Mildly Oxidized Low Density Lipoprotein Induces Contraction of Human Endothelial Cells through Activation of Rho/Rho Kinase and Inhibition of Myosin Light Chain Phosphatase*

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Mildly oxidized low density lipoprotein (mox-LDL) is critically involved in the early atherogenic responses of the endothelium and increases endothelial permeability through an unknown signal pathway. Here we show that (i) exposure of confluent human endothelial cells (HUVEC) to mox-LDL but not to native LDL induces the formation of actin stress fibers and intercellular gaps within minutes, leading to an increase in endothelial permeability; (ii) mox-LDL induces a transient decrease in myosin light chain (MLC) phosphatase that is paralleled by an increase in MLC phosphorylation; (iii) phosphorylated MLC stimulated by mox-LDL is incorporated into stress fibers; (iv) cytoskeletal rearrangements and MLC phosphorylation are inhibited by Ca2+ from transferase from Clostridium botulinum, a specific Rho inhibitor, and Y-27632, an inhibitor of Rho kinase; and (v) mox-LDL does not increase intracellular Ca2+ concentration. Our data indicate that mox-LDL induces endothelial cell contraction through activation of Rho and its effector Rho kinase which inhibits MLC phosphatase and phosphorylates MLC. We suggest that inhibition of this novel cell signaling pathway of mox-LDL could be relevant for the prevention of atherosclerosis.

The response to injury hypothesis of atherosclerosis proposes that the first step in atherogenesis is an endothelial dysfunction induced by stimuli such as mildly oxidized LDL (mox-LDL)1 (1). Vascular endothelium exposed to oxidized LDL in vitro and in vivo shows an increased permeability (2, 3), a hallmark of endothelial dysfunction. The signal pathways by which mox-LDL and oxidized LDL reduce endothelial barrier function are unknown. Confluent endothelial cells challenged with vasoactive substances, such as thrombin, show a rapid reorganization of their actin cytoskeleton, i.e. stimulation of myosin light chain (MLC) phosphorylation and actin stress fiber formation, leading to cell contraction and intercellular gaps (4, 5). Such cytoskeletal rearrangements reduce endothelial barrier function (6) and are typically found in vivo in endothelium overlying early atherosclerotic lesions (7).

Thrombin induces endothelial cell contraction and increased endothelial permeability through binding to a serpentine receptor which is coupled via heterotrimeric G-proteins of the Gq family to phospholipase Cb to yield inositol 1,4,5-trisphosphate, which mobilizes Ca2+ from intracellular stores and thus increases cytosolic Ca2+ concentration (8). This increase in intracellular Ca2+ concentration leads to activation of Ca2+/calmodulin-dependent myosin light chain kinase (MLCK), which phosphorylates Thr-18 and Ser-19 of the MLC of myosin II to enable actin-myosin interaction and cell contraction (9). We and others recently found an additional pathway by which thrombin regulates endothelial cell contraction. Thrombin, probably through coupling of the thrombin receptor to heterotrimeric G-proteins of the G12/13 family (10), was shown to activate the Ras-related GTPase Rho and its effector p160 Rho kinase in endothelial cells (5). Rho kinase phosphorylates myosin binding subunit of MLC phosphatase which is thereby inactivated (11–13). Indeed, we observed that thrombin transiently inactivated MLC phosphatase in human endothelial cells and that phosphatase inactivation correlated with peak levels in MLC phosphorylation. Hence, the Rho/Rho kinase pathway seems to regulate endothelial contractility in concert with Ca2+/calmodulin-dependent MLCK. A similar regulatory system was reported in smooth muscle cells (14–16).

This study was aimed to investigate whether mox-LDL would mimic the action of vasoactive substances on human endothelial cells. We found that (i) mildly oxidized LDL induced a rapid reorganization of the actin cytoskeleton, the formation of intercellular gaps, and the stimulation of MLC phosphorylation within minutes and (ii) that mox-LDL induced these changes through stimulation of the Rho/Rho kinase pathway with the subsequent inhibition of MLC phosphatase and without increasing the cytosolic Ca2+ concentration.

EXPERIMENTAL PROCEDURES

Materials—Rho kinase inhibitor Y-27632 was kindly provided by Yoshitomi Pharmaceuticals, 3–7-25 Kayota, Iruma-Shi Saitama, Japan. Anti-phospho-MLC antibody was a generous gift from Dr. James Staddon, EISAI Co., London, UK. All other materials not further specified were from Sigma, Deisenhofen, Germany.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described previously (5). Before exposure to mox-LDL or native LDL, cells were serum deprived for 15 min. Medium was then replaced by fresh serum-free medium containing mox-LDL or native LDL as indicated.

