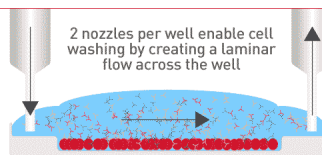


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Lipoic Acid Affects Cellular Migration into the Central Nervous System and Stabilizes Blood-Brain Barrier Integrity¹

Gerty Schreibelt,* René J. P. Musters,[†] Arie Reijerkerk,* Lody R. de Groot,* Susanne M. A. van der Pol,* Esther M. L. Hendriks,* Ed D. Döpp,* Christine D. Dijkstra,* Benjamin Drukarch,[‡] and Helga E. de Vries^{2*}

Reactive oxygen species (ROS) play an important role in various events underlying multiple sclerosis (MS) pathology. In the initial phase of lesion formation, ROS are known to mediate the transendothelial migration of monocytes and induce a dysfunction of the blood-brain barrier (BBB). In this study, we describe the beneficial effect of the antioxidant α -lipoic acid (LA) on these phenomena. *In vivo*, LA dose-dependently prevented the development of clinical signs in a rat model for MS, acute experimental allergic encephalomyelitis (EAE). Clinical improvement was coupled to a decrease in leukocyte infiltration into the CNS, in particular monocytes. Monocytes isolated from the circulation of LA-treated rats revealed a reduced migratory capacity to cross a monolayer of rat brain endothelial cells *in vitro* compared with monocytes isolated from untreated EAE controls. Using live cell imaging techniques, we visualized and quantitatively assessed that ROS are produced within minutes upon the interaction of monocytes with brain endothelium. Monocyte adhesion to an *in vitro* model of the BBB subsequently induced enhanced permeability, which could be inhibited by LA. Moreover, administration of exogenous ROS to brain endothelial cells induced cytoskeletal rearrangements, which was inhibited by LA. In conclusion, we show that LA has a protective effect on EAE development not only by affecting the migratory capacity of monocytes, but also by stabilization of the BBB, making LA an attractive therapeutic agent for the treatment of MS. *The Journal of Immunology*, 2006, 177: 2630–2637.

Infiltration of leukocytes into the CNS is a crucial event in the development of multiple sclerosis (MS),³ a chronic inflammatory disease of the CNS. In MS, infiltrated monocyte-derived macrophages form the major cell type in perivascular infiltrates and are central to the process of demyelination and axonal damage, which are characteristic features of MS (1). To enter the CNS and exert their damaging effects, leukocytes have to cross the blood-brain barrier (BBB), which mainly consists of highly specialized brain endothelial cells (ECs) and their tight junction complexes. Transendothelial migration of leukocytes requires the active participation of brain ECs to rearrange their cytoskeleton and tight junctions, processes that involve intracellular signaling events for instance through the family of the Rho-GTPases (2, 3).

For the migration of monocytes, we previously showed that reactive oxygen species (ROS) are crucial and that superoxide was the pertinent ROS facilitating monocyte trafficking (4). ROS not

only mediate cellular migration that initiates lesion development, but also contribute to lesion persistence in MS by degradation and phagocytosis of myelin, and the induction of axonal and oligodendrocyte damage (5–10). To counteract the detrimental effects of ROS, an appropriate antioxidant therapy is considered beneficial for patients who have MS by the limitation of both cellular influx and lesion progression. In the animal model for MS, experimental allergic encephalomyelitis (EAE), protective effects of antioxidants such as flavonoids (11), catalase (12), *N*-acetyl-L-cysteine (13), bilirubin (14), and α -lipoic acid (LA) (15, 16) have been described. These ROS scavengers were shown to affect cellular migration, demyelination, and axonal damage leading to reduced clinical signs.

To serve as a potential therapeutic agent, antioxidants must exert a broad range of activity, thereby changing the redox balance. Of interest is LA, also referred to as thioctic acid (reviewed in Refs. 17 and 18). LA is a ROS-scavenging molecule and a naturally occurring cofactor found in several multi-enzyme complexes involved in energy formation in tissues with high metabolic activity, such as liver, heart, and kidney (19). LA forms a redox couple along with its reduced form, dehydrolipoic acid. Both LA and dehydrolipoic acid serve as antioxidants via several mechanisms, including metal chelation, ROS scavenging, regeneration of vitamins, and the induction of endogenous antioxidants, such as glutathione (18). In addition, LA induces the expression of endogenous phase II detoxication enzymes, like NAD(P)H:quinone oxidoreductase and GST, enhancing the capacity of cells to scavenge ROS and resist oxidative damage (20).

In this study, we examined the effects of LA on initial phases of lesion development in MS. We demonstrate that LA potently inhibits monocyte migration across the BBB *in vitro* as well as *in vivo* under inflammatory conditions. We provide evidence that LA strongly reduces monocyte infiltration into the CNS in acute EAE, not only by affecting the migratory capacity of monocytes, but also

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³ Abbreviations used in this paper: MS, multiple sclerosis; BBB, blood-brain barrier; EC, endothelial cell; ROS, reactive oxygen species; EAE, experimental allergic encephalomyelitis; LA, α -lipoic acid; LPA, lysophosphatidic acid; EGFP, enhanced GFP; DHR, dihydrorhodamine.

via a stabilizing effect on the BBB to resist an oxidative attack generated by monocyte adhesion, as shown in the present study. Our results indicate the suitability of LA as potential new candidate drug to limit MS lesion formation and progression.

