Oxidized LDL and its Compound Lysophosphatidylcholine Potentiate AngII-Induced Vasoconstriction by Stimulation of RhoA

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Abstract. RhoA stimulates vascular tone by increasing smooth muscle Ca\(^{2+}\) sensitivity, e.g., in atherosclerosis. This study was an investigation of the influence of oxidized LDL (Ox-LDL), which accumulates in atherosclerotic plaques, on vascular tone induced by angiotensin II (AngII), with particular emphasis on the RhoA pathway. OxLDL had no influence on unstimulated vascular tone of isolated rabbit aorta, but it potentiated contractile responses induced by AngII. The Ca\(^{2+}\)-antagonist felodipin partially prevented potentiation of contractile responses, whereas the AT\(_1\) receptor antagonist losartan blunted AngII responses in presence and in absence of OxLDL. Rho-kinase inhibition by Y27632 abolished potentiation of contractile responses, and RhoA inhibition by C3-like transerase partially prevented it, suggesting that OxLDL activated RhoA. Activation of RhoA was further analyzed by detection of its translocation to the cell membrane after stimulation with OxLDL. Western blot analysis of aorta homogenates, as well as direct visualization in cultured smooth muscle cells using confocal laser scan microscopy, revealed that OxLDL potently activated RhoA. The effect of OxLDL was mimicked by its compound lysophosphatidylcholine, and C3 inhibited both lysophosphatidylcholine and OxLDL-induced RhoA stimulation. In conclusion, OxLDL stimulates the RhoA pathway, resulting in potentiation of AngII-induced vasoconstriction. Lysophosphatidylcholine mimics the OxLDL effect, consistent with a causal role of this OxLDL compound. Stimulation of RhoA by OxLDL may contribute to vasospasm in atherosclerotic arteries.

Vascular reactivity is altered under the conditions of atherosclerosis and hypercholesterolemia. In particular, attenuation of endothelium-dependent dilation has frequently been described in animal models of hypercholesterolemia (1), but it has also been found in the human forearm or in the human coronary circulation of individuals with known hypercholesterolemia or atherosclerosis (2–4). Attenuation of endothelium-dependent dilations results in an increase in vascular smooth muscle tone, but enhanced smooth muscle tone can also be caused by enhanced responses to constrictor agonists. While the majority of investigations concentrated on the impact of atherosclerosis and hypercholesterolemia on endothelium-dependent, particularly NO-mediated, dilations, it is well known that alterations of vascular reactivity are not restricted to endothelial mechanisms. Enhanced vascular smooth muscle contractility has been observed in hypercholesterolemic monkeys (5), dogs (6), miniature swine (7), and rabbits (1,8). On a molecular basis, it has been suggested that effects of LDL on Ca\(^{2+}\) homeostasis may contribute to enhanced vascular contractility, beside its known negative impact on NO-mediated dilations. Although native and oxidized LDL increase intracellular Ca\(^{2+}\) in endothelial cells (9), which is thought to enhance NO-synthase activity, LDL inactivate NO at the same time (8). On the other hand, they further increase Ca\(^{2+}\) in smooth muscle cells (10,11). Both mechanisms lead to an enhanced contraction of smooth muscle cells. Indeed, in a previous study from our laboratory, we could demonstrate that oxidized LDL (OxLDL) potentiates agonist-induced constrictor responses (CR) and that this effect could be diminished by various Ca\(^{2+}\)-antagonists (12). Of note, the inhibitory effect of the Ca\(^{2+}\)-antagonists on potentiation of CR was only approximately 50 to 70%, and the OxLDL-induced potentiation of CR was clearly independent of the vascular endothelium. Thus, additional mechanism(s) beside increased intracellular Ca\(^{2+}\) levels seemed to be relevant.

