FEBS Letters 466 (2000) 70–74 FEBS 23187

Mildly oxidised low density lipoprotein induces platelet shape change via Rho-kinase-dependent phosphorylation of myosin light chain and moesin

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Received 25 November 1999

Edited by Philip Randle

Abstract Oxidised low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. Here we demonstrate that mildly oxidised (mox) LDL engages the GTPase Rho and its effector molecule p160 Rho-kinase to induce phosphorylation of myosin light chain and of moesin leading to platelet shape change. Pretreatment of platelets with the selective Rho inhibitor C3-transferase from *Clostridium botulinum* or with the Rho-kinase inhibitor Y-27632 blocked mox-LDL-induced myosin light chain phosphorylation, moesin phosphorylation and shape change. Mox-LDL did not induce an increase in cytosolic Ca^{2+} during shape change. We propose that Rho/Rho-kinase inhibition could be a strategy for prevention of the pathologic platelet activation during atherogenesis.

Key words: Platelet shape change; Rho-GTPase; Oxidized low density lipoprotein; Rho-kinase; Myosin light chain phosphorylation; Moesin phosphorylation

1. Introduction

Oxidative modification of low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis [1]. Previous studies indicate that oxidatively modified LDL, but not native LDL activates platelets, mildly oxidised LDL (mox-LDL) being more active than heavily oxidised LDL [2,3]. Oxidised LDL accumulates in lipid rich atherosclerotic plaques. Upon plaque rupture it is exposed to circulating platelets, leading to platelet activation and aggregation, intravascular plug formation and potentially to stroke or myocardial infarction [4,5]. The first response of platelets upon activation by mox-LDL and other agonists is platelet shape change [6]. Shape change is a contractile event, triggered by myosin light chain (MLC) phosphorylation [7,8]. Phosphorylation induces a conformational change in MLC that enables actin–myosin interaction and cell contraction. In order to exert the mechanical tension required for shape change actomyosin bundles have to be anchored to the platelet membrane. One actomyosin filament/plasma membrane crosslinker molecule is moesin. Upon phosphorylation on Thr-558, moesin changes its conformation and is then capable of binding to the plasma membrane via the N-terminus and to F-actin with the C-terminus [9]. It was reported recently that MLC phosphorylation and shape change are regulated by two pathways in platelets: Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and Rho-kinase [8,10]. Each of the two pathways can be activated independently of the other dependent on the type and concentration of the platelet agonist [8]. Upon stimulation by GTP-bound Rho, Rho-kinase directly phosphorylates MLC [11] and phosphorylates and thereby inactivates MLC-phosphatase [12–14] to induce shape change [8,10] or contraction of smooth muscle cells [15], fibroblasts [16] or endothelial cells [17,18]. Rho-kinase also phosphorylates and thereby activates the actin filament/plasma membrane crosslinker protein moesin [19,20]. Here we report that (i) mox-LDL induces shape change and MLC phosphorylation via the Rho/Rho-kinase pathway but not via activation of the Ca^{2+}/calmodulin-dependent MLCK, and that (ii) moesin is rapidly phosphorylated in a Rho-kinase-dependent manner under these conditions.

2. Materials and methods

2.1. Materials

The Rho-kinase inhibitor Y-27632 was kindly provided by Akiko Yoshimura, Yoshitomi Pharmaceuticals, Iruma-Shi, Saitama, Japan. Anti-phospho-MLC antibody was a gift from J.M. Staddon, Eisai Company, London, UK, anti-phospho-Thr-558 moesin antibody was a gift from Kozo Kaibuchi, Nara Institute of Science and Technology, Ikoma, Japan. All materials not specified were purchased from Sigma Chemical Co., Deisenhofen, Germany.

