Cholesterol efflux, an important mechanism by which high density lipoproteins (HDL) protect against atherosclerosis, is initiated by docking of apolipoprotein A-I (apoA-I), a major HDL protein, to specific binding sites followed by activation of ATP-binding cassette transporter A1 (ABC1) and translocation of cholesterol from intracellular compartments to the exofacial monolayer of the plasma membrane where it is accessible to HDL. In this report, we investigated potential signal transduction pathways that may link apoA-I binding to cholesterol translocation to the plasma membrane and cholesterol efflux. By using pull-down assays we found that apoA-I substantially increased the amount of activated Cdc42, Rac1, and Rho in human fibroblasts. Moreover, apoA-I induced actin polymerization, which is known to be controlled by Rho family G proteins. Inhibition of Cdc42 and Rac1 with Clostridium difficile toxin B inhibited apoA-I-induced cholesterol efflux, whereas inhibition of Rho with Clostridium botulinum C3-exoenzyme exerted opposite effects. Adenoviral expression of a Cdc42(T17N) dominant negative mutant substantially reduced apoA-I-induced cholesterol efflux, whereas dominant negative Rac1(T17N) had no effect. We further found that two downstream effectors of Cdc42/Rac1 signaling, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK), are activated by apoA-I. Pharmacological inhibition of JNK but not p38 MAPK decreased apoA-I-induced cholesterol efflux, whereas anisomycin and hydrogen peroxide, two direct JNK activators, could partially substitute for apoA-I in its ability to induce cholesterol efflux. These results for the first time demonstrate activation of Rho family G proteins and stress kinases by apoA-I and implicate the involvement of Cdc42 and JNK in the apoA-I-induced cholesterol efflux.

Numerous studies demonstrated a protective role of high density lipoproteins (HDL) against development of atherosclerosis. HDL is believed to carry cholesterol from peripheral tissues to the liver for eventual elimination from the body in a process referred to as “reverse cholesterol transport” (1, 2). Cholesterol efflux, in which excess cellular cholesterol is released from cells and transferred to HDL particles, is the first step of reverse cholesterol transport. HDL seems to stimulate cholesterol efflux by two distinct mechanisms (3, 4). One mechanism involves unspecific passive diffusion of cholesterol to the HDL particle. The second mechanism is mediated by interaction of apolipoprotein A-I (apoA-I), either on HDL or as a free apoprotein, with specific binding sites that in turn directly mobilize cholesterol from both intracellular compartments and the plasma membrane. We and others (5–7) have recently shown that apoA-I-mediated specific cholesterol efflux is critically dependent on the function of ABC1, a member of the ATP-binding cassette family of proteins, which is defective in Tangier disease. How exactly HDL or apoA-I activate ABC1 and thereby initiate cholesterol efflux remains a matter of debate. By analogy to other agonists, cellular signaling responses generated as a result of interaction of apoA-I with cells could provide a link to ABC1 activation and mobilization of intracellular cholesterol. HDL was shown previously to induce a variety of cellular signals, none of which, with the exception of protein kinase C, could be unequivocally shown to be associated with removal of cellular cholesterol (8–16). Moreover, most of these responses could not be mimicked by apoA-I, thus precluding their direct involvement in the specific cholesterol efflux (11, 14, 16).

The goal of the present study was to specifically investigate the apoA-I-induced signal transduction and its relationship to cholesterol efflux. Our results show that apoA-I activates Rho family small G proteins (Cdc42, Rac1, and Rho) and stress kinases (JNK and p38 MAPK) and suggest that activation of Cdc42 and JNK is relevant to apoA-I-induced cholesterol efflux.