Contraction of smooth muscle cells is not only regulated by the cytosolic calcium concentration via Ca\(^{2+}\)-dependent myosin light chain phosphorylation, but also by changes in the
activity of the smooth muscle myosin phosphatase (SMMP) that is regulated by the RhoA-Rho-kinase pathway (13). We recently showed that OxLDL stimulates the constrictor apparatus in isolated resistance arteries (14), and we found evidence that OxLDL activates both a Ca$^{2+}$-dependent and a Rho- and Rho-kinase-dependent mechanism in the microcirculation. Interestingly, it has been demonstrated that Ca$^{2+}$ sensitization by upregulation of Rho-kinase plays a key role for vasospasm in large conduit arteries, for example in porcine coronary arteries (15); in the latter study, serotonin induced coronary spasm at the site of locally upregulated Rho-kinase. More recently, it has been shown that Rho-kinase inhibitors prevent agonist-induced vasospasm in human internal mammary artery (16), that Rho-kinase activity contributes to enhanced cerebral vascular tone (17), and that Rho-kinase is involved in agonist-induced contraction of human atherosclerotic arteries (18). Thus, the activity of the RhoA/Rho-kinase pathway may be of particular relevance for the vascular response to constrictor agonists in large arteries, especially in those segments that exhibit atherosclerotic plaques known to contain large quantities of OxLDL.

The aim of the present study was to analyze the impact of OxLDL — and of lyso phosphatidylcholine (LPC), a compound of OxLDL formed during the lipoprotein oxidation process (19,20) on vascular tone in large conduit arteries (isolated rabbit aortic rings). Angiotensin II (AngII), as well as OxLDL, is present in atherosclerotic plaques of large arteries (21,22). Thus, a potential interaction between OxLDL and AngII on CR is of particular pathophysiologic relevance, and we therefore investigated the impact of OxLDL on AngII-induced CR. To further analyze the effect of OxLDL on the RhoA pathway, we specifically blocked Rho-kinase by Y27632 (23) and inhibited RhoA in isolated arteries using the C3 exoenzyme from Clostridium limosum, which inactivates RhoA by ADP-ribosylation (24). Furthermore, activation of RhoA was analyzed by studying its translocation from the cytosolic to the membranous fraction, which has been shown to be associated with Ca$^{2+}$ sensitization of smooth muscle cells (25). For this purpose, we investigated the cytosolic and membrane distribution of RhoA in homogenates of aortic rings. In addition, we performed immunocytochemistry with a RhoA-specific antibody in cultured bovine aortic smooth muscle cells (SMC) using confocal laser scan microscopy for direct visualization of RhoA translocation. To exclude that the potential interaction between OxLDL and AngII on CR is due to an alteration of endothelium-derived nitric oxide, we also performed experiments in endothelium-denuded segments and in aortic rings treated with the NO-synthase inhibitor N$^{3}$-nitro-L-arginine.

**Materials and Methods**

**Reagents**

Krebs-Heps buffer was of the following composition: 1.8 mmol/L CaCl$\times 2$ H$_2$O, 2.6 mmol/L KCl, 0.5 mmol/L MgCl$\times 6$ H$_2$O, 137 mmol/L NaCl, 9.1 mmol/L Hepes, 2.8 mmol/L glucose, 0.35 mmol/L NaH$_2$PO$_4$, pH 7.3. Pentobarbital was from Byk Gulden (Konstanz, FRG). Phenylephrine (PE) was from Hoechst (Frankfurt, FRG). AngII, lyso phosphatidylcholine (LPC), sodium nitroprusside (SNP), ace-tycholine (ACh), ethylene-diamine-tetraacetic acid (EDTA), N$^{3}$-nitro-L-arginine, and butylated-hydroxy-toluene (BHT) were from Sigma (Munich, FRG). Y27632 [((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl))] was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd., Osaka, Japan. Felodipin was a generous gift from Astra Chemicals (Wedel, FRG). Losartan was a generous gift from Merck, Sharp & Dome (Munich, FRG). All drugs if not indicated otherwise were further diluted in Krebs-Heps buffer.

**Isolation and Oxidation of LDL**

Pooled human LDL was obtained from healthy subjects from the local blood bank, isolated by ultracentrifugation, oxidized by CuSO$_4$, and analyzed as described recently (14). Lipoproteins were prepared freshly every 2 wk. The degree of oxidation was quantified by the increase in relative mobility on agarose gel (lipidophor electrophoresis kits, IMMUNO, Heidelberg, FRG), indicating an enhanced negative charge of oxidized LDL (26). The mobility of OxLDL on agarose gel electrophoresis as an index for lipoprotein oxidation was 2.5-fold to 3.0-fold increased compared with native LDL.