2.2. Isolation of platelets and measurement of platelet shape change

Human platelets were treated with acetylsalicylate, isolated by centrifugation in the presence of apyrase as described [8] and resuspended in a buffer containing 20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, and 0.6 U/ml apyrase. Platelet shape change was measured by recording the light transmission in a LABOR aggregometer as described at a cell density of $(2–4)\times10^8$/ml. Y-27632 was added at the given concentration 30 min before stimulation followed by incubation at 37°C. In experiments using C3-transferase, platelets were resuspended in buffer exactly as described [21] and incubated with C3-transferase (400 μg/ml) for 4 h at 37°C. After incubation with C3-transferase or vehicle, platelets were diluted to 10^9/ml for measurement of shape change.

2.3. Preparation of mildly oxidised LDL

LDL was isolated in the continuous presence of EDTA as described [3], dialysed at 4°C using a N_2-saturated buffer (pH 7.4) and then stored at 4°C in darkness under N_2. All LDL concentrations are given in terms of their protein content. Mox-LDL was prepared from EDTA-free LDL by Cu^{2+}-triggered oxidation exactly as described [3].

2.4. Measurement of cytosolic Ca^{2+} concentrations

For measurement of the cytosolic Ca^{2+} concentration, washed platelets were loaded with 4 μM FURA2-AM as described [8]) and resuspended at a cell density of 2×10^8/ml. Ca^{2+} was measured in a thermostatically controlled (37°C) chamber whilst stirring using a Delta-Scan-1 double beam fluorescence spectrophotometer (Photon

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Abbreviations: C3, C3-transferase from *Clostridium botulinum*; mox-LDL, mildly oxidised low density lipoprotein; LPA, lysophosphatidic acid; MLC, myosin light chain; MLCK, MLC-kinase

