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ORIGINAL PAPER

Ouabain-induced apoptosis and Rho kinase: a novel caspase-2 cleavage site and fragment of Rock-2

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Abstract Ouabain, a specific Na^+/K^+-ATPase inhibitor, has recently been identified as a mammalian hormone. Its elevated concentrations in human plasma have also been associated with pathogenesis of several diseases. Recent studies have shown that ouabain induces aponecrotic cell death in a cell-type- and dose-dependent manner. However, the exact mechanism of ouabain-induced cell death is not fully understood. The Rho GTPase effectors Rho kinases-1 and -2 (Rock-1 and Rock-2) which play central roles in the organization of the actin cytoskeleton, involve in several models of apoptosis. In this study, we investigated the possible involvement of Rocks in ouabain-induced human umbilical vein endothelial cell (HUVEC) apoptosis. Ouabain treatment resulted in loss of cell–cell and cell–substratum adhesion and apoptotic blebbing. Pretreatment of cells with Y-27632, a specific Rock inhibitor, resulted in the inhibition of cell-to-cell detachment and formation of membrane blebs. However, Y-27632 did not prevent ouabain-induced cell–substratum detachment. Instead, treatment with Y-27632 actually accelerated this process. Ouabain treatment induced cleavage of Rock-1 and Rock-2, which was prevented by caspase-3 and caspase-2 specific inhibitors z-DEVD-fmk and z-VDVAD-fmk, respectively. Ouabain-induced Rock-2 cleavage generated a fragment of approximately 140 kDa corresponding to the consensus sequence of caspase-2 on the carboxy terminus of Rock-2. Although it has been previously shown that Rock-2 was cleaved by caspase-2, we have identified here a novel cleavage site and fragment of Rock-2. Our data indicate that ouabain induces both Rock-1 and Rock-2 cleavage via caspase-dependent mechanisms and provide evidence that Rocks are involved in ouabain-induced cell-to-cell detachment and apoptosis.

Keywords Ouabain · Apoptosis · Rho kinase · Rock-1 cleavage · Rock-2 cleavage

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Introduction

Na^+/K^+-ATPase (NKA) is a membrane-bound enzyme that maintains the transmembrane ion balance and volume of a cell. Its functional integrity is crucial for cell homeostasis and survival [1]. Ouabain, a cardiotonic steroid, is a specific inhibitor of NKA and also influences various cytosolic signaling events [2]. Hamlyn et al. [3] demonstrated for the first time that ouabain or a closely related compound exists in the human plasma, and their findings were confirmed by other researchers [4, 5]. Since the discovery of endogenous ouabain, much attention has been focused on the physiological and pharmacological activities of this compound. It was found that endogenous ouabain levels increase in pathological conditions such as arterial hypertension, chronic cardiac and renal failure [6]. In addition, ouabain induces aponecrotic cell death in a cell-type- and dose-dependent manner. Ouabain-induced apoptosis involves the activation of caspase-3, cytochrome c release from mitochondria, and production of reactive oxygen radicals [7].

It is widely accepted that NKA plays a pivotal role in the formation and maintenance of adherent junctions and that if the cells are subjected to ouabain they detach from each other and from the substrate, leading to their death. Contreras et al. [8] have shown that E-cadherin, a membrane protein of the adherent junctions, is removed from the membrane by ouabain treatment. Moreover, NKA is co-localized with adherens junctions and after the treatment of cells with ouabain, NKA α_1 and β_1 subunits are endocytosed [9, 10].

Akimova et al., however, reported that ouabain and other cardiotonic steroids killed renal epithelial and vascular endothelial cells independently of the $[Na^+]_i/[K^+]_i$ ratio. They examined several signaling pathways, including PI3K, MAPK ERK1/2 kinase, tyrosine kinases and Ras signaling, in ouabain-treated renal epithelial cells, but none of the signaling systems tested affected ouabain-induced cell death [11]. However, in a more recent study, Akimova et al. [12] indicated that p38 MAPK signaling was involved in ouabain-induced renal epithelial cell death but not in HUVEC. In spite of this extensive research, the exact mechanism of ouabain-induced cell death is not fully understood.

Rho kinases (Rock-1 and Rock-2, also termed ROKβ and ROKα), the most studied effectors of the small G protein Rho, are serine/threonine kinases with a molecular mass of $\sim$160 kDa [13, 14]. It has been shown Rocks regulate the following major physiological functions: smooth muscle contractions, including ouabain-induced and non-muscle cell contraction, migration, proliferation, and gene expression and differentiation [15, 16]. In addition to their physiological roles, Rocks are involved in a wide range of pathological conditions such as hypertension [17], cerebral vasospasm [18], preeclampsia [19], and carcinogenesis [20].

Rocks have an N-terminal kinase domain and a C-terminal inhibitory domain that are connected to each other through a coiled-coil structure containing a Rho-binding domain. Two types of activation modes of Rocks have been determined. The interaction between the Rho-binding domain and an active Rho disrupts the interaction between the catalytic domain and the inhibitory C-terminal domain of Rock and activates the enzyme [13, 14]. Rock can also be activated by proteolytic cleavage of the inhibitory C-terminal domain. This second mode of activation occurs during apoptosis. Although both Rock isoforms become active via proteolytic processing, the mechanisms of these processes are different for Rock-1 and Rock-2. Rock-1 is cleaved by caspase-3 at the cleavage site DETD 1113 [21, 22], whereas Rock-2 is cleaved by granzyme B at the IGLD 1131 site during cytotoxic lymphocyte granule-induced apoptosis [23].

Activated Rock induces myosin light chain (MLC) phosphorylation [24, 25] either directly or through the inhibition of MLC phosphatase [24, 26, 27], which inhibits the activity of MLC kinase. MLC phosphorylation leads to the contraction of the cortical actin ring and formation of dynamic membrane blebs, as seen in apoptotic cells [28]. Inhibition of bleb formation with Y-27632, a specific Rock inhibitor, indicates that the occurrence of apoptotic membrane blebbing is Rock-dependent [21, 22]. In a recent study, van der Heijden et al. [29] showed that the prevention of f-actin rearrangement by Rock inhibition attenuated ischemia/reperfusion-induced endothelial cell apoptosis. Similar results were obtained for cardiac myocyte apoptosis. In that study, Rock-1 null mice (Rock-$1^{(-/-)}$) showed a marked reduction in myocyte apoptosis associated with pressure overload [30]. In another study, Petrache et al. [31] demonstrated that Rho kinase inhibition attenuates TNF-α-induced endothelial cell apoptosis.

Taken together, these studies raise the possibility that the Rho/Rho kinase pathway and/or proteolytic activation of Rho kinase may be involved in the ouabain-induced cell detachment and death. Therefore, in this work, we examined the possible involvement of Rho kinase in the mechanisms underlying ouabain-induced cell death in human umbilical vein endothelial cell (HUVEC).

Materials and methods

Cell culture and drug treatments

Our protocol was approved by the Ethics Committee of the Zekai Tahir Burak Maternity Hospital. HUVECs were isolated and cultured in our laboratory from human umbilical cord as described by Baudin et al. [32]. The cells were harvested by collagenase Type II (PAA) digestion and seeded on plastic culture flasks and 1% gelatin-coated plates in M199 containing 10% fetal bovine serum (PAA), 0.135% $NaHCO_3$, 15 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 lg/ml streptomycin. Cultures were placed in a humidified chamber containing 95% air and 5% CO_2 at 37°C. The following day, non-adherent cells were removed by changing the culture medium. The culture medium was changed every 2 days until the cell culture reached confluence. To avoid phenotypic changes and deterioration of the attachment by passaging, only primary cells were used. Before the treatments, 100% confluent HUVEC monolayers were kept for 48 h in M 199 containing 10% fetal bovine serum. Cells were treated with ouabain (Sigma), specific Rho kinase inhibitor Y-27632 (Sigma), and caspase-2, caspase-3, and pan-caspase inhibitors (Tocris Bioscience) at varying concentrations and time points.