**Preparation of the C3-Like Exoenzyme**

C3 and C3-like transferases such as the C. limosum C3-like exoenzyme specifically inactivate RhoA by an ADP-ribosylation step (27). To enable uptake of the C3-like exoenzyme into the cells of aortic rings, a fusion toxin consisting of the N-terminal part of C2IN (binding component of the C. botulinum C2 toxin; characterized in reference 28), and the C3-like exoenzyme from C. limosum was constructed, expressed, and purified as described (24). The binding and translocation component C2II from the C. botulinum C2 toxin was purified as recombinant protein and activated with trypsin as described (29). Uptake into cells and efficacy of the C2II/fusion toxin complex has been described elsewhere (24).

**Blood Vessel Preparation and Measurement of Tension**

The care of the animals and the experimental procedures performed in this study were in strict accordance with the standards and guidelines provided by German animal protection laws. Isolated rabbit aortic rings were superfused with Krebs-Heps buffer under isometric conditions, and force development was measured as described previously in more detail (20). Briefly, the thoracic aorta was removed from New Zealand white rabbits of either sex (4 to 5 mo old, 2.5 to 3.5 kg, sacrificed by exsanguination after an intraperitoneal injection of pentobarbital, 300 to 400 mg/ml) and cut into rings (3- to 4-mm long). In some experiments, the endothelium was removed mechanically as described (20). The ring segments were mounted into organ baths (volume 2 ml) and connected to a strain gauge transducer (Hugo Sachs, Hugstetten, FRG) to record changes in isometric tension. The aortic rings were superfused at a constant rate (3 ml/min) with Krebs-Heps buffer (37°C, bubbled with a 95% O$_2$ and 5% CO$_2$ gas mixture). At the beginning of each experiment, rings were stretched to a resting tension of 2.5 g to optimize vasconstrictor responses and then contracted with phenylephrine (PE, 1 μmol/L). Once the contractions had reached a plateau, the endothelial integrity of the preparations was verified by adding 1 μmol/L ACh to the superfusate. Only arteries with a vaso dilator response of > 70% reversal of preconstriction were considered endothelium-intact.

**Analysis of Constrictor Responses**

To determine a threshold dose for AngII that elicits only minimal constrictor responses, a wide range of concentrations of AngII (0.001 nmol/L to 0.1 μmol/L) was used. According to the resulting concen-
The concentration response curves, 0.01 to 0.3 nmol/L AngII was identified as threshold dose.

To study the influence of OxLDL (100 μg/mL) on basal tone and on AngII-induced constrictor responses, rings were initially superfused with OxLDL in the absence of AngII, and eventual changes of basal tone were noted. After washout of OxLDL for approximately 30 min, AngII was added at threshold dose of 0.3 nmol/L. When a stable constrictor response was induced by AngII, OxLDL was again added to the superfusate. In another set of experiments, complete concentration-response curves to AngII (0.001 nmol/L to 0.1 μmol/L) were constructed in the absence or presence of OxLDL. In part of the experiments, LPC (100 μg/mL) was used instead of OxLDL. The impact of felodipin, losartan, Y27632, or C3-like transerase on CR elicited by OxLDL and AngII was determined by adding the substances or their solvents as controls to the superfusate. The concentrations and pretreatment periods of the various substances are given in the results section.

Cell Cultures

Bovine aortic SMC were purchased from Clonetics (Walkersville, MD) and cultured in smooth muscle cell basal medium supplemented with gentamycin (50 μg/ml), amphotericin B (50 ng/ml), human recombinant epidermal growth factor (0.5 μg/ml), human fibroblast growth factor (1.0 μg/ml), 5 mg/ml bovine insulin (all from Clonetics), and 5% fetal calf serum (Life Technologies, Karlsruhe, Germany). Cells (approximately 10⁶) were incubated with OxLDL (5 μg/ml) or its buffer as control and taken for immunocytochemistry.

Analysis of RhoA Stimulation

Western Blotting. Translocation of RhoA from the cytosolic to the particulate fraction as parameter of its stimulation was determined as described by Gong et al. (25). Briefly, pieces of aorta were homogenized in ice-cold buffer (10 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM EDTA, 250 mM sucrose, 1 mM DTT) + protease inhibitors (Complete; Roche, Mannheim, Germany). For separation of particulate and cytosolic fractions homogenates were centrifuged at 100,000 × g for 30 min at 4°C, and the supernatants were collected as the cytosolic fraction. Pellets (the membrane fraction) were resuspended in the same buffer. Samples (5 μg of protein each) were separated on 15% SDS-polyacrylamide gels and then blotted onto polyvinylidene difluoride membrane and labeled with monoclonal anti RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody (1:1000), followed by peroxidase labeled anti-mouse secondary antibody. Blots were detected with enhanced chemiluminescence (ECL, Amersham, Pharmacia, Biotech).