PII: S0014-5793(99)01762-7

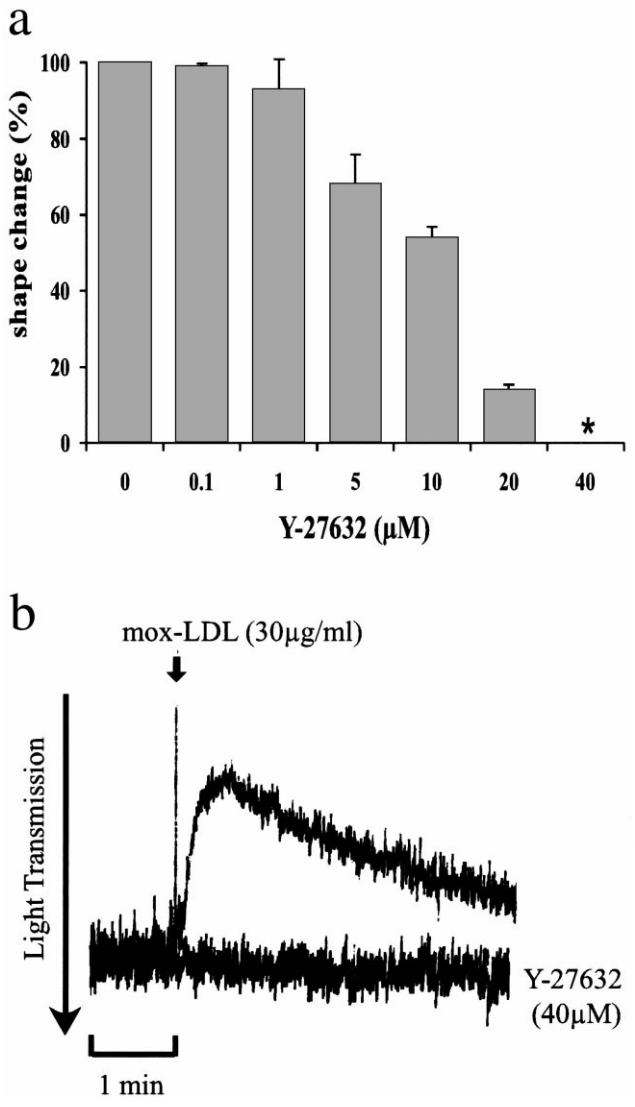

Fig. 1. Effect of Rho-kinase inhibition on mox-LDL-induced platelet shape change. a: Suspensions of washed human platelets were placed in aggregometer cuvettes and incubated with the indicated concentrations of Y-27632 for 30 min and then stimulated with mox-LDL. Values are % of maximal shape change induced by mox-LDL (10–40 μg/ml) in the absence of Y-27632. Data are mean ± S.E.M. of six experiments. b: Shape change tracings of platelets stimulated with mox-LDL (30 μg/ml) in the presence or absence of Y-27632 (30 min, 40 μM). The decrease in light transmission together with the disappearance of rapid oscillations is indicative of shape change.

Technology International). Fluorescence measurement and calculation of cytosolic Ca^{2+} concentrations were performed as described [8].

2.5. Measurement of MLC and moesin phosphorylation

Platelets were stimulated as indicated and measurement of shape change was carried out as described above. Portions of 50 μl were taken at the given time points, transferred to equal amounts of boiling sample buffer (final concentration 62.5 mM Tris, 3% SDS, 5% glycerol, 5% 2-mercaptoethanol, 1 mM EDTA, pH 6.8) and boiled for 5 min. Proteins were then separated by SDS 12.5% PAGE (MLC phosphorylation) or SDS 10% PAGE (moesin phosphorylation) and electroblotted onto PVDF membranes using Mini Protean II electrophoresis cells (Bio-Rad). Membranes were incubated overnight with anti-phospho-MLC antibody (1/500) or anti-phospho-moesin antibody (1/500) in Tris-buffered saline containing 0.05% Tween 20, washed three times, incubated for 1 h with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Amersham) (1/7500 for detecting phospho-MLC, 1/3000 for detecting phospho-moesin) in Tris-buffered saline, washed three times and then developed with Luminol solution (Pierce) and exposed to Kodak X-OMAT films. The amount of phosphorylated MLC or phosphorylated moesin was determined by densitometric analysis of the protein bands that reacted with anti-phospho-MLC or anti-phospho-moesin antibody, using a Sharp XL-325 densitometer and Pharmacia Image Master software. Phosphorylated MLC and phosphorylated moesin appeared as a single band of 20 kDa and 78 kDa, respectively. These 20 kDa and 78 kDa bands were also detected by a mouse monoclonal antibody to total MLC (Sigma, Deisenhofen, Germany) or moesin (Transduction Laboratories, Hamburg, Germany). The range of linearity of the densitometric measurements of phosphorylated myosin and moesin was determined by a dilution series of protein samples of mox-LDL-stimulated platelets (4–40 μg of protein per lane).

3. Results

3.1. Rho-kinase inhibition blocks mox-LDL-induced shape change

To investigate whether mox-LDL induces MLC phosphorylation and platelet shape change via Rho/Rho-kinase or