**EXPERIMENTAL PROCEDURES**

**Materials—** Phosphospecific antibodies against MKK3/MKK6, MKK4, JNK, p38 MAPK, ATF-2, Elk-1, and c-Jun were purchased by New England Biolabs. Phosphospecific antibodies against MLC and antibodies against PAK-1 were from Santa Cruz Biotechnology. Anti-Cdc42 antibodies were from BD Transduction Laboratories. Glutathione S-transferase-p21 binding domain (GST-PBD) beads, GST- rhodotin beads, anti-Rac1, and anti-Rho (A, B, -C) antibodies, and Clostridium botulinum C3-exoenzyme were purchased by Upstate Biotechnology, Inc. ApoA-1, SB202190, JNKi-2, 276386, and Clostridium difficile toxin B were from Calbiochem-Novabiochem. BODIPY-phalloidin was from Molecular Probes. PD189318 was from Alexis Corp. [53S]Orthophosphoric acid, GTPy[S], and [3H]Cholesterol were from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine protein kinase; GEFs, guanine nucleotide exchange factors; GAPs, GTPase-activating proteins; GTPy[S], guanosine 5’-3-O-(thio) triphosphate; GST-PBD, glutathione S-transferase-p21 binding domain.

---

* This work was supported by an Innovative Medizinische Forschung grant (to J.-R. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Institut fur Klinische Chemie und Laboratoriumsmedinizin, Westfälische Wilhelms-Universität, D-48129 Münster and the Institut für Arterioskleroseforschung an der Universität Münster, D-48149 Münster, Germany. Tel./Fax: 49-251-8356276; E-mail: nofer@uni-muenster.de.

‡ The abbreviations used are: HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; LPA, lysophosphatidic acid; MLC, myosin light chain; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; PIPES, 1,4-piperazinediethanesulfonic acid; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GEFs, guanine nucleotide exchange factors; GAPs, GTPase-activating proteins; GTPy[S], guanosine 5’-3-O-(thio) triphosphate; GST-PBD, glutathione S-transferase-p21 binding domain.
serum, and bovine serum albumin (BSA) were from PAA Laboratories. All other chemicals were from Sigma.

**Cell Culture**—Human dermal fibroblasts were purchased from American Type Cell Culture Collection and grown to confluence in DMEM supplemented with fetal bovine serum (10%, v/v). Cells were enriched with cholesterol by incubation in serum-free DMEM with 2 mg/ml BSA and 30 µg/ml of nonlipoprotein cholesterol for 24 h.

**Adenoviral Transduction of Fibroblasts**—The adenoviral stocks Ad-N17Rac1, Ad-N17Cdc42, and Ad-β-Gal were the generous gifts from Dr. Anne Ridley (Ludwig Institute for Cancer Research, Royal Free and University College School of Medicine, London, UK). The adenoviruses were transfected into 293HEK cells, which express E1 to allow purification of adenoviral particles. The titers of adenoviral stocks were determined by a plaque assay with an anti-hexon antibody (Chemicon). Confluent fibroblasts were infected with Ad-N17Rac1, Ad-N17Cdc42, and Ad-β-Gal at a multiplicity of infection of 6 in medium containing 2% heat-inactivated fetal calf serum. After 90 min of incubation, 0.5 ml of serum-supplemented DMEM was added per 24-well plate, and the incubation was continued for an additional 24 h.

**Assay for Actin Polymerization**—Actin polymerization was quantified as described by Ha and Exton (17). Briefly, cells were treated with agonists and fixed with 4% formaldehyde containing 2.5% octyl glucopyranoside for 15 min on ice. Cells were then stained with 0.6 ml/well of 15 nM BODIPY-phalloidin for 30 min, and bound BODIPY-phalloidin was extracted with 0.1 ml of methanol for 1 h on ice. The fluorescence intensity measurements were performed using a Hitachi F-2000 fluorometer with excitation and emission wavelengths of 505 and 515 nm, respectively.

**Fluorescence Microscopy**—Fibroblasts grown on coverslips were treated with agonists for 5 min. The cells were fixed, permeabilized, and stained with BODIPY-phalloidin as described above. Coverslips were examined under a fluorescence microscope (Leica) equipped with an excitation filter (470–490 nm), a dichroic mirror (505 nm), and an emission filter (515 nm).