Immunocytochemistry. SMC were seeded on coverslips and cultured in serum-free media for 20 h. After stimulation with OxLDL for various time points, cells were fixed in 4% paraformaldehyde at 4°C for 10 min, washed, incubated in 50 mM NH₄Cl for 10 min, and washed again. After incubation in 0.1% Triton X100 in PBS for 10 min, cells were blocked with 10% goat serum in PBS containing 0.1% Triton X100 for 30 min. Immunostaining was carried out with monoclonal mouse anti RhoA (Santa Cruz, CA) diluted 1:200 in 10% goat serum in PBS containing 0.1% Triton X 100 for 60 min at 37°C. After washing, Cy 3–conjugated goat anti-mouse (Dianova, Hamburg, Germany) diluted 1: 2000 in 10% goat serum in PBS containing 0.1% Triton X100 was used as a secondary antibody. Specimens were embedded with Mowiol (Calbiochem, La Jolla, CA) and examined using an argon/krypton laser confocal microscope (Leica, Solms, Germany) with an excitation wavelength of 514 nm.

Statistical Analyses

Data are presented as means ± SEM. The n-value refers to the number of animals from which aortic segments were obtained. In each experiment, three to five rings obtained from one animal were compared in different treatments. Comparisons in the Figures 1 to 3 were performed using paired t test. EC₅₀ values were calculated by non-linear regression analysis using the PRISM statistical software (Graph Pad Software, San Diego, CA). Differences were considered signifi-

**Figure 1.** Oxidized LDL (OxLDL) and lysophosphatidylcholine (LPC) potentiate angiotensin II (AngII)-induced constrictor responses in rabbit aorta. (Top) Original recording of vascular tone (g) of an isolated rabbit aortic ring. Stimulation with OxLDL (100 μg/mL) alone had no effect on vascular tone. After washout of OxLDL, a threshold concentration of AngII (0.3 nmol/L) induced a weak constrictor response. In the presence of AngII, OxLDL induced a strong increase in vascular tone. (Middle) Bar graph summary of the effect of OxLDL (100 μg/mL) on constrictor responses induced by a threshold concentration of AngII (0.3 nmol/L). OxLDL significantly increased AngII-induced vascular tone. n = 14 experiments, * P < 0.05 AngII versus OxLDL + AngII. (Bottom) Bar graph summary of the effect of LPC (100 μg/mL) on constrictor responses induced by a threshold concentration of AngII (0.3 nmol/L). LPC significantly increased AngII-induced vascular tone. n = 7 experiments, * P < 0.05 AngII versus LPC + AngII.

![Figure 1](image-url)
Results

OxLDL and LPC Potentiate AngII-Induced Constrictor Responses

In contrast to its effect on vascular tone in the microcirculation, OxLDL or LPC had no influence on resting tone of the aortic rings in the absence of AngII. However, when the segments were prestimulated with a threshold concentration of AngII (0.3 nmol/L) that elicited a weak contraction, stimula-
OxLDL strongly increased vascular tone (representative force record in Figure 1, top). A similar effect was induced by LPC (100 μg/mL). The middle and bottom panels of Figure 1 summarize the enhancement of CR induced by threshold AngII-concentrations in the presence of OxLDL or LPC. Native LDL had no impact on resting tone or on constrictor responses induced by AngII (data not shown). To analyze whether OxLDL or LPC also increased the efficacy of AngII-induced constrictor responses, full concentration-response curves were obtained for AngII (0.001 nmol/L to 0.1 μmol/L; n = 6 experiments). However, OxLDL did not increase maximal CR induced by AngII, and EC50 values of the concentration-response curves did not differ (4.6 × 10−10 mol/L for AngII alone [CI, 3.64 to 5.73 × 10−10 mol/L], and 4.9 × 10−10 mol/L for AngII + OxLDL [CI, 3.99 to 6.05 × 10−10 mol/L]). Thus, OxLDL and LPC increased CR induced by low AngII concentrations without influencing its efficacy.