Phase-contrast microscopy-cell counts

At the end of the different treatment periods, pictures were taken through a phase-contrast microscope at random sites.

Using the pictures, total cells and cells with blebs were counted in three fields randomly selected from six flasks. The percentage of cells with blebs was calculated by dividing the number of cells with blebs by the total number of cells.

Cell-detachment assay

An xCELLigence Real-Time Cell Analyzer (RTCA) DP system (Roche) was used to evaluate ouabain-induced detachment of HUVECs. This method uses impedance technology as an indicator of cell adhesion. The system produces an electronic readout of impedance to quantify cell adhesion, proliferation and viability in real time. The presence of cells on the E-plate electrodes leads to an increase in electrode impedance, which results in a record of cell density, i.e., the cell index. The more cells attached to the substratum, the larger the increase in cell index. If the cells detach from the substratum the cell index will decrease.

Freshly isolated HUVECs were plated on 16-well E-plates at a density of 1.5×10^5 cells/well. Cell growth was monitored continuously using the RTCA DP instrument. After cells reached a steady state, the cells were treated with ouabain (0.1–10 μM), Y-27632 (10 μM), ouabain + Y-27632 (1–10 μM, 30 min before the ouabain treatment), ouabain + Z-VDVAD-fmk (50 μM), ouabain + Z-DEVD-fmk (50 μM), ouabain + Z-VAD-fmk (50 μM) (2 h before the ouabain treatment), Z-VDVAD-fmk (50 μM), Z-DEVD-fmk (50 μM) and Z-VAD-fmk (50 μM). As an indicator of cell detachment, the cell index was recorded continuously and analyzed by RTCA software version 1.2. Slopes of the time-versus-cell index curves were evaluated as an index of the rate of detachment.

Immunofluorescence

HUVEC were grown on coverslips and treated as indicated in Figs. 4 and 10. After treatments, cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 5 min. Cells were incubated with 1% BSA in PBS for 45 min to block unspecific binding of the antibodies. Monolayers were then incubated in pERM (1:50, Cell Signaling) primary antibody in a humidified chamber for 1 h at room temperature and with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:50, Pierce) in 1% BSA for 1 h at room temperature, in the dark. For f-actin labeling, the cells were incubated with 50 μg/ml Rhodamine-phalloidin (Sigma) for 45 min. The nuclei were counterstained with 0.1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma) for 5 min. Coverslips were mounted with the SlowFade Antifade Kit (Invitrogen). Immunofluorescence was visualized using a Leica DMIL inverted fluorescence microscope and recorded with a Leica (DFC 420C) digital camera. Images were overlayed and merged using Photoshop CS 3 (Adobe) (Fig. 4) and Image J 1.42q (Fig. 10).

TUNEL staining

The TUNEL (terminal dUTP nick end labeling) assay was performed with the in situ cell apoptosis detection kit (Roche) according to the manufacturer's instructions. TUNEL-positive nuclei and DAPI-stained nuclei were counted in each well in three different fields. An apoptotic index was calculated as a ratio of the number of TUNEL-positive nuclei to DAPI-stained nuclei in each slide.

Caspase activity assays

Caspase-3 and caspase-2 activities were measured by using the Caspase-3/CPP32 Colorimetric Assay Kit and Caspase-2 Colorimetric Assay Kit from Biovision following the manufacturer's protocol. After experimental treatment, endothelial cells were scraped in culture medium, pelleted and resuspended in lysis buffer. 130 μg of cytosolic extract were used for each assay. Each sample were incubated with the substrates (DEVD-pNA and VDVAD-pNA) at 37°C for 2 h. The *p*NA light emission was quantified at 405 nm on a microplate reader. Fold-increase in caspase-3 and caspase-2 activities were determined by comparing the results with level of the control.

Western blot

After experimental treatment, the confluent HUVEC monolayers were scraped in media and centrifuged. Pellets were resuspended and lysed with lysis buffer: 50 mM Tris-HCL pH 7.5, 400 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Roche). The cells were then sonicated. The proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. Five percent non-fat dry milk and 3% BSA (for phospho-proteins) in TBS-T were used for blocking. Membranes were incubated with Rock-1 (1:500, BD Biosciences), Rock-2 (1:750, BD Biosciences), pERM (1:500, Cell Signaling), pMYPT (1:500, Millipore), ERM (1:1,000, Cell Signaling), MYPT (Santa Cruz, 1:500) and pan-actin (loading control) (1:10,000, Neo Markers) antibodies. After being washed, membranes were then incubated with a corresponding anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:1,000, Pierce). The ECL (Amersham Biosciences) system was used for the detection. Signals were quantified by densitometric analysis using the Scion Image computer program.

Lung and brain whole tissue lysates were used for Rock-1 and Rock-2 as positive controls, respectively.

In vitro caspase-2 cleavage assay

HUVEC from T25 flask were washed in PBS and scraped in 1 ml buffer [20 mM Tris, pH 7.4, 0.9% sucrose, 1 mM EDTA, protease inhibitors (Roche)]. After centrifugation at 1,000 × *g* at 4°C for 10 min, cells were sonicated in denaturing lysis buffer (1% SDS, 5 mM EDTA, 10 mM dithiothreitol, protease inhibitors) and boiled for 1 min. 20 μg cell lysates were mixed with caspase reaction buffer [50 mM Hepes (pH 7.4), 50 mM NaCl, 10 mM EDTA, 5% glycerol, 0.1% CHAPS, 10 mM DTT] at a dilution rate of 1:10 and incubated with 5 units active recombinant human caspase-2 (Bio Vision) for 24 h at 37°C. The reaction was stopped by putting the samples on ice. The reaction product was analyzed by western blot with Rock-2 antibody .

Statistical analysis

All values were expressed as the mean ± sem and analyzed by Student's unpaired *t* test, and ANOVA. $P \leq 0.05$ was considered statistically significant.

Results

Effects of ouabain on endothelial cell morphology and detachment

Confluent unstimulated HUVECs exhibit a cobblestone appearance. Treatment of endothelial cells with ouabain resulted in the concentration-dependent detachment of the cells from the substratum and one another and produced apoptotic blebbing (Fig. 1). To evaluate the possible involvement of Rock in these effects, cells were pretreated for 30 min with Y-27632, a highly specific Rock inhibitor. Y-27632 significantly inhibited bleb formation and partially inhibited cell-to-cell detachment. However, pretreatment of cells with Y-27632 did not prevent but actually accelerated ouabain-induced cell–substratum detachment (Figs. 1, 3b).