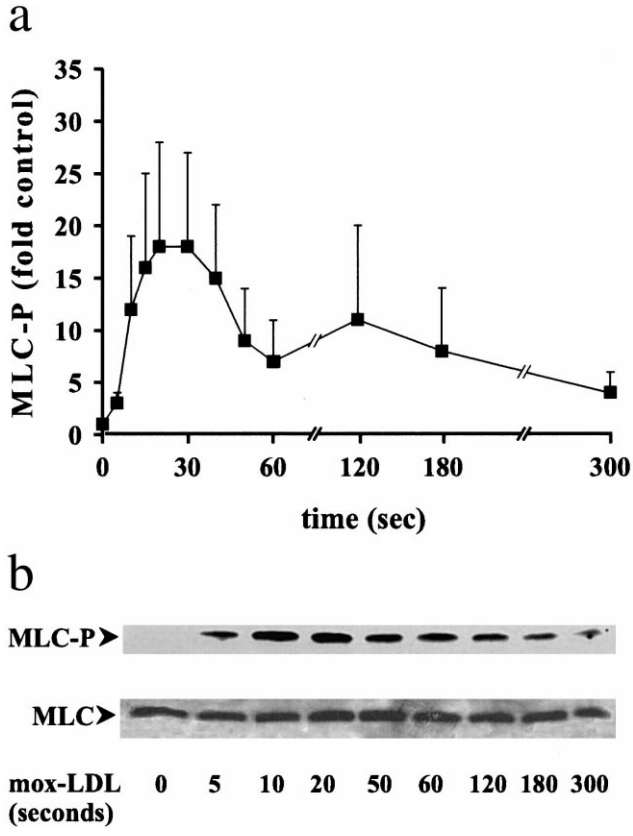

Fig. 2. Stimulation of MLC phosphorylation by mox-LDL during platelet shape change. Suspensions of washed platelets were exposed to mox-LDL (10–40 μg/ml). In parallel to shape change MLC phosphorylation was determined by Western blot using a specific antibody to phosphorylated MLC. The 20 kDa band detected by the anti-phospho-MLC antibody was then analysed densitometrically. a: Time course of MLC phosphorylation in mox-LDL-stimulated platelets. Data are mean of three experiments ± S.E.M. b: Representative Western blot demonstrating the time course of MLC phosphorylation in mox-LDL-stimulated platelets. Equal protein load of MLC was checked by Western blot using an antibody to total MLC (phosphorylated and unphosphorylated MLC).

Ca^{2+}/calmodulin-dependent MLCK we treated platelets with the selective Rho-kinase inhibitor Y-27632 [22,23] as indicated and then stimulated platelets with mox-LDL. Fig. 1a indicates that Y-27632 at concentrations of 5 μM or higher significantly reduced mox-LDL-induced shape change, and that 40 μM Y-27632 induced a complete inhibition. Fig. 1b depicts representative shape change tracings of platelets stimulated with mox-LDL in the presence or absence of Y-27632. These results suggest that mox-LDL induces shape change via activation of Rho-kinase.

3.2. Stimulation of MLC phosphorylation by mox-LDL during shape change

Phosphorylation of the light chain enables myosin to interact with and to slide against F-actin filaments in stress fibres, which are the contractile organelles of non-muscle cells. To measure mox-LDL-induced MLC phosphorylation during platelet shape change we performed Western blots using a specific antibody to phosphorylated MLC. Fig. 2a indicates that MLC phosphorylation in unstimulated platelets was very low. In mox-LDL-treated platelets MLC phosphorylation was elevated about 18-fold after 20 s of stimulation; then MLC phosphorylation fell to a plateau before it reached almost baseline levels after 5 min. Fig. 2b depicts representative Western blots demonstrating the time course of MLC phosphorylation and equal protein load of MLC. Our data indicate that mox-LDL like other platelet activators induces shape change by a rapid and reversible increase in MLC phosphorylation.