At high concentrations, OxLDL as well as LPC are known to induce endothelial dysfunction, and endothelial dysfunction might enhance vascular contractility due to diminished dilator influences. To investigate whether OxLDL-induced potentiation of CR was caused by induction of endothelial dysfunction, we performed experiments with endothelium-denuded aortic segments and with segments treated with N-G-nitro-L-arginine (1 mmol/L, 30 min) to inhibit the influence of nitric oxide mechanically or pharmacologically, as shown in previous studies (12,20). However, the effect of OxLDL and LPC on CR was unaltered by these measures (data not shown). Furthermore, preliminary experiments revealed that acetylcholine-induced, endothelium-dependent dilations were not attenuated by the relatively low concentrations of OxLDL and of LPC used in this study (data not shown).

**Impact of Losartan and Felodipin on Potentiation of Constrictor Responses**

We analyzed whether the potentiating effect of OxLDL involved the AT1-receptor signaling pathway by testing the effect of the AT1-receptor antagonist losartan. Preincubation (30 min) with losartan (0.1 μmol/L) completely inhibited AngII-induced constrictor responses. Under these conditions, OxLDL did not increase vascular tone in response to AngII (data not shown).

OxLDL has been reported to increase the intracellular Ca2+ concentration in vascular cells (10,11). We therefore studied the influence of the dihydropyridine-type Ca2+-antagonist felodipin on constrictor responses. Felodipin (1 μmol/L, 30 min preincubation) had no impact on threshold CR induced by AngII, but it partially prevented OxLDL-induced potentiation of CR (by 41%).

**Effect of the Rho-Kinase Inhibitor Y27632 on Potentiation of Constrictor Responses**

The top panel of Figure 2 depicts a force record of vascular tone similar to the one shown in the top panel of Figure 1. As shown in this force record as well as in the summaries in the middle and bottom panels of Figure 2, the Rho-kinase inhibitor Y27632 (0.3 μmol/L) virtually abolished OxLDL- and LPC-induced potentiation of constrictor responses. Interestingly, Y27632 also inhibited constrictor responses induced by low concentrations of AngII alone.

**C Limosus C3-Like Transferase Partially Prevents Potentiation of Constrictor Responses**

Next, we attempted to analyze whether OxLDL and LPC acted via direct stimulation of Rho-kinase or whether it involved activation of RhoA upstream. For this purpose, we specifically inhibited RhoA using a C3-like exoenzyme from *C. limosus*, which acts as a C3-like transferase and inactivates RhoA via ADP-ribosylation. Membrane permeation of the C3-like exoenzyme was achieved by *C. botulinum* C2 toxin (27). While the single components of the fusion toxin were without effect on potentiation of CR (data not shown), 200 ng/ml of the complete fusion toxin (binding component + C3-like exoenzyme) significantly reduced the combined effect of AngII + OxLDL (by 49%; Figure 3, top) or AngII + LPC (by 46%; Figure 3, bottom) on vascular tone, indicating that OxLDL and LPC lead to a stimulation of RhoA.

**OxLDL Induces RhoA Translocation**

Finally, we aimed to demonstrate directly that OxLDL stimulates RhoA. RhoA stimulation was assessed by means of its translocation from the cytosolic to the particulate membrane fraction, an effect that is associated with Ca2+ sensitization of smooth muscle cells (25). Representative blots of rabbit aortic ring homogenates in the top panel of Figure 4 depict the relative amount of RhoA in the pellet (P, membrane fraction) and in the supernatant (C, cytosolic fraction), showing the translocation of RhoA after stimulation with 100 μg/mL OxLDL, with AngII 0.3 nmol/L, with 100 μg/mL LPC, with the combination of OxLDL and AngII in the presence or in the absence of C3, or with buffer as control. In comparison with controls, OxLDL, AngII, as well as LPC, induced translocation of RhoA. Combined treatment of aortic rings with OxLDL and AngII resulted in almost complete RhoA translocation. The specific RhoA inhibitor C. limosus C3-like transerase prevented OxLDL-induced and AngII-induced RhoA translocation.