We also evaluated the effects of caspase inhibition on ouabain-induced detachment and bleb formation. As shown in Figs. 2 and 3c none of the caspase inhibitors inhibited the formation of blebs and detachment in ouabain-treated HUVECs. Mills et al. and Huot et al. have published similar results in PC12 cells and HUVECs, respectively. They demonstrated that dynamic membrane blebbing is present in cells treated with the pan-caspase inhibitor z-VAD-fmk [28, 33]. Consistent with their studies, our findings indicate that membrane blebbing and caspase

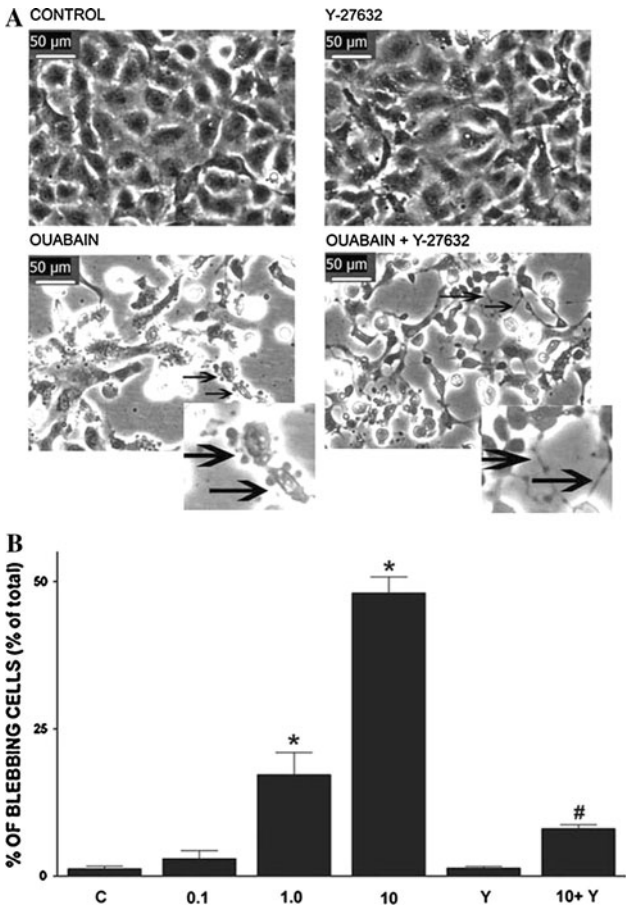

Fig. 1 Effects of ouabain on endothelial cell morphology. Confluent endothelial cells were treated with 0.1–10 μM ouabain for 16 h in the absence and presence of 10 μM Y-27632 for 30 min. Ouabain produced cell detachment and membrane blebbing. Y-27632 significantly inhibited bleb formation. **a** Representative phase-contrast micrographs of HUVECs. Scale bars indicate 50 μm. *Arrows* indicate blebbing cells in ouabain-treated groups and intercellular actin meshwork in ouabain + Y-27632-treated groups. (CONTROL: control cells, OUABAIN: 10 μM ouabain, OUABAIN + Y-27632: 10 μM ouabain + 10 μM Y-27632Y, Y-27632: 10 μM Y-27632.) **b** Effects of Rock inhibition in ouabain-induced bleb formation. Total cells and cells with blebs were counted in three randomly selected fields in six flasks. The percentage of cells with blebs was calculated by dividing the number of cells with blebs with the number of total cells. Data are means ± sem from 6 determinations; (*) indicates difference from control, (#) indicates difference from ouabain-treated groups. $P < 0.05$ was considered significant. (C: control cells, 0.1: 0.1 μM, 1.0: 1 μM, 10: 10 μM ouabain, Y: 10 μM Y-27632, 10 + Y: 10 μM ouabain + 10 μM Y-27632)

activation may follow different pathways in ouabain-induced HUVEC apoptosis.

To confirm the phase contrast microscopy observations and to evaluate possible changes of the actin cytoskeleton, we examined the effects of Rock inhibition in the ouabain-treated HUVECs by fluorescence microscopy. Y-27632-treatment alone prevented the formation of f-actin bundles and relaxed the cells. In addition, there was no significant cell detachment and death in Y-27632 treated cells.

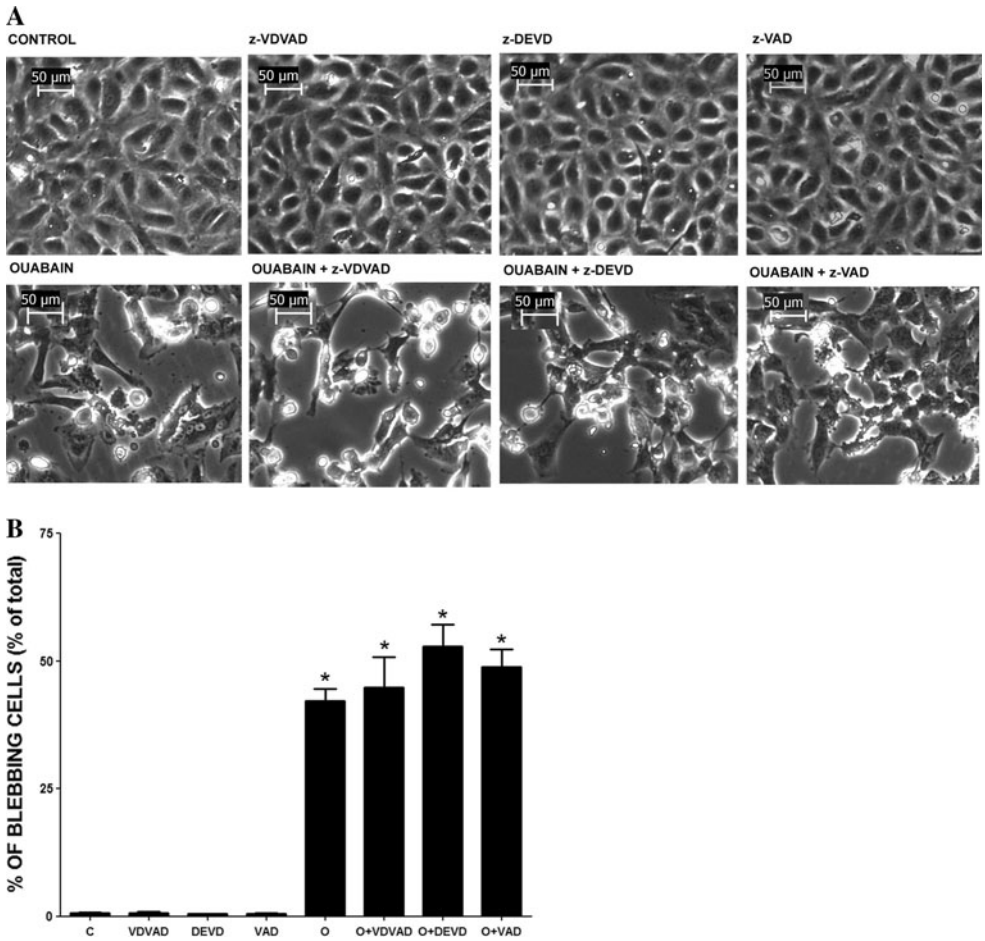

Fig. 2 Ouabain-induced membrane blebbing is insensitive to caspase inhibition with z-VAD-fmk, z-DEVD-fmk, z-VDVAD-fmk in HUVECs. Cells were preincubated with z-VDVAD-fmk, z-DEVD-fmk, z-VAD-fmk (50 µM, 2 h) and then treated with ouabain (10 µM, 16 h). **a** Representative phase-contrast micrographs of HUVECs. Scale bars indicate 50 µm. **b** Since, ouabain-treatment causes bleb formation and caspase-inhibition does not affect it. Total cells and cells with blebs were counted in three randomly selected fields in six flasks. The percentage of cells with blebs were calculated by dividing the number of cells with blebs with the total number of cells. Data are means ± sem from 6 determinations; (*) indicates difference from control. $P < 0.05$ was considered significant. (C: control cells, VDVAD: 50 µM z-VDVAD-fmk, DEVD: 50 µM z-DEVD-fmk, VAD: 50 µM z-VAD-fmk, O: 10 µM ouabain, O + VDVAD: 10 µM ouabain + 50 µM z-VDVAD-fmk, O + DEVD: 10 µM ouabain + 50 µM z-DEVD-fmk, O + VAD: 10 µM ouabain + 50 µM z-VAD-fmk)

However, consistent with the phase-contrast observations, ouabain treatment induced cell detachment and formation of blebs (Fig. 4).

Moreover, as shown in Fig. 4, combinations of ouabain and Y-27632 bring about the formation of an intercellular actin meshwork. The prevention of cell contraction by Rock inhibition may be responsible for the inhibition of cell-to-cell detachment and the subsequent formation of an actin meshwork. However, the exact mechanism of this effect remains to be elucidated.