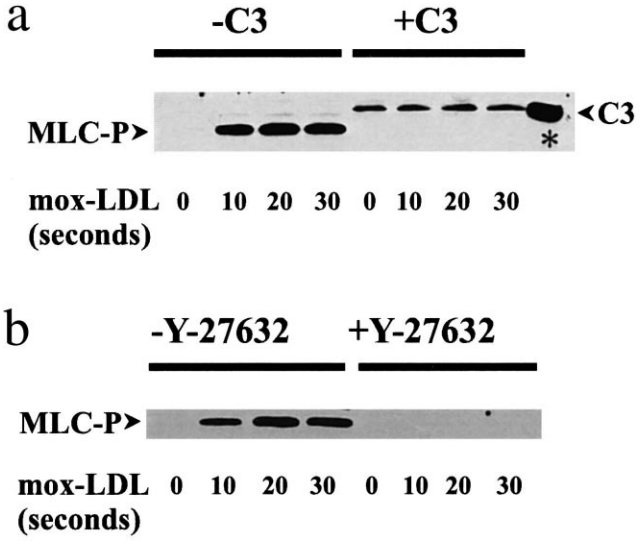

Fig. 3. Effect of Rho/Rho-kinase inhibition on mox-LDL-induced MLC phosphorylation. Platelet suspensions were placed in aggregometer cuvettes and incubated with (a) C3-transferase (4 h, 400 μg/ml) or (b) Y-27632 (30 min, 40 μM) and stimulated with mox-LDL (10–80 μg/ml). MLC phosphorylation was determined in parallel to shape change. Aliquots of the platelet suspensions were transferred to boiling sample buffer for detection of MLC phosphorylation by Western blot using a specific antibody to phosphorylated MLC. Blots are representative of three experiments. C3-transferase cross-reacted with the antibody to phosphorylated MLC. The asterisk indicates a lane where only C3-transferase was loaded (50 μg) to demonstrate the crossreaction.

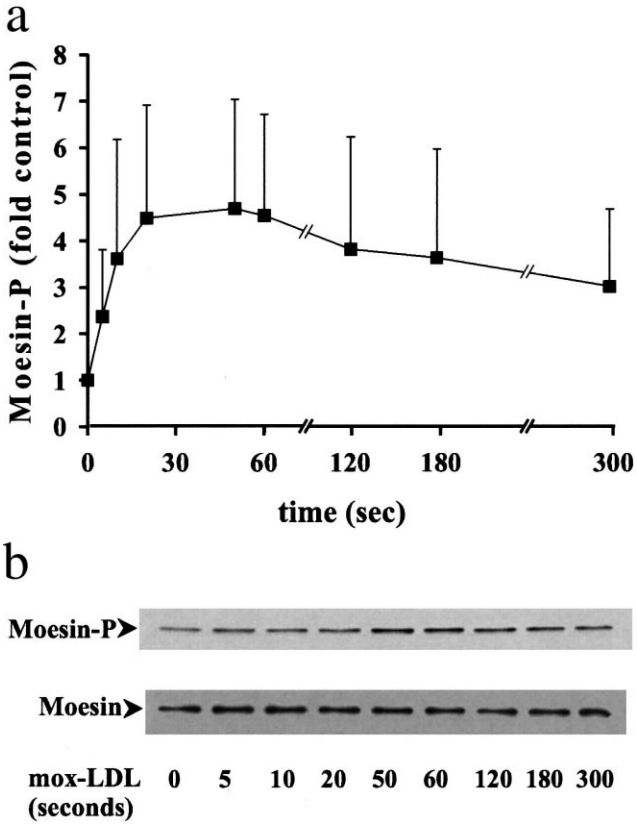

Fig. 4. Stimulation of Thr-558 moesin phosphorylation by mox-LDL during shape change. Suspensions of washed platelets were exposed to mox-LDL (10–40 μg/ml). In parallel to shape change Thr-558 moesin phosphorylation was determined by Western blot using a specific antibody to phosphorylated Thr-558. a: Time course of moesin phosphorylation during mox-LDL-induced shape change. Data are means of three experiments ± S.E.M. b: Representative Western blot indicating moesin phosphorylation during mox-LDL-induced shape change. Equal protein load of moesin was checked by Western blot using an antibody to total moesin (phosphorylated and unphosphorylated moesin).

3.3. Mox-LDL induces MLC phosphorylation via Rho/Rho-kinase

As inhibition of Rho-kinase prevents mox-LDL-induced shape change, inhibition of Rho or Rho-kinase should also block MLC phosphorylation under these conditions. Consequently we treated platelets with C3-transferase from *Clostridium botulinum* to inhibit Rho specifically [24] or with the Rho-kinase inhibitor Y-27632. Thereafter we stimulated platelets with mox-LDL for different time periods as indicated, and then measured MLC phosphorylation. Fig. 3 indicates that inhibition of Rho as well as inhibition of Rho-kinase completely blocked mox-LDL-induced MLC phosphorylation, indicating that mox-LDL activates Rho/Rho-kinase to induce MLC phosphorylation and shape change.