To visualize RhoA translocation directly and to study the time course of OxLDL-induced RhoA translocation, we additionally performed immunocytochemistry with the RhoA-specific antibody in cultured bovine aortic SMC using confocal laser scan microscopy. As shown in the bottom panel of Figure 4, RhoA remained distributed homogenously in the cellular cytosol under control conditions. Translocation of RhoA to the cell membrane was visible already after 1 min of OxLDL stimulation and peaked after 5 min of stimulation. After 30 min, RhoA enrichment in the cell membrane decreased but was still clearly above control levels.

**Discussion**

In this study, we investigated the impact of OxLDL and of its compound LPC on vascular tone and on CR induced by AngII in isolated rabbit aortic rings. OxLDL and LPC had no influence on basal vascular tone, but it strongly increased...
contractile responses induced by low concentrations of AngII without influence on its efficacy. This effect was not due to attenuation of endothelial function and could at best be partially explained by the Ca$^{2+}$-increasing effects of OxLDL, as the Ca$^{2+}$-antagonist felodipin was only partially effective. However, agents interfering with the Ca$^{2+}$-sensitizing cascade (inhibitors of Rho and Rho-kinase) prevented potentiation of constrictor responses. Furthermore, OxLDL and AngII directly stimulated RhoA, as demonstrated by its translocation from the cytosol to the membrane. We therefore conclude that OxLDL and AngII increase the vascular tone by stimulating the RhoA/Rho-kinase cascade.

Our rationale for studying the impact and interaction of OxLDL and AngII on the vasculature is based on the observation that both substances colocalize in the atherosclerotic plaque (21,22), that vascular reactivity is altered in hypercholesterolemia and atherosclerosis (2–4), and that OxLDL and AngII individually influence vascular tone. Our investigation focused on potential effects these two compounds directly exerted on smooth muscle cells. It is well known that OxLDL and probably also AngII attenuate endothelium-dependent dilations (30–32), and it is well established that attenuation of endothelial function may increase CR to norepinephrine and other agonists (33). However, the potentiation of vascular tone as observed in this study was clearly independent of endothelial function. The effect of OxLDL on AngII-induced CR occurred in the presence as well as in the absence of the vascular endothelium, and the NO-synthase inhibitor N$^{0}$-nitro-L-arginine did not significantly alter OxLDL-induced enhancement of vascular tone. This may be due to the absence of the stimulator effects of blood flow on the endothelium, thereby reducing the basal NO production to a functionally insignificant amount. However, beside its influence on endothelial function, OxLDL seems to directly stimulate smooth muscle cells. These results are in accordance with a previous study from our laboratory, which also showed potentiation of agonist-induced CR by OxLDL in isolated endothelium-denuded arteries, which linearly correlated with the concentration and degree of oxidation of LDL (12). In that study, CR to norepinephrine, phenylephrine, and serotonin were enhanced. Therefore, the effect of OxLDL as analyzed here may not be specific for AngII-induced contractions, but may rather be of general nature. However, the interaction between OxLDL and AngII may be of particular pathophysiologic significance, because both compounds co-localize in the atherosclerotic plaque.

It is known that OxLDL may increase intracellular Ca$^{2+}$ in smooth muscle cells, potentially leading to increased vascular tone (10,11); we therefore analyzed whether the Ca$^{2+}$-antagonist felodipin abolished OxLDL-induced potentiation of CR. Felodipin only partially prevented the impact of OxLDL on potentiation of CR; therefore, other mechanisms contributing to the observed phenomenon must be considered.

It has become apparent in recent years that the effect of constrictor agonists may be not only explained by an elevation of intracellular free calcium, but also by a decrease of the activity of the SMMP; finally, both events lead to an increased phosphorylation of myosin light chain (13). The regulation of the SMMP is not yet completely understood. However, recent data identified SMMP as a target of Rho kinase that is regulated by the small GTP-binding protein, RhoA (13). Consequently, stimulation of this signaling cascade involving RhoA, Rho-kinase, and SMMP has been termed Ca$^{2+}$ sensitization (34). Ca$^{2+}$ sensitization seems to be of major relevance for regulation of vascular tone and to be critically involved in the development of vasospasm in large arteries (15–17). Interestingly, AngII — besides its impact on intracellular Ca$^{2+}$ increase — stimulates the Rho pathway, e.g., in rat mesenteric resistance arteries (35), and stimulates RhoA in rat cardiac myocytes (36). Our observation that AngII induces translocation of RhoA in aortic tissue is in line with these studies and sheds new light on the potential role of AngII as a modulator of vascular tone. Of note, Y27632 completely blunted constrictor responses induced by AngII alone (Figure 2). This observation raises the question whether an AngII-induced rise in intracellular Ca$^{2+}$ — besides its stimulation of myosin light chain kinase — influences Rho-kinase activity. This topic, however, was beyond the scope of our study.