Ouabain induces apoptosis in HUVEC

To evaluate whether ouabain induces cell death in HUVECs via apoptosis, we performed the TUNEL assay. As shown in Fig. 5, ouabain increased the percentage of TUNEL positive nuclei, indicating that ouabain induced apoptosis in HUVECs. Pretreatment with Y-27632 does not abrogate that effect.

Cleavage of Rock-1 and Rock-2 in ouabain-treated HUVECs

To elucidate whether the proteolytic activation of Rocks accompanies ouabain-induced apoptotic cell death, we analyzed the proteolytic processing of Rock-1 and 2 in HUVECs with an antibody that detects both uncleaved and cleaved forms of the enzyme. Ouabain induced cleavage of both Rock-1 and 2 and generated truncated forms of the enzyme that were ~130 and ~140 kDa, respectively,

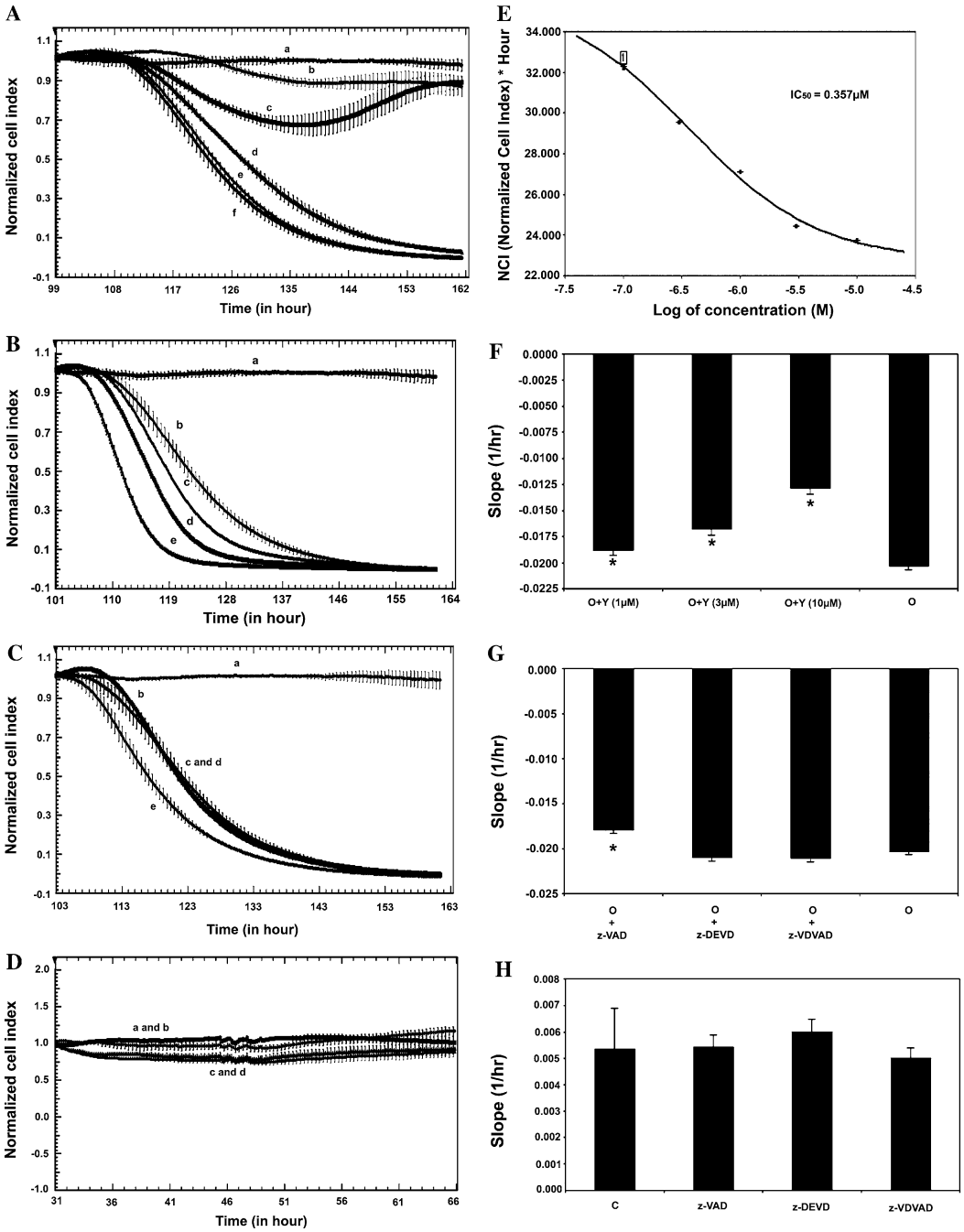

Fig. 3 Dynamic monitoring of cell detachment induced by ouabain using the xCELLigence system. **A** An original trace of ouabain-induced detachment recorded by RTCA DP instrument (a:control cells, b: 0.1 μM ouabain, c: 0.3 μM ouabain, d: 1 μM ouabain, e: 3 μM ouabain, f: 10 μM ouabain). **B** An original trace of the effects of Y-27632 on ouabain-induced detachment (a: control cells, b: 10 μM ouabain, c: 10 μM ouabain + 1 μM Y-27632, d: 10 μM ouabain + 3 μM Y-27632, e: 10 μM ouabain + 10 μM Y-27632). **C** An original trace of the effects of caspase inhibition on ouabain-induced detachment (a: control cells, b: 10 μM ouabain, c: 10 μM ouabain + 50 μM z-VDVAD-fmk, d: 10 μM ouabain + 50 μM z-DEVD-fmk, e: 10 μM ouabain + 50 μM z-VAD-fmk). **D** An original trace of the effects of caspase inhibitors alone on detachment (a: control cells, b: 50 μM z-VDVAD-fmk, c: 50 μM z-DEVD-fmk, d: 50 μM z-VAD-fmk). **E** Concentration-cell index curve of ouabain in endothelial cells. **F** Slope of the curves in (**B**) as an index of the cell detachment rate [O + Y(1 μM): 10 μM ouabain + 1 μM Y-27632, O + Y(3 μM): 10 μM ouabain + 3 μM Y-27632, O + Y(10 μM): 10 μM ouabain + 10 μM Y-27632, O: 10 μM ouabain]. **G** Slope of the curves in (**C**) as an index of the cell detachment rate (O + z-VAD: 10 μM ouabain + 50 μM z-VAD-fmk, O + z-DEVD: 10 μM ouabain + 50 μM z-DEVD-fmk, O + z-VDVAD: 10 μM ouabain + 50 μM z-VDVAD-fmk, O: 10 μM ouabain)**. H** Slope of the curves in (D) as an index of the cell detachment rate (c: control cells, z-VAD: 50 μM z-VAD-fmk, z-DEVD: 50 μM z-DEVD-fmk, z-VDVAD: 50 μM z-VDVAD-fmk)**.** Data are means ± sem from 3 determinations. (*) indicates difference from ouabain-treated groups. $P < 0.05$ was considered significant. *CI* normalized at the time of 103 h