**Pull-down Assay for Cdc42, Rac1, and Rho Activation**—Stimulated fibroblasts were lysed in a buffer containing 20 mmol/liter HEPES (pH 7.4), 150 mmol/liter NaCl, 2% Nonidet P-40, 20% glycerol, 8 mmol/liter EDTA, 1 mmol/liter MgCl2, 1 mmol/liter ortho- vanadate, and the Complete® protease inhibitor mixture. Cell lysates were homogenized by three thaw-freeze cycles, cleared by centrifugation (14,000 rpm, 4 °C), and incubated for 1 h at 4 °C with 10 µg/sample of GST-PBD beads for Cdc42 or Rac1 precipitation or with GST-rhotkin beads for Rho precipitation. The beads were collected by centrifugation (14,000 rpm, 4 °C) and washed, and captured proteins were removed by boiling for 5 min in Laemmli sample buffer. Samples were then subjected to Western blotting with antibodies against Cdc42, Rac1, and Rho.

**Measurement of GTP/GDP Exchange**—Measurement of GTP/GDP exchange was performed in permeabilized fibroblasts as described by de Vries et al. (18) and Downward (19) with minor modifications. Briefly, confluent and serum-starved fibroblasts were stimulated with apoA-I (10 µg/ml) or LPA (20 µg/ml) for 1 min (Cdc42) or 10 min (Rac1). Cells were then cooled rapidly by addition of ice-cold buffer containing 150 mmol/liter glutamate, 20 mmol/liter PIPES, 2 mmol/liter EDTA, 2 mmol/liter EDTA (pH 7.0), and exposed to streptolysin O (100 hemolytic units/ml) in the same buffer containing ATP (2.2 mmol/liter) and Mg2+ (1 mmol/liter) for 10 min at 4 °C. Permeabilization by streptolysin O was induced by addition of buffer containing free Ca2+ (15 µmol/liter) at 37 °C. GTPγS (2.5 µCi/ml) was added immediately thereafter for 30 min. Cells were washed and lysed, and lysates were immunoprecipitated overnight with monoclonal antibodies against Cdc42 and Rac1 or with GST-rhotkin beads (2 µg/300 µl). GST-bound forms of Cdc42 and Rac1 were eluted from immunoprecipitates with Laemmli buffer at 68 °C for 20 min. Recovered radioactivities were measured by scintillation counting. Data were normalized to protein content of each sample.

**SDS-PAGE and Western Blotting**—SDS-PAGE and Western blotting were performed exactly as described previously (14). For each blot with an antibody against a ubiquitously expressed protein (α-actin), PAK-1 Immunoprecipitation—Fibroblasts were incubated in phosphate-free Krebs-Ringer buffer containing 0.1 mM [32P]phosphate for 2 h at 37 °C and stimulated with agonists. Cells were then scraped into 0.5 ml of lysis buffer containing 50 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 2 mmol/liter EDTA, 2 mmol/liter NaF, 10 mmol/liter Na3P04, 50 µg/ml phenylmethylsulfonyl fluoride, and Complete® protease inhibitor mixture. Lysates were pre-

---

protein was performed overnight, and the phosphorylation of PAK-1 was analyzed by autoradiography after proteins were captured by incubation for 2 h with protein A/G-agarose, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Equal amounts of immunoprecipitated PAK-1 protein were confirmed by Western blotting.

**Efflux of Cellular Cholesterol**—Cholesterol efflux was measured according to established methods. Briefly, [3H]cholesterol (1 µCi/ml) was added to cells grown in 24-well plates for 24 h. Cells were washed and incubated for 4 h at 37 °C with DMEM containing BSA (0.2%, v/v) and apoA-I at desired concentrations. The efflux medium was collected and centrifuged to remove cell debris. Cells were solubilized in 0.1 M NaOH. Radioactivity in efflux media and cell lysates was determined by scintillation counting. Data were normalized to protein content of each sample. The results are reported as percentage of efflux relative to the radioactivity fraction present in efflux medium in the absence of apoA-I.

**General Procedures**—Data are presented as means ± S.D. for at least three separate experiments or as representative immunoblots for at least three repetitions, unless indicated otherwise.