3.4. Mox-LDL induces moesin phosphorylation during platelet shape change

The actin filament/plasma membrane crosslinker protein moesin is phosphorylated during platelet activation by thrombin [9]. Therefore moesin could also be phosphorylated upon platelet activation by mox-LDL. To address this question we measured moesin phosphorylation in platelets stimulated with

mox-LDL, using a specific antibody to moesin phosphorylated on Thr-558. As indicated by Fig. 4 we found that moesin is partly phosphorylated in unstimulated platelets similar as described previously [9]. During shape change induced by mox-LDL, moesin phosphorylation was elevated about 5-fold within a few seconds of stimulation and remained elevated for at least 5 min after stimulation (Fig. 4a). Fig. 4b depicts representative Western blots demonstrating the time course of moesin phosphorylation and equal protein load of moesin. Mox-LDL-induced moesin phosphorylation could be blocked by inhibition of Rho or Rho-kinase. Fig. 5a shows moesin phosphorylation in cells pretreated with C3-transferase and then stimulated with mox-LDL. Fig. 5b depicts moesin phosphorylation in platelets pretreated with Y-27632 and then stimulated with mox-LDL. Both inhibitors markedly reduced mox-LDL-induced moesin phosphorylation. Our data show for the first time that mox-LDL induces moesin phosphorylation via Rho/Rho-kinase during platelet shape change.

3.5. mox-LDL does not increase cytosolic Ca^{2+} during platelet shape change

MLC phosphorylation is regulated by Ca^{2+}/calmodulin-dependent MLCK or by Rho-kinase during platelet shape change [8,10]. To investigate whether also Ca^{2+}/calmodulin-dependent MLCK at least partly contributes to mox-LDL-induced MLC phosphorylation we measured cytosolic Ca^{2+} concentrations. As indicated in Fig. 6, mox-LDL at the concentrations used (10–100 μg/ml) did not increase the cytosolic Ca^{2+} concentration. Under the same conditions thrombin (1 U/ml) induced a significant elevation of the cytosolic Ca^{2+}. These data indicate that MLCK is most likely not involved in mox-LDL-induced MLC phosphorylation in human platelets.

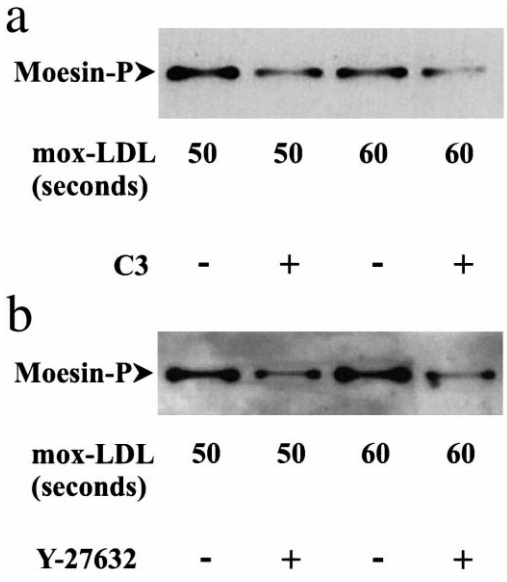

Fig. 5. Effect of Rho/Rho-kinase-inhibition on mox-LDL-induced moesin phosphorylation. Platelet suspensions were placed in aggregometer cuvettes and incubated with (a) C3-transferase (4 h, 400 μg/ml) or (b) Y-27632 (30 min, 40 μM) and stimulated with mox-LDL (10–80 μg/ml). Moesin phosphorylation was determined in parallel to shape change. Aliquots of the platelets were transferred to boiling sample buffer for detection of moesin phosphorylation by Western blot using a specific antibody to phosphorylated moesin. Blots are representative of three experiments.