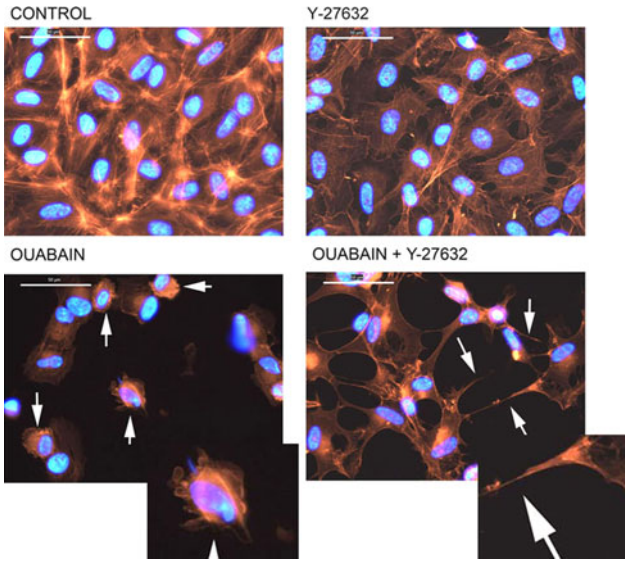

Fig. 4 Ouabain mediated changes of the actin cytoskeleton in **HUVECs**. Ouabain treatment induced membrane blebbing and Y-27632 prevented the formation of blebs. Representative fluorescence micrographs of HUVECs that were stained by rhodamine-phalloidin and DAPI. The cells were fixed and double-stained with rhodamine-phalloidin for actin bundles (*red labeling*) and DAPI for nuclei (*blue labelling*). *Arrows* indicate blebbing cells in ouabain-treated groups and intercellular actin meshwork in ouabain + Y-27632-treated groups. Scale bars indicate 50 μm. (CONTROL: control cells, OUABAIN: 10 μM ouabain, OUABAIN + Y-27632: 10 μM ouabain + 10 μM Y-27632Y, Y-27632: 10 μM Y-27632)

which was absent in control cells (Fig. 6a, b). We also determined that the relative amount of the cleaved form of Rock-2 was significantly less than that of Rock-1 (Fig. 6e).

Inhibition of proteolytic processing of both Rock-1 and Rock-2 by the pan-caspase inhibitor Z-VAD-fmk indicated that caspases are responsible for this phenomenon (Fig. 7a, c). To determine which caspases are responsible for Rock-1 and Rock-2 cleavage, we used the caspase-2 and caspase-3 specific inhibitors z-VDVAD-fmk and z-DEVD-fmk, respectively. Pretreatment of cells with the caspase-2 specific inhibitor z-VDVAD-fmk prevents ouabain-induced proteolytic cleavage of Rock-2 indicating that caspase-2 is responsible for this cleavage (Fig. 7c). As for Rock-1, its cleavage was inhibited by the caspase-3 inhibitor z-DEVD-fmk as shown previously by several groups in apoptotic cells [22] (Fig. 7a).

We also determined that ouabain-induced Rock-2 cleavage was inhibited by the caspase-3 inhibitor Z-DEVD-fmk (Fig. 7c). Li et al. [34] have shown that activation of caspase-3 in apoptotic cells results in cleavage and activation of caspase-2, and a caspase-3 preferred peptide inhibitor DEVD-CHO inhibits caspase-2 activation. Taken together, it is possible that ouabain induces caspase-3, which induces caspase-2 activation causing proteolytic cleavage of Rock-2.

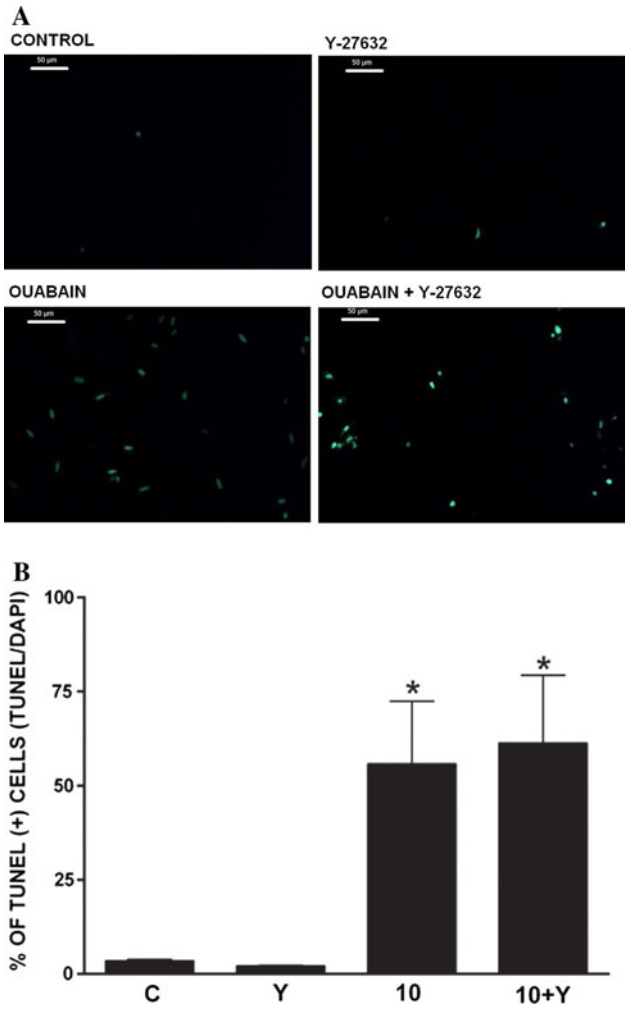

Fig. 5 Ouabain-induced apoptotic cell death in HUVECs. The cells were pretreated with 10 μM Y-27632 for 30 min before exposing them to 10 μM ouabain, and incubated for 16 h. **a** Representative fluorescence micrographs of endothelial cells that were stained by TUNEL assay to detect apoptotic cells. Scale bars indicate 50 μm. **b** Apoptotic index. The apoptotic index, or the ratio of TUNEL-positive nuclei to DAPI-stained nuclei, was determined by analyzing three randomly selected areas in each slide. Data are means ± sem from 3 determinations; (*) indicates difference from control. $P < 0.05$ was considered significant (C: control cells, Y: 10 μM Y-27632, 10: 10 μM ouabain, 10 + Y: 10 μM ouabain + 10 μM Y-27632 O: 10 μM ouabain, O + Y: 10 μM ouabain + 10 μM Y-27632)

To test this assumption we evaluated caspase-3 and caspase-2 activations in ouabain-treated HUVEC. Ouabain induced both caspase-3 and caspase-2 activations (Fig. 8). Ouabain-induced caspase-2 activation was inhibited in the presence of caspase-3 inhibitor, z-DEVD-fmk (Fig. 8b).

Rock-2 cleavage by caspase-2 in vitro

To determine whether caspase-2 cleaves Rock-2, we incubated endothelial cell lysates with active recombinant human caspase-2 for 24 h at 37°C in vitro. In Fig. 7g, it is