The abbreviations used are: mox-LDL, mildly oxidized low density lipoprotein; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; LPA, lysophosphatidic acid; MLC, myosin light chain; MLCK, myosin light chain kinase; PBS, phosphate-buffered saline.

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Mildly Oxidized LDL Regulates MLC Phosphatase via Rho Kinase

**RESULTS**

**Mox-LDL Stimulates Actin Stress Fiber Formation via Rho/Rho Kinase**—As shown in Fig. 1, exposure of confluent endothelial monolayers to mox-LDL (250 μg/ml) for 2 min changed their cobblestone morphology with a polygonal cell shape and a dense peripheral actin ring to a contracted phenotype with rounded cells, prominent actin stress fibers, and intercellular gaps (Fig. 1b). To test whether mox-LDL induces actin stress fibers and cell contraction via Rho and Rho kinase, we stained control cells or cells stimulated with mox-LDL for F-actin using rhodamine-phallolidin. As indicated by Fig. 1a, control cells showed a dense peripheral actin ring and almost no stress fibers. Endothelial cells stimulated with mox-LDL (2 min, 250 μg/ml) showed prominent actin stress fibers and a contracted phenotype with intercellular gaps (Fig. 1b). To investigate whether these actin rearrangements are because of activation of Rho/Rho kinase, we pretreated cells with the selective Rho-inhibitor C3 transferase from Clostridium botulinum (24 h, 5 μg/ml) or the Rho kinase inhibitor Y-27632 (30 min, 10 μM) and then stimulated the cells with mox-LDL. C3 transferase (Fig. 1c) or Y-27632 (Fig. 1d) did not influence the actin distribution in unstimulated HUVEC, but C3 transferase (Fig. 1d) and Y-27632 (Fig. 1f) completely blocked the mox-LDL-induced actin stress fiber formation and cell contraction, indicating that mox-LDL induced these cytoskeletal rearrangements through activation of Rho and Rho kinase. In contrast to mox-LDL, native LDL had no effect on the actin cytoskeleton in human endothelial cells (Fig. 1g).

**Mox-LDL Induces an Increase in Endothelial Permeability That Is Mediated by Rho/Rho Kinase**—Endothelial cell con-
traction and intercellular gap formation are expected to increase endothelial permeability. Indeed in our experimental setting, mox-LDL (2 min, 250 μg/ml), but not native LDL, markedly increased transendothelial diffusion of horseradish peroxidase (Fig. 2). Pretreatment of cells with C3 transferase (24 h, 5 μg/ml) or Y-27632 (30 min, 10 μM) blocked the mox-LDL-induced increase in permeability. These findings suggest that activation of Rho/Rho kinase is critically involved in mox-LDL-induced increase in endothelial permeability.

Mildly Oxidized LDL Regulates MLC Phosphatase via Rho Kinase

**Fig. 2.** Mox-LDL increases transendothelial diffusion of HRP in a Rho/Rho kinase-dependent manner. HUVEC were incubated without or with 5 μg/ml C3 transferase (C3) from *C. botulinum* for 24 h or for 30 min with 10 μM Rho kinase inhibitor Y-27632 and exposed to mox-LDL or native LDL (2 min, 250 μg/ml). Transendothelial diffusion of HRP was determined spectrophotometrically as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of 5–8 measurements.

**Fig. 3.** Mox-LDL stimulates MLC phosphorylation and inhibits MLC phosphatase activity. *a*, MLC phosphatase activity was measured in HUVEC stimulated with mox-LDL (250 μg/ml) for different time periods. Phosphatase inactivation was maximal between 30 s and 2 min of stimulation. Results are mean ± S.E. of three experiments. *b*, HUVEC were stimulated with mox-LDL (250 μg/ml) for different time periods. MLC phosphorylation was determined by Western blot using a specific antibody to phosphorylated MLC. MLC phosphorylation was maximal after 2 min of stimulation. Results are the mean ± S.E. of seven experiments. The asterisk (*) indicates MLC phosphorylation after stimulation of endothelial cells with native LDL (2 min, 250 μg/ml).