**RESULTS**

**ApoA-I Induces Actin Polymerization in Human Fibroblasts**—Rho family G proteins (Cdc42/Rac1/Rho) regulate actin cytoskeleton reorganization in response to extracellular signals. Therefore, we first examined the effect of apoA-I on the actin polymerization. Actin polymers were visualized with a BODIPY-phalloidin and observed under a fluorescence microscope. No polymerized actin could be seen in serum-starved cells (Fig. 1A). However, exposure of fibroblasts to 10 µg/ml apoA-I was accompanied by the appearance of intracellular actin fibers. The changes were evident after 1 min and reached maximum after ~5 min. LPA, a compound inducing actin polymerization in various cell systems, evoked similar morphological changes as apoA-I (Fig. 1A). To investigate the time and concentration dependence of apoA-I-induced actin polymerization, the GST-PBD-phalloidin bound to actin fibers was extracted, and the fluorescence was measured. ApoA-I rapidly increased the intracellular content of polymerized actin with a maximum at 5 min after stimulation. The levels decreased steadily afterward and reached basal levels after 30 min. The maximal stimulation was seen at an apoA-I concentration of ~5 µg/ml. ApoA-I stimulated actin polymerization about half as effectively as 20 µg/ml LPA.

**ApoA-I Activates Small G Proteins Cdc42 and Rac1**—The induction of actin polymerization in the presence of apoA-I suggested the involvement of Cdc42/Rac1/Rho proteins in this process. To assess directly whether apoA-I affects the activity of the Rho family G proteins, we examined the amounts of activated Cdc42 and Rac1 in fibroblasts treated with apoA-I. The GST-PBD beads precipitated only marginal amounts of Cdc42 and Rac1 from unstimulated fibroblasts. By contrast, exposure of cells to 10 µg/ml apoA-I caused a strong increase in the amounts of both active Cdc42 and Rac1. However, both GTPases showed clearly distinctive activation kinetics. Whereas maximal activation of Cdc42 was detectable after 5–10 min of apoA-I exposure, Rac1 activation reached its maximum 30 min after stimulation. The comparison of the apoA-I-induced Cdc42/Rac1 response with that of 20 µg/ml LPA showed that apoA-I was able to cause a substantial stimulation of G proteins.

The activation of Cdc42 and Rac1 is regulated either by guanine nucleotide exchange factors (GEFs), which catalyze exchange of GDP into GTP and mediate activation, or may result from inhibition of GTPase-activating proteins (GAPs), which stimulate GTP hydrolysis leading to inactivation. To investigate the possible involvement of GEFs and GAPs in apoA-I-induced Cdc42 and Rac1 activation, we investigated the GTP loading of Cdc42 and Rac1 after stimulation with apoA-I. Permeabilized fibroblasts were incubated with non-hydrolyzable analogue of GTP, GTPγS, in the presence of apoA-I (10
of Cdc42 and Rac1, were used as positive controls. Fibroblasts were incubated with 20 μg/ml apoA-I for 1 or 10 min markedly increased the amount precipitated afterward. As shown in Fig. 1A, cells were fixed, permeabilized, and stained with BODIPY-phalloidin, as described under “Experimental Procedures.” Cells treated with LPA, a potent stimulator of Cdc42 and Rac1, were used as positive controls. Fibroblasts were incubated with 20 μg/ml LPA for 1 or 10 min prior to Cdc42 and Rac1 precipitation with GST-PBD, respectively. Captured proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-Cdc42 and anti-Rac1 antibodies. Blots were analyzed by densitometry. Shown are the results from three independent experiments.

Activation of Cdc42 and Rac1—As sensitivity toward toxin B varies considerably among various cell lines, we first performed controls to assess whether toxin B effectively inhibited GTPases under our experimental conditions. Fig. 2A shows that preincubation of fibroblasts for 2 h with 50 ng/ml toxin B markedly reduced the apoA-I-induced activation of both Cdc42 and Rac1 as well as PAK-1 autophosphorylation. To verify these results with an independent method, we measured apoA-I-induced actin polymerization and found that it was suppressed in toxin B-pretreated cells (Fig. 2A).

Next, we examined whether a similar pretreatment with toxin B could affect the apoA-I-induced cholesterol efflux. As shown in Fig. 2B, addition of apoA-I for 4 h to human fibroblasts led to a concentration-dependent efflux of cellular cholesterol into the cell medium. The maximal efflux was observed at an apoA-I concentration of 10 μg/ml. In the absence of apoA-I, toxin B treatment did not affect cholesterol efflux. However, toxin B significantly mitigated the apoA-I-evoked cholesterol efflux; in five experiments ~70% reduction in the overall response was observed.