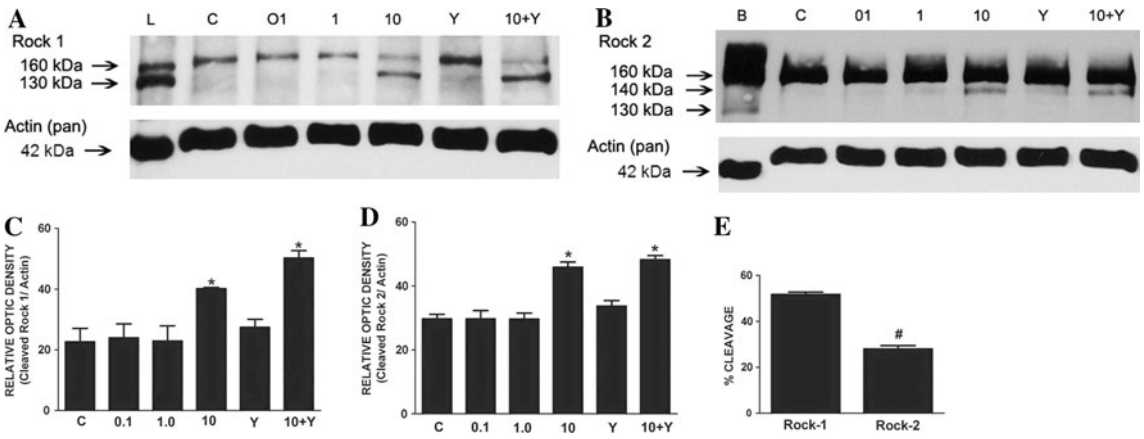

Fig. 6 Rock-1 and Rock-2 are cleaved in ouabain-induced apoptosis. Confluent endothelial cells were incubated with ouabain (0.1–10 μM, 16 h) in the absence and presence of Y-27632 (10 μM, 30 min). Lysates were immunoblotted with the antibody against Rock-1 and Rock-2. **a** Representative immunoblot for Rock-1 cleavage in HUVECs. **b** Representative immunoblot for Rock-2 cleavage in HUVECs. **c** Densitometric analysis of blots for cleaved Rock-1. **d** Densitometric analysis of blots for cleaved Rock-2. **e** Percent cleavage of Rocks. Percent cleavage of Rock-1 and Rock-2 was calculated by the formula; % cleavage = (band density of the cleaved form/band density of uncleaved + cleaved forms of Rocks) × 100. Data are means ± sem from 4 determinations; (*) indicates difference from control, (#) indicates difference from Rock-1 cleavage. $P < 0.05$ was considered significant (L: lung whole tissue lysates, B: brain whole tissue lysates, C: control cells, 01: 0.1 μM, 1: 1 μM, 10: 10 μM ouabain, Y: 10 μM Y-27632, 10 + Y: 10 μM ouabain + 10 μM Y-27632)

shown that incubation of cell lysates with active recombinant caspase-2 resulted in Rock-2 cleavage and this cleavage generated a 140 kDa fragment of Rock-2.

Rock activation in ouabain-treated HUVECs

Rho kinases specifically phosphorylate COOH-terminal threonine residues of ezrin-radixin-moesin (ERM) proteins [35] and phosphorylate myosin phosphatase target subunit 1 (MYPT) of MLC phosphatase at threonine 696 [24], and regulate their functions. Therefore, to determine Rock activity in ouabain-treated HUVECs, we evaluated the phosphorylation levels of the two different Rock targets, MYPT and ERM. We observed that there was profound basal Rock activity in HUVECs. Ouabain treatment induced the phosphorylation of MYPT and ERM proteins and these activations were prevented by Y-27632 in ouabain-treated HUVECs (Fig. 9).

It has been shown that ERM proteins are involved in the Rock dependent apoptosis of jurkat cells [36] and that they play a pivotal role in the formation of membrane blebs [37]. Therefore, we evaluated the localization of phosphorylated ERM proteins in ouabain-treated HUVECs. In contrast to immunoblotting data, there was no detectible fluorescence for phosphorylated ERM in control cells. Treatment of HUVECs with ouabain induced phosphorylation of ERM in membrane blebs and Rock inhibition with Y-27632 significantly reduced this phosphorylation. However, caspase inhibition did not alter the phosphorylation and localization of ERM proteins. Moreover, as shown in Fig. 10 ouabain-treated HUVECs showed a colocalization with f-actin-pERM on membrane blebs.

These observations, taken together with the lack of effects of caspase inhibition on the ouabain-induced detachment and formation of blebs, indicate that ouabain-induced detachment and blebbing do not depend on the caspase-induced Rock cleavage but depend on the activity of uncleaved Rock forms. Therefore, it may be thought that uncleaved Rock activity is both necessary and sufficient to produce membrane blebbing in ouabain-treated HUVECs.

Discussion

The endothelium is involved in several vital functions of the cardiovascular system, such as the regulation of blood flow, coagulation, inflammatory responses, and angiogenesis. In addition, the vascular endothelium is the first cell line to interact with circulating endogenous ouabain as well as the cardiac glycosides that are used in the treatment of heart failure. For this reason, we evaluated the chronic effects of ouabain in human endothelial cells.

Our results showed that ouabain induces caspase-3 activation, Rock-1 cleavage, and produces apoptotic blebbing in HUVECs. Interestingly, we also detected the cleaved form of Rock-2 in ouabain-treated cells, which is thought to be resistant to caspase-induced processing in several cell lines. The inhibition of Rho kinases with a highly specific Rock inhibitor Y-27632 prevents bleb formation and inhibits ouabain-induced cell-to-cell detachment partially. These