**Fig. 4.** Mox-LDL-induced MLC phosphorylation is mediated by Rho/Rho kinase. HUVEC were stimulated with mox-LDL (250 μg/ml) as indicated. MLC phosphorylation was then determined by Western blot using an antibody to phosphorylated MLC. The lower molecular weight band is because of an unspecific cross-reaction with the secondary antibody. In cells treated with C3 transferase (24 h, 5 μg/ml) or with Y-27632 (Y) (30 min, 10 μM), mox-LDL-induced (2 min, 250 μg/ml) MLC phosphorylation was completely blocked. A Western blot representative of three similar experiments is shown.

**Fig. 5.** Mox-LDL induces incorporation of phospho-MLC into stress fibers. Human endothelial cells were not stimulated (*a*), stimulated with mox-LDL (2 min, 250 μg/ml) (*b*), treated with C3 transferase (24 h, 5 μg/ml) and then stimulated with mox-LDL (*c*), pretreated with Y-27632 (30 min, 10 μM) and then stimulated with mox-LDL (*d*), or treated with n-LDL (2 min, 250 μg/ml) (*e*). Cells were then fixed and stained for phosphorylated MLC.

**Fig. 6.** Mox-LDL does not increase cytosolic Ca²⁺ concentration. HUVEC were loaded with Fura 2-AM and stimulated with mox-LDL (150 μg/ml). Cytosolic Ca²⁺ concentration was then traced in 38 single cells. mox-LDL yielded no increased intracellular Ca²⁺-levels, whereas cells responded well to thrombin (1 unit/ml). LDL-induced increase in permeability. These findings suggest that activation of Rho/Rho kinase is critically involved in mox-LDL-induced increase in endothelial permeability.

**Mox-LDL Inactivates MLC Phosphatase and Enhances MLC Phosphorylation in Human Endothelial Cells**—Phosphorylation of the light chain enables myosin to interact with and to slide against F-actin filaments in stress fibers, which are con-
sidered the contractile organelles of non-muscle cells such as endothelial cells (9). If in fact mox-LDL stimulates Rho/Rho kinase, this should lead to inhibition of MLC phosphatase followed by MLC phosphorylation (5). Indeed, MLC phosphatase activity was found to be rapidly reduced to about 60% of the basal activity between 30 s and 2 min of mox-LDL treatment, which was followed by a return to base-line values after 5 min (Fig. 3a). To determine the corresponding levels of phosphorylated MLC, we performed Western blots using a phospho-MLC specific antibody. Fig. 3b shows a time course of MLC phosphorylation in endothelial cells stimulated with mox-LDL. MLC phosphorylation rose to a peak within 2 min of stimulation and then fell to base-line levels within 15 min. In contrast to mox-LDL, native LDL did not induce MLC phosphorylation in HUVEC. To demonstrate that the mox-LDL-induced MLC phosphorylation is because of Rho/Rho kinase activation, we pretreated HUVEC with C3 transferase (24 h, 5 μg/ml) or Y-27632 (30 min, 10 μM). The results presented in Fig. 4 demonstrate that inhibition of Rho or Rho kinase completely blocked mox-LDL-induced MLC phosphorylation. To investigate the intracellular distribution of phosphorylated MLC in HUVEC after mox-LDL stimulation, cells were stained for phosphorylated MLC and MLCK were 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 Co., London, UK.