As toxin B inhibits both Cdc42 and Rac1, it does not allow the discrimination between the effects of each of these G proteins. To estimate the contribution of Cdc42 and Rac1 to apoA-I-induced cholesterol efflux, we used metabolically inactive dominant negative mutants, which specifically inhibit different
subclasses of small G proteins. As shown in Fig. 2C, adenovirus-mediated overexpression of Cdc42(T17N) and Rac1(T17N) led to complete inhibition of apoA-I-induced Cdc42 and Rac1 activation, respectively. In cells overexpressing dominant negative Cdc42(T17N), the reduction of apoA-I-induced cholesterol efflux corresponded to an ~60% decrease in the overall response. By contrast, adenoviral introduction of the dominant negative Rac1(T17N) into fibroblasts had no impact on the apoA-I-induced cholesterol efflux.

Lack of Involvement of Rho Activation in ApoA-I-induced Cholesterol Efflux—In addition to Cdc42 and Rac1, Rho proteins were shown to participate in actin polymerization by extracellular signals. Therefore, we next examined the influence of apoA-I on Rho activation. As shown in Fig. 3A, only small amounts of active Rho could be precipitated by rhotekin-GST beads from lysates of untreated fibroblasts. By contrast, exposure of cells to apoA-I (10 μg/ml) led to a marked increase in the amount of active Rho, which was detectable 1 min after addition of an agonist and persisted throughout the entire length of stimulation. Activated Rho has been demonstrated to inhibit the myosin light chain phosphatase thereby causing phosphorylation of myosin light chains (MLC). To provide further evidence for apoA-I-induced activation of Rho, we examined the effect of apoA-I on MLC phosphorylation. Fig. 3A demonstrates that the exposure of fibroblasts to apoA-I led to MLC phosphorylation in a time- and concentration-dependent fashion. The maximal MLC phosphorylation was detected at 10 μg/ml apoA-I.

Next, we evaluated whether apoA-I-induced Rho activation and MLC phosphorylation are related to the efflux of intracellular cholesterol. To this goal, we used *C. botulinum* C3-exoenzyme that specifically inactivates Rho but not Cdc42 or Rac1. In addition, we used compound Y27632, an inhibitor of MLC phosphorylation. First, the efficiency of both inhibitors in our experimental system was verified. As shown in Fig. 3B, treatment of cells with 5 μg/ml C3-exoenzyme for 12 h substantially reduced the amount of activated Rho, which could be precipitated from apoA-I-stimulated fibroblasts. Similarly, preincubation of fibroblasts for 30 min with 10 μmol/liter Y27632 abolished apoA-I-induced MLC phosphorylation. The effect of both compounds on apoA-I-induced cholesterol efflux was shown in Fig. 3C. Increasing concentrations of C3-exoenzyme enhanced whereas increasing concentrations of Y27632 had no effect on apoA-I-induced cholesterol efflux.

**Effect of ApoA-I on Activation of JNK and p38 MAPK**—The downstream effectors linked to the activation Cdc42 and Rac1 include several hierarchically organized kinases as follows: phosphorylation of MKK4 activates JNK, which in turn phosphorylates transcription factor c-Jun, whereas phosphorylation of MKK3/6 induces consecutive phosphorylation and activation of p38 MAPK and transcription factor Elk-1. The transcription factor ATF-2 is phosphorylated by both JNK and p38 MAPK. We were interested to examine whether apoA-I-induced activation of Cdc42 and Rac1 is associated with activation of JNK and p38 MAPK signaling cascades in human fibroblasts. The activation status of the signaling elements in both cascades was examined by using antibodies specifically reacting with phosphorylated epitopes. As shown in Fig. 4A, exposure of fibroblasts to 10 μg/ml apoA-I led to a phosphorylation/activation of both MKK4/JNK/c-Jun and MKK3/6/p38 MAPK/Elk-1 signaling cascades with the onset of activity at 1 min and the maximum at 10–30 min after stimulation. In addition, apoA-I-stimulated phosphorylation of ATF-2. The effect of apoA-I on kinase activation was concentration-dependent with the phosphorylations detectable at 1 μg/ml apoA-I. To determine whether apoA-I-induced activation of JNK and p38 MAPK was causally linked to Cdc42/Rac1 activation, we examined the phosphorylation of JNK and p38 MAPK after inhibition of Rho.
family small G proteins with toxin B. As shown in Fig. 4B, apoA-I-induced phosphorylations of JNK and p38 MAPK were both markedly attenuated in fibroblasts pretreated with 50 ng/ml toxin B.