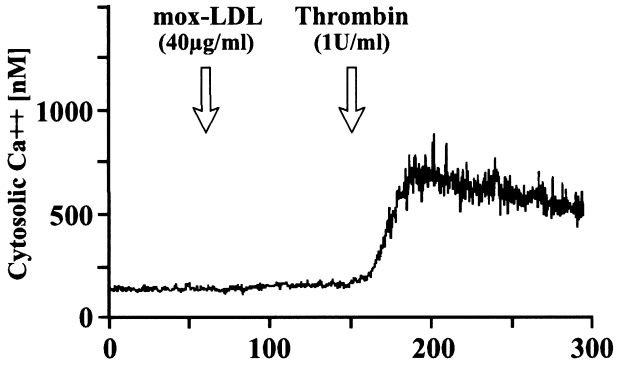

Fig. 6. Effect of mox-LDL on cytosolic Ca^{2+} concentration. Suspensions of Fura2-AM-loaded platelets were placed in fluorimeter cuvettes and exposed to mox-LDL (10–100 μg/ml). Mox-LDL induced no increase in cytosolic Ca^{2+} concentration, whilst platelets responded well to thrombin (1 U/ml). Data are representative of six experiments.

4. Discussion

In the present study we report for the first time that mox-LDL induces platelet shape change via activation of Rho/Rho-kinase. Upon stimulation with mox-LDL, Rho-kinase induces MLC phosphorylation allowing the myosin head interacting with actin to enable the formation of contractile actin/myosin bundles and in parallel phosphorylates moesin which then mediates the interaction of the newly formed bundles with the plasma membrane. Both processes are likely required for induction of shape change. It has been found in this regard that phosphorylation of MLC precedes shape change [25] and that moesin-Thr-558 is phosphorylated during platelet activation by thrombin [9]. Our results further show that Ca^{2+}/calmodulin-dependent MLCK is very likely not activated during mox-LDL-induced shape change, because under the conditions described mox-LDL did not increase the cytosolic Ca^{2+} concentration. On the other hand it could be speculated that basal activities of MLCK could be sufficient to induce MLC phosphorylation leading to shape change when MLC-phosphatase is phosphorylated and inhibited by Rho-kinase.

It is well documented that activation of platelets by mox-LDL is not a toxic effect, but is initiated by activation of receptor-mediated signal pathways [26]. In particular we reported recently that mox-LDL activates the LPA (lysophosphatidic acid) receptor to induce platelet shape change [26]. Therefore LPA should activate platelets by the same signal pathways as mox-LDL. Indeed we found that LPA induces MLC and moesin phosphorylation in a Ca^{2+}-independent manner via Rho/Rho-kinase (unpublished observation). Furthermore we showed recently that mox-LDL engages Rho/Rho-kinase to increase endothelial permeability [18]. This means that the Rho/Rho-kinase pathway could contribute to the pathologic platelet activation and to endothelial injury during cardiovascular diseases. We therefore propose that inhibition of Rho-kinase or Rho could be a preventive or therapeutic strategy to treat cardiovascular diseases.

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft GRK 438, by the Ernst-und-Berta-Grimmke

Stiftung and by the August Lenz-Stiftung. We thank Carola Meister and Barbara Böhlig for expert technical assistance and Prof. P.C. Weber, Institut für Prophylaxe der Kreislaufkrankheiten for support, as well as Martin Aepfelbacher, Max-von-Pettenkofer-Institut für Medizinische Mikrobiologie for helpful discussions and critically reading the manuscript. Anti-phospho-moesin antibody was a generous gift from Kozo Kaibuchi, Nara Institute of Science and Technology, Ikoma, Japan. Anti-phospho-MLC antibody was kindly provided by Dr. J.M. Staddon, Eisai Company, London, UK.