**REFERENCES**

1. Ross, R. (1999) *N. Engl. J. Med.* 340, 115–126
2. Rangaswamy, S., Penn, M. S., Saidel, G. M., and Chisolm, G. M. (1997) *Circ. Res.* 80, 73–74
3. Liao, L., Aw, T. Y., Kvietys, P. R., and Granger, D. N. (1995) Arterioscler. *Thromb. Vasc. Biol.* 15, 2305–2311
4. Wojick-Stohard, B., Entwistle, A., Garg, R., and Ridley, A. J. (1998) *J. Cell. Physiol.* 176, 150–165
5. Essler, M., Amano, M., Kruse, H-J., Kaibuchi, K., Weber, P. C., and Aeppelbacher, M. (1998) *J. Biol. Chem.* 273, 21867–21874
6. van Erpdenburg, V. W. M. (1997) Arterioscler. *Thromb. Vasc. Biol.* 17, 1018–1023
7. Colangelo, S., Langille, B. L., Steiner, G., and Golieb, A. I. (1997) Arterioscler. *Thromb. Vasc. Biol.* 18, 52–56
8. Grund, R. J. A., Turnell, A. S., and Graham, P. W. (1996) Biochem. *J.* 313, 353–368
9. Hoeckler, Z. M., and Wysolmerski, R. B. (1995) *J. Cell Biol.* 130, 613–627
10. Offermanns, S., Laugwitz, K. L., Spicher, K., and Schultz, G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 504–508
11. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Teng, Y., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *Science* 273, 245–248
12. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kikunaka, A., Morii, N., and Narumiya, S. (1996) *EMBO J.* 15, 1865–1873
13. Leung, T., Shen, X., Manser, E., and Lim, L. (1996) *Mol. Cell. Biol.* 16, 5313–5327
14. Gong, M. C., Izuka, K., Nixon, G., Broene, J. P., Hall, A., Eccleston, J. F., Sugui, M., Kobayashi, S., Somlyo, A. V., and Somlyo, A. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1340–1345
15. Fu, X., Gong, M. C., Jia, T., Somlyo, A. V., and Somlyo, A. P. (1998) *FEBS Lett.* 440, 183–187
16. Sosimo, A. P. (1997) *Nature* 389, 908–909
17. Shirazi, A., Lizuoka, K., Fadden, P., Mosse, C., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. (1994) *J. Biol. Chem.* 269, 31596–31606
18. Weidmann, A., Scheithle, K., Hrbezick, N., Petesch, A., Lorenz, R., and Siess, W. (1995) Arterioscler. *Thromb. Vasc. Biol.* 15, 1131–1138
19. van IJzendoorn, S. C., van Gool, R. G., Reutelingsperger, C. P., and Aepfelbacher, M. (1996) *Arterioscler. Thromb. Vasc. Biol.* 16, 3382–3385
20. Essler, M., Hermann, K., Amano, M., Kaibuchi, K., Heesemann, J., Weber, P. C., and Aeppelbacher, M. (1996) *J. Immunol.* 157, 2453–4559
21. Göhlke, A., Harhammer, R., and Schultz, G. (1996) *J. Biol. Chem.* 271, 24583–459
22. Chrzanzowska-Wodnicka, M., and Burridge, K. (1996) *J. Cell Biol.* 133, 1403–1415
23. Auge, N., Fitoussi, G., Bascan, J-L., Pieraggi, M-T., Junquero, D., Valet, P., Girolami, J-P., Salvayre, R., and Negre-Salvayre, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 871–880
24. Uehata, M., Ishizaki, T., Sato, H., Uno, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) *Nature* 389, 990–994

**DISCUSSION**

Here we describe a novel signal pathway of mox-LDL in human endothelial cells. Mox-LDL induces cell contraction, i.e. formation of actin stress fibers and intercellular gaps, leading to an increase in endothelial permeability, through activation of the Rho/Rho kinase pathway. This mechanism potentially contributes to endothelial dysfunction in early atherosclerotic lesions. Down-regulation of MLC phosphatase could also be a mechanism by which mox-LDL enhances the effects of Ca^{2+}-mobilizing vasoactive substances. Conceivably, continuous stimulation of the vascular endothelium by subendothelial mox-LDL may eventually lead to an enhanced susceptibility of the endothelium to such vasoactive substances.

Our data indicate that mox-LDL utilizes physiologic signal mechanisms to activate the endothelium. Although we have not determined in the present study the mox-LDL-stimulated signal pathways upstream of Rho, we suggest that mox-LDL stimulates the Rho/Rho kinase pathway in endothelial cells through activation of heterotrimeric G-protein-coupled receptors such as the lysophosphatidic acid (LPA) receptor. We have indeed recently found that LPA is formed during mild oxidation of LDL in the absence of cells and that LPA was the main ingredient in mox-LDL responsible for the induction of platelet shape change and endothelial cell activation (21). In fibroblasts, LPA induces Rho-dependent stress fiber formation through activation of G_{13} (22) and enhances contractility by this way (23).

Activation of Rho and Rho kinase by mox-LDL may not be restricted to endothelial cells. It is likely that mox-LDL activates the same pathway in smooth muscle cells and fibroblasts. Indeed, it is well documented that Rho kinase plays a pivotal role for Ca^{2+} sensitization of vascular smooth muscle cells. Furthermore mox-LDL was found to induce vascular smooth muscle cell contraction (24). Hence mox-LDL could contribute to elevated levels of blood pressure by activation of Rho kinase and thereby enhanced contractility of smooth muscle cells. Indeed, the Rho kinase inhibitor Y-27632 has previously been found to reduce the blood pressure in hypertensive rats (25).

In conclusion, we propose that the Rho/Rho kinase pathway is critical for the activation of endothelial cells by mox-LDL and could be a new target to prevent atherogenesis and cardiovascular disease.

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