We next investigated whether signaling via JNK or p38 MAPK was related to apoA-I-induced cholesterol efflux. To this end, we pretreated fibroblasts for 30 min with increasing concentrations of the following inhibitors: JNKI-2 to selectively block JNK, SB202190 to inhibit p38 MAPK, and PD169316, an inhibitor with dual selectivity for both JNK and p38 MAPK. As shown in Fig. 4C, JNKI-2 and PD169316 at concentrations 10 and 50 μmol/liter, respectively, completely blocked JNK activation by 10 μg/ml apoA-I. ApoA-I-induced p38 MAPK activation was effectively blocked by 10 μmol/liter SB202190. In the applied concentration ranges, JNKI-2 exerted no effects on p38 MAPK activation, and SB202190 exerted no effects on JNK activation (not shown). PD169313 effectively inhibited p38 MAPK activation at concentrations 10 times lower than those required for the inhibition of JNK (not shown). The examination of the effect of inhibitors on apoA-I-induced cholesterol efflux revealed that pretreatment of fibroblasts with JNKI-2 or PD169316 in concentrations found to block apoA-I-induced JNK activation led to an ∼50–60% decrease in cholesterol efflux. By contrast, SB202190 at a concentration inhibiting apoA-I-induced p38 MAPK activation exerted no effect on cholesterol efflux (Fig. 4C).

Effect of Direct JNK Activation on Cholesterol Efflux—The potential role of JNK in cholesterol efflux was further examined using anisomycin and hydrogen peroxide, two compounds commonly used to directly activate JNK. As shown in Fig. 5A, stimulation of fibroblasts with anisomycin or hydrogen peroxide led to a time- and concentration-dependent activation of JNK. Within the same concentration range, both compounds induced cholesterol efflux (Fig. 5B). To test whether stimulatory effects of anisomycin and hydrogen peroxide were indeed related to JNK stimulation, we examined the effect of both compounds on JNK activation and cholesterol efflux in fibroblasts pretreated with 10 mmol/liter JNKI-2 or 50 μmol/liter PD169316. As shown in Fig. 5C, both inhibitors effectively blocked JNK activation and cholesterol efflux evoked by exposure of fibroblasts to 50 μmol/liter anisomycin or 0.5 mmol/liter hydrogen peroxide.

**DISCUSSION**

HDL has been shown to activate a multitude of intracellular signaling pathways including kinases (i.e., protein kinase C, ERK1/2, and Akt) (8–14), phospholipases (i.e., phosphatidylinositol-specific phospholipase C and phosphatidylycholine-specific phospholipase D) (15, 16), and mobilization of intracellular Ca²⁺ (16). However, work from our laboratory and other laboratories has demonstrated that these signaling events are generally not related to cholesterol efflux but rather account for other potentially anti-atherogenic effects of HDL, such as stimulation of cell proliferation or inhibition of apoptosis (12–14, 16). Moreover, induction of these signaling pathways is not linked to apoA-I but rather to active lipids carried by HDL (10–14, 16). The focus of the present study was to investigate intracellular signaling events specifically related to interaction of cells with apoA-I. We show that apoA-I induces the activation of Rho family G proteins (i.e., Cdc42, Rac1, and Rho) and stress kinases (i.e., JNK and p38 MAPK). By applying various tools including pharmacological inhibitors and activators, bacterial toxins, and dominant negative mutants, we further demonstrate that activation of Cdc42 and JNK is relevant to the apoA-I-induced cholesterol efflux.