The studies in the aorta are in line with our previous findings that OxLDL induced Ca$^{2+}$ sensitization of smooth muscle cells in the hamster microcirculation (14). Moreover, it has been demonstrated that mildly oxidized LDL could stimulate the RhoA/Rho-kinase pathway in endothelial cells (37). In the present study, we aimed to analyze whether stimulation of the RhoA/Rho-kinase cascade could influence the vascular response to the constrictor agonist AngII, which has a central role in the pathogenesis of hypertension and vascular remodeling. Our evidence for the assumption that OxLDL indeed stimulates the AngII-induced effects by activating the Rho pathway is threefold. First, the specific inhibitor of Rho-kinase, Y27632 (23), abolished potentiation of vascular tone. Second, specific inhibition of RhoA by a fusion toxin transporting a C. limosum C3-like transferase into vascular cells prevented the
OxLDL effect on vascular tone by approximately 50%. And thirdly, OxLDL induced translocation of RhoA from the cytosol to the plasma membrane. This translocation is correlated with Ca\(^{2+}\) sensitization of vascular smooth muscle cells (25). Thus, this is the first study to show that OxLDL acts via stimulation of RhoA. According to the immunostaining data shown in the top panel of Figure 4, C. limosum C3-like transferase almost completely prevented AngII-induced and OxLDL-induced translocation of RhoA. However, C3 attenuated potentiation of constrictor responses only by 50%. This observation indicates that the effect of OxLDL on the Rho cascade is an important, but probably not the sole mechanism for the enhancement of constrictor responses. More likely, OxLDL acts via both an increase in intracellular Ca\(^{2+}\) and a Ca\(^{2+}\) sensitization. However, it should be pointed out that the incomplete effect of C3 on constrictor responses also may have been due to an incomplete ADP ribosylation of RhoA.

To examine which compound of OxLDL may be responsible for potentiation of CR and stimulation of the RhoA/Rho-kinase pathway, we studied whether the OxLDL effects could be mimicked by its compound LPC. LPC is a byproduct of cholesterol esterification and accumulates in LDL during its oxidative modification (38). The rationale to perform these experiments is based on the fact that only oxidized LDL increased vascular tone, whereas the native form was without effect (data not shown; reference 12); the latter observation hints at the lipid peroxidation process itself. As shown here, synthetic LPC has principally the same impact as OxLDL on vascular tone, and its effect can be prevented by the same inhibitors of the RhoA/Rho-kinase pathway. Thus, the data are consistent with the idea that LPC may be the or at least one of the compounds of OxLDL that are responsible for potentiation of constrictor responses to AngII. However, it remains to be determined by which mechanism LPC and OxLDL interfere with the RhoA cascade.

Clinical Outlook

Hypercholesterolemia and atherosclerosis are associated with endothelial dysfunction, and it is believed that attenuation of endothelial function increases vascular tone. However, other mechanisms besides disturbed endothelial dilator responses may be clinically relevant in this setting. It has been recently demonstrated that local upregulation of Rho-kinase in pig coronary arteries results in vascular spasm, but only when the arteries were additionally stimulated with a constrictor agonist (15). The present study clearly shows that OxLDL — independent of any potential effects on endothelial function — increases the Ca\(^{2+}\) sensitivity of myofilaments in vascular smooth muscle through stimulation of the small GTPase RhoA and the Rho-associated kinase, resulting in enhanced CR to AngII. Thus, this observation provides a new mechanistic explanation for vasospasm as well as hypertension due to increased vascular resistance as observed in hypercholesterolemic patients. In particular, since AngII and OxLDL colocalize in the atherosclerotic plaque (21,22), OxLDL-induced (and AngII-induced) Ca\(^{2+}\) sensitization of the vasculature may be of particular importance for the development of vascular spasm in the region of atherosclerotic plaques.

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