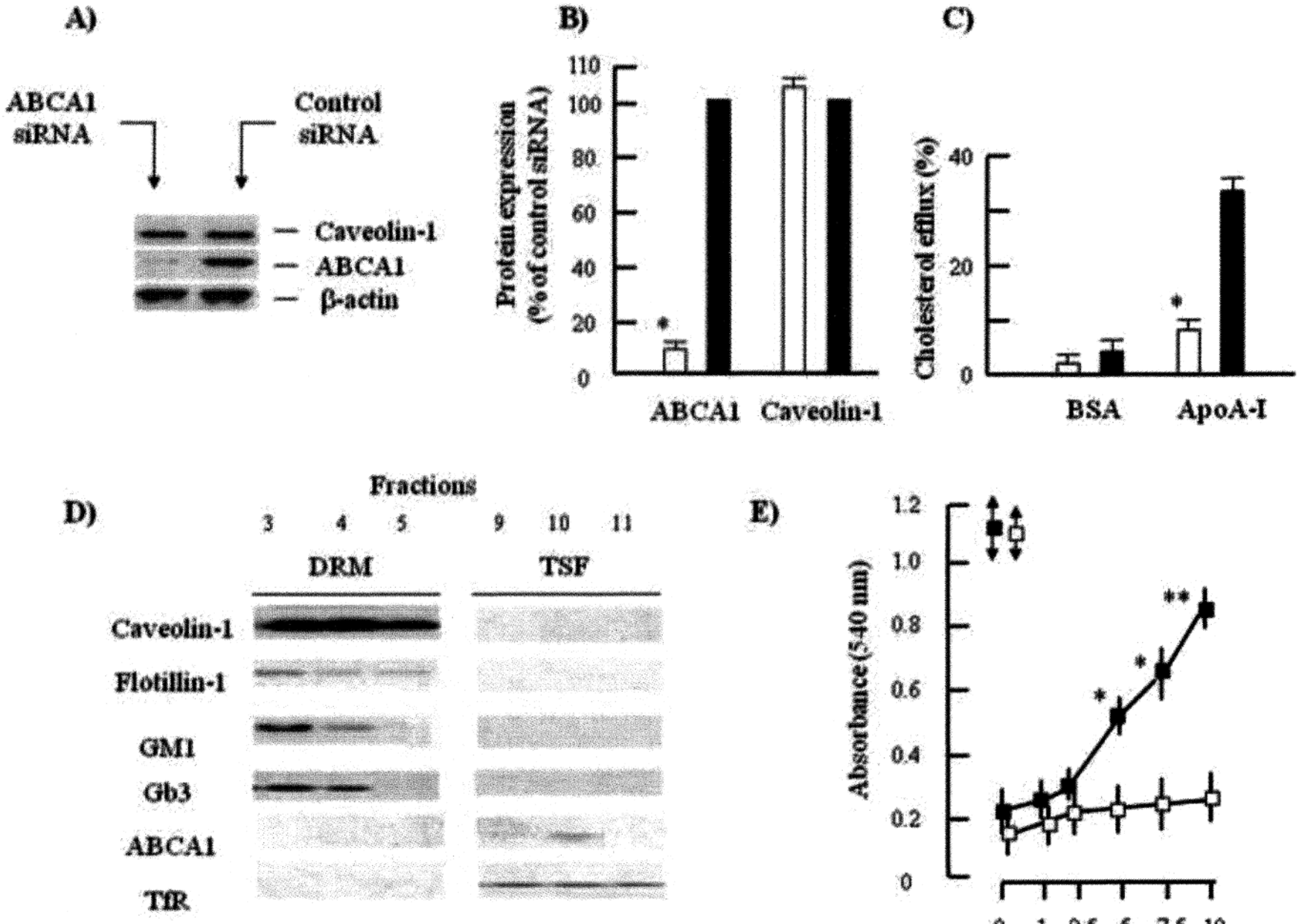

Fig. 6