The present study demonstrates that apoA-I induces a marked stimulation of Cdc42, Rac1, and Rho activity in human fibroblasts. The concomitant activation of all three GTPases may be a consequence of GEFs stimulation, as incubation of fibroblasts with apoA-I promoted GTP loading of Cdc42 and Rac1. However, the activation of GAPs by apoA-I is not entirely excluded in this study. Several GEFs are known to have specificity for more than one GTPase (20). Thus, activation of GEFs by apoA-I would be expected to induce Cdc42, Rac1, and Rho in a close temporal sequence. Furthermore, Cdc42, Rac1, and Rho tightly regulate each other, both in a positive and negative

---

**Fig. 3.** **ApoA-I activates Rho in human fibroblasts.** A, cells were stimulated with 10 μg/ml apoA-I for the indicated times or for 10 min with increasing concentrations of apoA-I. Active Rho was precipitated from cell lysates using GST-rhotekin beads as described under "Experimental Procedures." Captured proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-Rho antibodies. MLC phosphorylation was analyzed by Western blotting with phosphospecific anti-MLC antibodies (MLC). Shown are blots representative for one experiment out of three. B, cells were incubated with C. botulinum C3-exoenzyme (5 μg/ml) for 12 h or with 10 μmol/liter Y27632 for 30 min and stimulated with 10 μg/ml apoA-I for 10 min. The amount of active Rho and MLC phosphorylation was determined as described above. Shown are blots representative for one experiment out of three. For cholesterol efflux determination cells were labeled with [³H]cholesterol and then treated for 12 h or 30 min with increasing concentrations of C3-exoenzyme or Y27632, respectively. Cells were then incubated with 10 μg/ml apoA-I for 4 h. The radioactivity released into the medium or associated with cells was determined by liquid scintillation spectroscopy. Shown are results from three separate experiments. **Cont.** control.
In several cell types including fibroblasts, a hierarchical cascade of Cdc42, Rac1, and Rho was found (21, 22). In our experimental system, apoA-I-induced Cdc42 activation consistently preceded activation of Rac1 suggesting a causal relationship between these two events. ApoA-I-induced activation of Rho, however, occurred simultaneously with Cdc42 activation and thus appears to be a separate event. Interestingly, in some cells Rho was found to inhibit Cdc42 activation (23). Such negative feedback could at least partially explain the enhanced Cdc42 activation and cholesterol efflux seen in the present study after inhibition of Rho with C3-exotoxin.

From the results of the present study, Cdc42 emerges as an important regulator of apoA-I-induced cholesterol efflux. Although apoA-I induced activation of all three Rho family G proteins, only selective inhibition of Cdc42 with a dominant negative form suppressed apoA-I-induced cholesterol efflux. By contrast, selective inhibition of Rac1 had no effect on cholesterol efflux, and even enhanced efflux of cholesterol was observed from cells, in which Rho activity was blocked. Previous studies (24) revealed that Cdc42 may be linked to intracellular cholesterol homeostasis. For instance, similar to other proteins involved in cholesterol efflux Cdc42 expression was reported to be up-regulated by cholesterol loading. Conversely, cellular levels of Cdc42 were decreased in cells from Tangier disease, in which defects of ABCA1 lead to severe impairment of cholesterol homeostasis and apoA-I-induced cholesterol efflux (25). More recently, Cdc42 was reported to interact directly with ABCA1, and this interaction was suggested to influence intracellular lipid traffic (26, 27). Given recent observations that ABCA1 serves as a docking molecule for apoA-I (28, 29), it would be attractive to envisage a signaling pathway in which apoA-I binding to ABCA1 results in Cdc42 activation followed by cholesterol efflux.