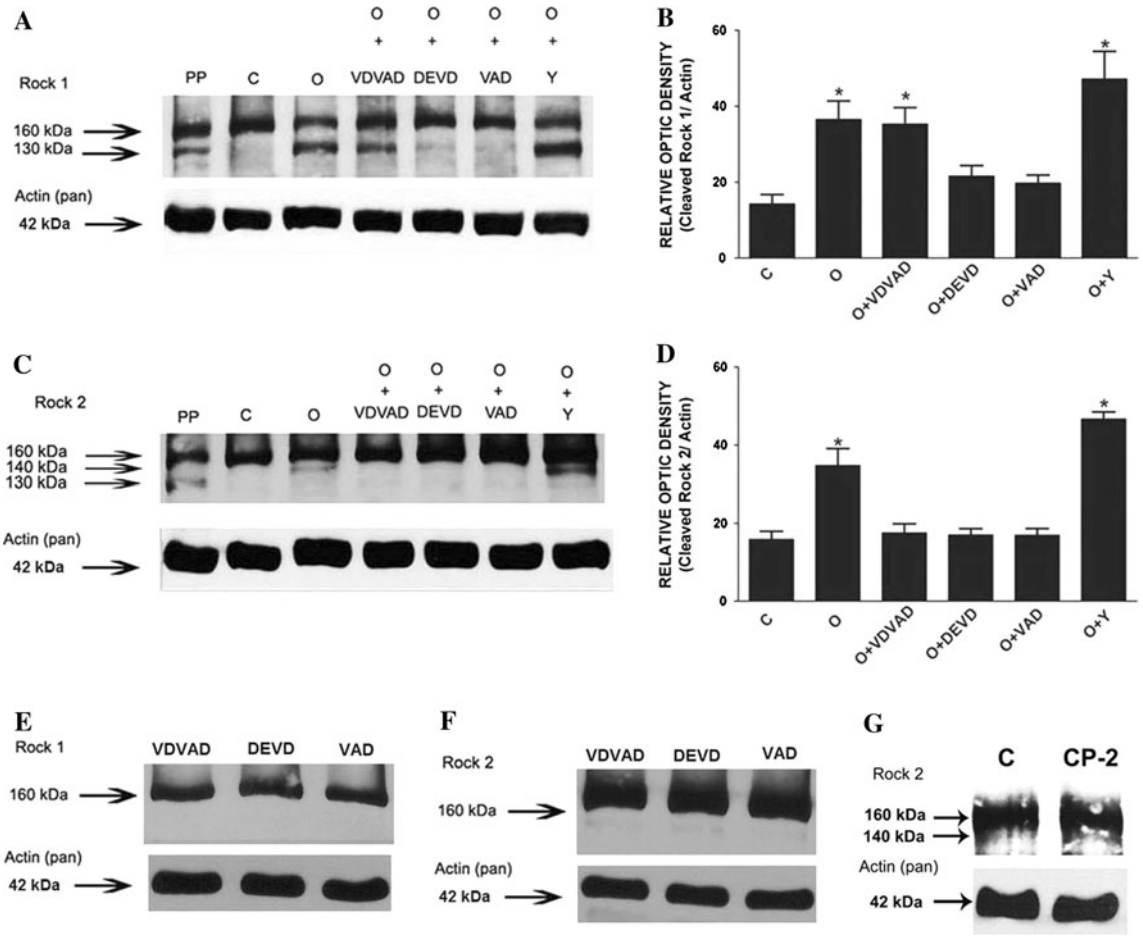

Fig. 7 Rock-1 and Rock-2 cleavage are caspase-dependent during ouabain-induced apoptosis. Confluent endothelial cells were incubated with 10 μM ouabain (16 h) in the absence and presence of 50 μM z-VDVAD-fmk (2 h), 50 μM z-DEVD-fmk (2 h), and 50 μM z-VAD-fmk (2 h), 10 μM Y-27632 (30 min). **a** Representative immunoblot for Rock-1 cleavage in HUVECs. **b** Densitometric analysis of blots for cleaved Rock-1. **c** Representative immunoblot for Rock-2 cleavage in HUVECs. **d** Densitometric analysis of blots for cleaved Rock-2. **e** Representative immunoblot for Rock-1 with caspase inhibitors alone. **f** Representative immunoblot for Rock-2 with caspase inhibitors alone. **g** Representative immunoblot for Rock-2 cleavage by recombinant caspase-2. Data are means ± sem from 4 determinations; (*) indicates difference from control. $P < 0.05$ was considered significant (PP: preeclamptic placenta whole tissue lysates, C: control cells, O: 10 μM ouabain, O + VDVAD: 10 μM ouabain + 50 μM z-VDVAD-fmk, O + DEVD: 10 μM ouabain + 50 μM z-DEVD-fmk, O + VAD: 10 μM ouabain + 50 μM z-VAD-fmk, O + Y: 10 μM ouabain + 10 μM Y-27632, CP-2: recombinant caspase-2)

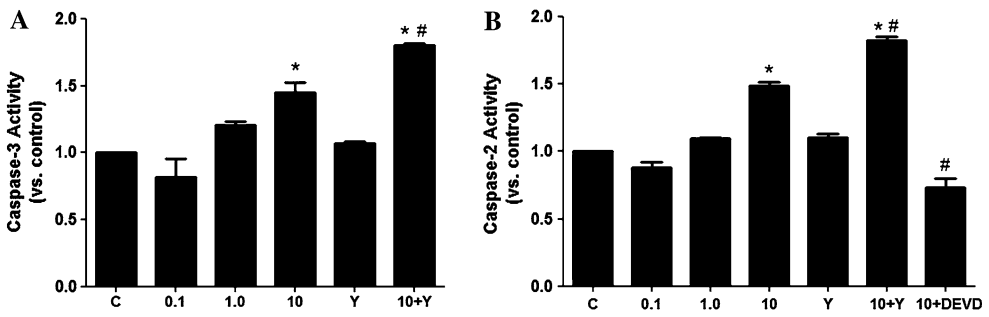

Fig. 8 Ouabain induces caspase-2 and caspase-2 activation in HUVEC. Caspase-3 and caspase-2 activities were assayed by the fluorometric substrate cleavage. **a** Caspase-3 activity. **b** Caspase-2 activity. Data are means ± sem from 4 determinations; (*) indicates difference from control, (#) indicates difference from ouabain-treated groups. $P < 0.05$ was considered significant (C: control cells, 0.1: 0.1 μM ouabain, 1.0: 1 μM ouabain, 10: 10 μM ouabain, Y: 10 μM Y-27632, 10 + Y: 10 μM ouabain + 10 μM Y-27632, 10 + DEVD: 10 μM ouabain + 50 μM z-DEVD-fmk)

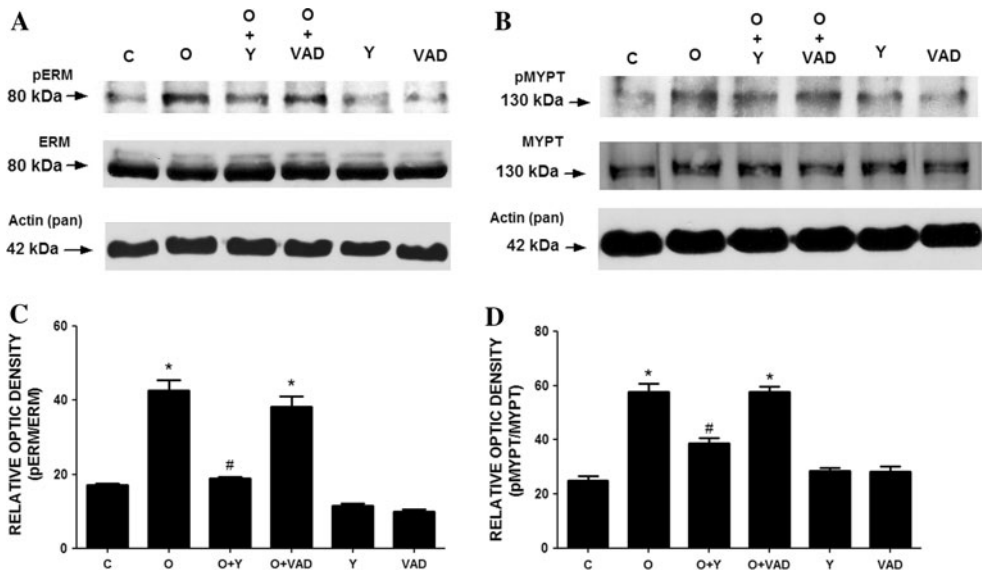

Fig. 9 Ouabain induces phosphorylation of ERM and MYPT as a result of Rock activation in HUVECs during apoptosis. Confluent endothelial cells were incubated with 10 μM ouabain (16 h) in the absence and presence of 10 μM Y-27632 (30 min). **a** Representative immunoblot for pERM in HUVECs. **b** Representative immunoblot for pMYPT in HUVECs. **c** Densitometric analysis of blots for pERM. **d** Densitometric analysis of blots for pMYPT. Data are means ± sem from 4 determinations; (*) indicates difference from control, (#) indicates difference from ouabain-treated groups. $P < 0.05$ was considered significant (C: control cells, O:10 μM ouabain, O + Y: 10 μM ouabain + 10 μM Y-27632, O + VAD: 10 μM ouabain + 50 μM z-VAD-fmk, Y: 10 μM Y-27632, VAD: 50 μM z-VAD-fmk)

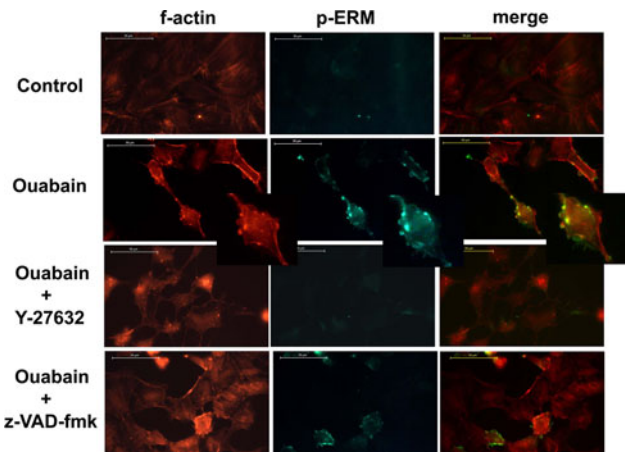

Fig. 10 Cellular localization of phosphorylated ERM proteins in ouabain-treated HUVECs. Representative immunofluorescence micrographs of HUVECs. Confluent endothelial cells were treated with 10 μM ouabain (16 h) in the absence and presence of 10 μM Y-27632 (30 min) and 50 μM z-VAD-fmk (2 h). Cells were fixed with 2% paraformaldehyde, then permeabilized. F-actin was stained with rhodamine-phalloidin (*red labeling*), pERM was immunostained with anti pERM antibody and visualized by FITC-labeled goat anti-rabbit IgG (*green labeling*) in endothelial cells. Please note that pERM was co-localized with f-actin in membrane blebs. Scale bars indicate 50 μm

results indicate that the Rho kinase pathway is involved in the ouabain-induced membrane blebbing and cell detachment in HUVECs.

Although it has been shown that granzyme B, a serine protease that is derived from cytotoxic T lymphocytes, induces both Rock-1 and Rock-2 cleavage in an indirect manner by activating caspase-3 and direct manner, respectively [23]; here, we show for the first time that an agent induces proteolytic activation of both Rock-1 and Rock-2 via caspase-dependent mechanisms in apoptosis.