revised Figure 7

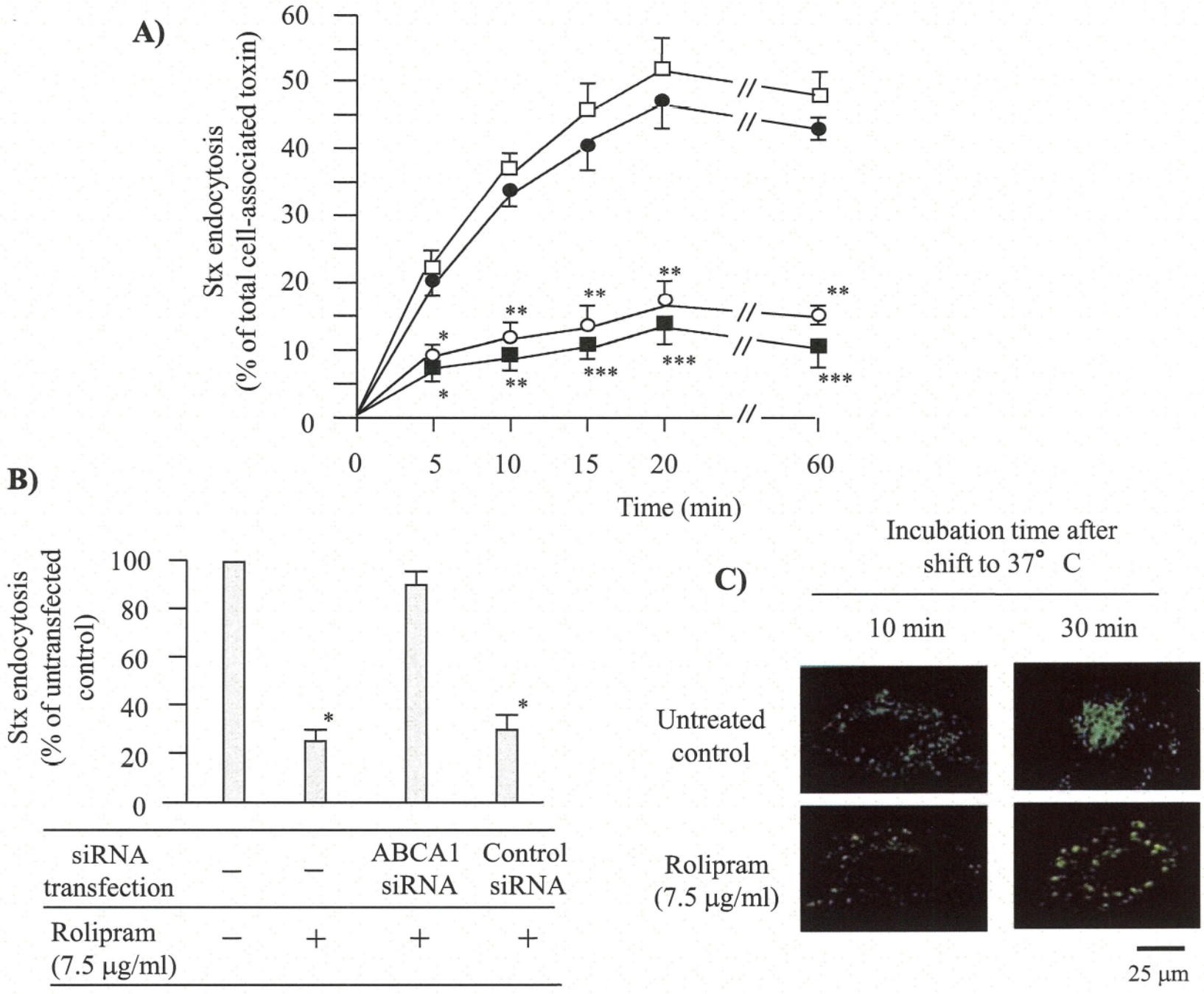

Fig. 7