Recent studies, in which ABCA1-containing vesicles were demonstrated to shuttle between the trans-Golgi network and the plasma membrane, suggested that vesicular transport may essentially contribute to the export of cellular cholesterol (30). Several other observations suggested that Cdc42 could be involved in vesicular trafficking of cholesterol between the trans-Golgi network and the plasma membrane. In contrast to other small G proteins such as Rac1, Cdc42 is enriched on the Golgi vesicles where it co-localizes with ABCA1 (30, 31). Moreover, Cdc42 is recruited to Golgi membranes through an interaction with COP-I, an essential element of the vesicle trafficking machinery, which is released from cells during apoA-I-induced cholesterol efflux (32, 33). Finally, Cdc42 is the only component of the Golgi compartment sensitive to brefeldin, which was shown previously to inactivate apoA-I-induced cholesterol efflux (31, 34). Thus, it is reasonable to assume that apoA-I-induced Cdc42 activation enhances vesicular transport and thereby promotes cholesterol export from cells. In contrast to Cdc42, Rac1 is not involved in the intracellular vesicle traffic. This may explain why inhibition of Rac1 does not affect apoA-I-induced cholesterol efflux.

The results of the present study demonstrates that apoA-I potently stimulates actin polymerization in a Cdc42/Rac1-de-
dependent manner. It cannot be excluded that Cdc42 affects cholesterol transport by stimulating actin polymerization-based propulsion of cholesterol-containing vesicles. However, the role of actin filaments in cholesterol trafficking is controversial. Although actin-perturbing compounds prevent delivery of cholesterol to the endoplasmic reticulum, they do not appear to influence the movement of cholesterol between other cell compartments (35–37). Other Cdc42 downstream effectors distinct from microfilaments may be potentially involved in the regulation of vesicular cholesterol transport. Stress-induced kinases represent widely recognized signaling targets of Cdc42/Rac1. Constitutively activated forms of Cdc42 and Rac1 were repeatedly reported to activate the JNK and p38 MAPK protein cascades, whereas elimination of Cdc42/Rac1 led to inhibition of JNK and p38 MAPK activation by extracellular signals (20, 38, 39). Thus, activation of stress kinases in the presence of apoA-I, as demonstrated for the first time in this study, is entirely consistent with the apoA-I-induced stimulation of Cdc42 and Rac1 activity. This notion is further strengthened by the observation that apoA-I is able to activate Pak-1, an important intermediate between Cdc42/Rac1 and JNK/p38 MAPK signaling pathways (40). Previous studies revealed that several kinases are activated in fibroblasts and other cells in the presence of apoA-I (11). However, with the exception of protein kinase C, the identity of these kinases remained obscured. It would be tempting to speculate that phosphorylation of at least some proteins observed previously in the presence of apoA-I reflects activation of kinases described in the present study. For instance, a 27-kDa protein phosphorylated under the influence of apoA-I in fibroblasts and endothelial cells could represent a heat shock protein 27, which is a canonical target of p38 MAPK signaling (11, 41).

To what extent activation of JNK and p38 MAPK contributes to the apoA-I-induced cholesterol efflux remains to be elucidated. The results of the present study suggest that JNK may play an auxiliary role. ApoA-I-induced cholesterol efflux was reduced in cells pretreated with two structurally unrelated JNK inhibitors. Conversely, JNK activators could partially substitute for apoA-I in inducing cholesterol export from cells. By which mechanism JNK could contribute to cholesterol efflux remains, however, open to speculation. Being a major player in cholesterol efflux ABCA1 would be an obvious target of JNK activity. However, we failed to observe phosphorylation of ABCA1 as a consequence of stimulation with anisomycin or hydrogen peroxide. Other as yet unidentified components of the cholesterol vesicular trafficking or transmembranous cholesterol transport machinery could serve as targets of JNK phosphorylation.

In conclusion, the present study demonstrates for the first time that activation of Rho family G proteins (Cdc42, Rac1, and Rho) and stress kinases (JNK and p38 MAPK) occurs under the influence of apoA-I. Moreover, our data suggest that activations of Cdc42 and JNK may be important steps in apoA-I-induced cholesterol efflux. However, because complete inhibition of both Cdc42 and JNK substantially inhibited but did not abolish apoA-I-induced cholesterol efflux, the existence of other as yet uncharacterized signaling pathways is not excluded by this study. Clearly, further work is necessary to fully elucidate the intracellular signaling pathways involved in cholesterol efflux.

REFERENCES
1. von Eckardstein, A., Nofer, J.-R., and Assmann, G. (2000) Curr. Opin. Lipidol. 15, 348–354

2. R. Feuerborn and J.-R. Nofer, unpublished observations.