We detected a potential caspase-2 cleavage sequence VDVAD in human Rock-2 at position 1211–1215 (VTQTD) (Supplementary Fig. 1). Similar to granzyme B, possible caspase-2 consensus sequence in Rock-2 is located in the inhibitor C-terminal portion of the enzyme indicating that this cleavage may activate Rock-2. However, if Rock-2 is cleaved by caspase-2 it will produce a higher molecular weight fragment than 130 kDa. To evaluate this possibility, we computed the theoretical molecular weight of granzyme B and caspase-2 cleaved fragments of Rock-2 with pI/Mw prediction software in the ExPASy web site (www.expasy.ch). Consistent with our assumption, the Mw of granzyme B and the caspase-2 cleaved fragment of Rock-2 were 131.31783 and 140.79244 kDa, respectively. In agreement with our assumptions, pretreatment of cells with the caspase-2 specific inhibitor z-VDVAD-fmk prevents the ouabain-induced proteolytic cleavage of Rock-2. Moreover, we then compared the cleavage pattern of Rock-2 in ouabain-treated HUVECs with that in preeclamptic placentas, which were previously demonstrated as having a 130 kDa fragment of Rock-2 [19]. Consistently, we determined that

the approximate molecular weight of ouabain-induced cleaved form of Rock-2 is higher than that in preeclamptic placentas. In addition, we observed that ouabain, only at concentrations producing cell detachment and death, was able to induce Rock-2 cleavage in HUVECs. Therefore, our current data indicate that Rock-2, like Rock-1, may be involved in ouabain-induced apoptotic cell death and that ouabain-induced Rock-2 cleavage may result from the caspase-2 induced proteolytic processing. However, we have shown here that the relative amount of the cleaved form of Rock-2 was significantly less than that of Rock-1. Thus, it is possible that the contribution of Rock-2 cleavage may be of secondary importance in ouabain-induced apoptosis.

Sapet et al. [38] have shown the involvement of caspase-2 in thrombin-induced Rock-2 but not Rock-1 cleavage, independent of cell death. To moreover, their results indicated that cleavage of Rock-2 by caspase-2 led to the generation of a fragment of approximately 130 kDa. However, in contrast to their findings we have demonstrated here that ouabain-induced Rock-2 cleavage generated a product of approximately 140 kDa, corresponding to the consensus sequence of caspase-2 on the carboxy terminus of Rock-2. It is possible that these two fragments of Rock-2 may be the same fragment, as only one recognition site for caspase-2 (VDVAD) exists in the amino acid sequence of Rock-2. Considering their results and ours, the possibility remains that ouabain-induced proteolytic activation of Rock-2 might be caspase-2-dependent and that it may also involve various cellular functions other than cell death. However, further studies are needed to understand whether this novel cleaved form of Rock-2 is active and is involved in apoptosis.

Based on the current findings and previously published data in which ouabain treatment internalizes the adherent junction proteins such as E-cadherin, β catenin, and NKA α_1 and β_1 subunits, it is possible that ouabain treatment, initially results in cell detachment, after which apoptosis is triggered. Then Rock-1 and Rock-2 are cleaved by activated caspases. At that point, the question is whether the cleaved forms of Rocks contribute to the formation of membrane blebbing. It has been shown that the activation of Rocks by proteolytic cleavage is involved in membrane blebbing in several cell types [21, 22]. However, our results do not support these findings, at least in ouabain-treated HUVECs. Indeed, current data indicate that the formation of ouabain-induced membrane blebbing requires uncleaved Rock activity since caspase inhibition did not prevent the formation of blebs. A model showing the proposed mechanisms involved in ouabain-induced cell death is summarized in Fig. 11.

In addition to directly triggering apoptosis, ouabain at high micromolar concentrations potentiates anti-FAS-induced apoptosis [39] and sensitizes cells to TNF-induced

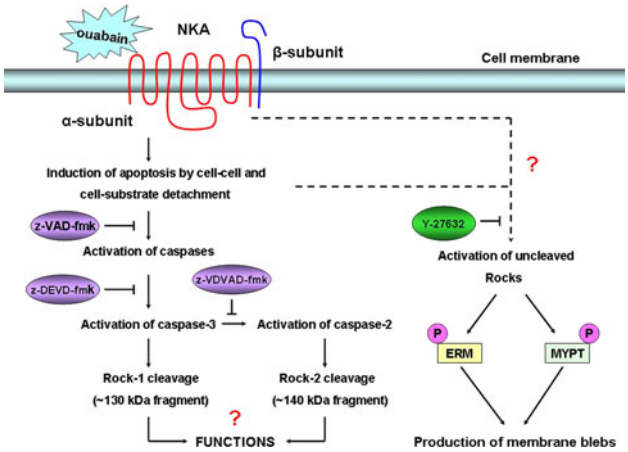

Fig. 11 A model that summarizes Rock pathways involved in ouabain-induced apoptosis. When ouabain is bound to NKA, it causes detachment of cells from each other and the substrate. This detachment triggers apoptosis. Once caspase-3 is activated, it activates caspase-2. Then active caspase-3 cleaves Rock-1 and active caspase-2 cleaves Rock-2, producing 130 and 140 kDa forms of the Rocks, respectively. Usage of caspase inhibitors (z-VAD-fmk, z-DEVD-fmk, Z-VDVAD-fmk) inhibits the cleavage of Rocks but membrane blebbing still exists. However, the functions of the cleaved forms of Rocks are unknown in ouabain-induced apoptosis. The interaction of ouabain and NKA and subsequently the induction of cell detachment also induces the activation of uncleaved forms of Rocks. Activated Rock phosphorylates the ERM and MYPT proteins. This activity is required for bleb formation as blebbing is inhibited by the Rock inhibitor Y-27632

apoptosis [40]. Moreover, Xiao et al. [41] have shown that combinations of sublethal concentrations of both C_2-Ceramide and β-amyloid with 0.1 μM ouabain, which did not cause cell death in our experimental conditions, caused apoptotic neuronal death. Their results indicate that ouabain, even in nanomolar concentrations, may produce apoptotic cell death via sensitization of cells to other apoptotic insults. Indeed, endogenous ouabain levels in the plasma and urinary TNF-α excretions were increased in essential hypertension [6, 42]. Considering that Rock activation involves both ouabain and TNF-α-induced apoptotic cell death [31], the treatment of hypertension with Rock inhibitors may provide an additional benefit to patients with high levels of ouabain and TNF-α.

Recent studies indicate that Rock inhibitors could be useful anti-metastatic and anti-angiogenic chemotherapeutic agents [43–45]. In addition, it is believed that the induction of apoptosis with ouabain and related cardiac glycosides could provide new strategies for anticancer drug development [46]. Here, we showed that Rock inhibition did not change ouabain-induced apoptosis but significantly decreased the detachment of cells from each other. Considering that loss of cell-to-cell adhesion is one of the indispensable processes of cancer cell invasion and metastasis [47], it seems plausible to propose that a combination

treatment of ouabain and Rock inhibitors may be a valuable tool to combat cancer cells. In this circumstance, ouabain will kill the cells and Rock inhibition will prevent the invasion of cancer cells. However, this possibility needs to be evaluated in cancer cell lines and animal models.

In conclusion, our current work includes some new findings on ouabain-induced apoptosis and the involvement of Rho kinase isoforms in the apoptotic machinery. We showed for the first time that ouabain induces both Rock-1 and Rock-2 cleavage via caspase-dependent mechanisms. Moreover, we reveal that a novel cleavage site for caspase-2 exists in the carboxy terminus of Rock-2. These results may be important in the treatment of several diseases such as hypertension and malignancies.

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