References

[1] Ross, R. (1999) New Engl. J. Med. 340, 115–126.
[2] Meraji, S., Moore, C.E., Skinner, V.O. and Bruckdorfer, K.R. (1992) Platelets 3, 155–162.
[3] Weidtmann, A., Scheidte, R., Hrboticky, N., Pietsch, A., Lorenz, R. and Siess, W. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1131–1138.
[4] Falk, E., Shah, P.K. and Fuster, V. (1995) Circulation 92, 657–671.
[5] Fernandez-Otiz, A., Badimon, J.J., Falk, E., Fuster, V., Meyer, B., Mailhac, A., Weng, D., Shah, P.K. and Badimon, L. (1994) J. Am. Coll. Cardiol. 23, 1562–1569.
[6] Siess, W. (1989) Physiol. Rev. 69, 58–178.
[7] Adelstein, R.S., Conti, M.A. and Barylko, B. (1978) Thromb. Haemost. 40, 241–244.
[8] Bauer, M., Retzer, M., Wilde, J.I., Maschberger, P., Essler, M., Aepfelbacher, M., Watson, S.P. and Siess, W. (1999) Blood 94, 1655–1672.
[9] Nakamura, F., Amieva, M.R. and Furthmayer, H. (1995) J. Biol. Chem. 270, 31377–31385.
[10] Klages, B., Brandt, U., Simon, M.I. and Offermanns, S. (1999) J. Cell Biol. 145, 745–754.
[11] Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996) J. Biol. Chem. 271, 20246–20249.
[12] Kimura, K., Fukata, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1998) J. Biol. Chem. 273, 5542–5548.
[13] Nakai, K., Suzuki, Y., Kihara, H., Wada, H., Fujioka, M., Ito, M., Nakano, T., Kaibuchi, K., Shiku, H. and Nishikawa, M. (1997) Blood 90, 3936–3942.
[14] Suzuki, Y., Yamamoto, M., Wada, H., Ito, M., Nakano, T., Sasaki, Y., Narumiya, S., Shiku, H. and Nishikawa, M. (1999) Blood 93, 3408–3417.
[15] Gong, M.C., Izuka, K., Nixon, G., Broene, J.P., Hall, A., Eccleston, J.F., Sugai, M., Kobayashi, S., Somlyo, A.V. and Somlyo, A.P. (1996) Proc. Natl. Acad. Sci. USA 93, 1340–1345.
[16] Chrzanowska-Wodnicka, M. and Burridge, K. (1996) J. Cell. Biol. 133, 1403–1415.
[17] Essler, M., Amano, M., Kruse, H-J., Kaibuchi, K., Weber, P.C. and Aepfelbacher, M. (1998) J. Biol. Chem. 273, 21867–21874.
[18] Essler, M., Retzer, M., Bauer, M., Heemskerk Aepfelbacher, M. and Siess, W. (1999) J. Biol. Chem. 274, 30361–30364.
[19] Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S. and Tsukita, S. (1998) J. Cell Biol. 140, 647–657.
[20] Shaw, R.J., Henry, M., Solomon, F. and Jacks, T. (1998) Mol. Biol. Cell 9, 403–419.
[21] Leng, L., Kashiwagi, H., Ren, X.D. and Shattil, S. (1998) Blood 91, 4206–4215.
[22] Somlyo, A.P. (1997) Nature 389, 908–908.
[23] Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S. (1997) Nature 389, 990–994.
[24] Aktories, K., Weller, U. and Chantwal, G.S. (1987) FEBS Lett. 212, 109–113.
[25] Daniel, J.L., Molish, I.R., Rigmaiden, M. and Stewart, G. (1984) J. Biol. Chem. 259, 9826–9831.
[26] Siess, W., Zangl, K.J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G. and Aepfelbacher, M. (1999) Proc. Natl. Acad. Sci. USA 96, 6931–6936.