Materials and Methods

Induction and LA treatment of acute EAE in Lewis rats

Acute EAE was induced in 8- to 11-wk-old male Lewis rats (200–250 g; Harlan), which were kept under standard laboratory conditions. Acute EAE was induced according to Floris et al. (21). At day 0, rats were injected s.c. in one hind footpad with 20 μ g of guinea pig myelin basic protein in PBS mixed with CFA (Difco) and *Mycobacterium tuberculosis* strain H37Ra (Difco). EAE induction was performed under isoflurane anesthesia. Control animals were injected with PBS mixed with CFA. Neurological aberrations were scored daily and graded from 1 to 5: 0, no clinical signs; 0.5 partial loss of tail tonus; 1, complete loss of tail tonus; 1.5, unsteady gait; 2, partial hind limb paralysis; 2.5 complete hind limb paralysis; 3, paralysis of the complete lower part of the body up to the diaphragm; 4, paraplegia; and 5, death due to EAE.

LA (DL-LA; Sigma-Aldrich) was dissolved in 2 M NaOH and further diluted in saline and sterile water. This solution was titrated with 2 M HCl to a pH of 7.2 and an approximate salt concentration of 0.9% (w/v). All solutions of LA were sterile filtered. Animals were injected daily s.c. from day 6 after immunization until the end of the experiment with vehicle or with 10, 30, or 100 mg/kg LA. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experiments of the VU University Medical Center (Amsterdam, The Netherlands).

Immunohistochemistry

Three animals per group were sacrificed for histological examination at the development of clinical symptoms at start of disease (day 13 after EAE induction; see Fig. 1), peak of disease (day 16), and end of disease (day 19). Brains and spinal cords of sacrificed animals were dissected, snap-frozen in liquid nitrogen, and stored at -80°C . Cryostat sections (7 μ m) were melted onto gelatin-coated glass slides and dried in containers with silica gel. Slides were fixed in acetone (10 min) and incubated with PBS supplemented with 10% FCS (BioWhittaker). Immunohistochemistry was performed as previously described (22). Infiltrated T cells were detected by mAb R7.3, directed against TCR $\alpha\beta$ (1/100, mIgG1; BD Pharmingen) (23). Monocyte infiltration was detected with the monocyte/macrophage marker ED1 (1/250, produced at the Department of Molecular Cell Biology and Immunology, VU University Medical Center and commercially available via Serotec) (24). Cerebral blood vessels were detected with an Ab directed against von Willebrand factor (1/100, rabbit anti-human; Dako-Cytomation). Binding of primary Abs was revealed using Texas Red-conjugated donkey anti-rabbit (1/1000; Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-conjugated goat anti-mouse (1/400; Molecular Probes). Sections were rinsed, dried, and mounted in Vectamount (Vector Laboratories). Sections were examined with a Nikon Eclipse E800 microscope and recordings were made with a digital Nikon DXM1200 camera.

Monocyte isolation

Monocytes were isolated at day 13 of EAE from vehicle-treated animals and animals treated with 100 mg/kg LA as described elsewhere (11). Monocytes were purified from PBMC by negative selection using OX33 mAb (directed against CD45RA, B cells) and R7.3 mAb, and goat anti-mouse Ig-coated magnetic beads (Biomag; Polysciences) as previously described (11). Granulocytes and remaining RBC were removed via FACS sorting on the basis of their morphology. This step results in a yield of around $20\text{--}30 \times 10^6$ monocytes per rat, with a purity of $>90\%$ ED8 (CD11b/CD18)-positive monocytes.

Monocyte migration

Monocyte migration was studied with time-lapse video microscopy using the well-characterized, immortalized Lewis rat brain EC line GP8.3 (25), as previously described (4). GP8.3 cells were cultured as described elsewhere (26). The $7.5 \times 10^5/\text{ml}$ freshly isolated monocytes were added to EC monolayers and the number of migrated monocytes was assessed after 4 h. The migration assay was conducted in the absence and presence of 100 or 300 μM LA. The 300 μM LA was the optimal concentration to reduce monocyte migration and this concentration was used in additional experiments. The migratory capacity of monocytes derived from control rats and vehicle- or LA-treated EAE animals was studied in the presence of 50% serum from the same animals (11).

Permeability of the BBB in vitro

The BBB was mimicked in vitro by culturing primary rat brain ECs (1.5×10^5), isolated as previously described (27), and astrocytes (4.5×10^4) onto an upper-side collagen/fibronectin-coated (Sigma-Aldrich) Costar Transwell filter (pore size, 0.4 μm ; Corning) as described previously (28). Cells were cultured in M199 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (all obtained from Invitrogen Life Technologies), 8 $\mu\text{g}/\text{ml}$ apo-transferrin, 5 $\mu\text{g}/\text{ml}$ putrescine, and 2.5 ng/ml sodium selenite (all obtained from Sigma-Aldrich) and mixed 1:1 with astrocyte-conditioned medium (28). Permeability for FITC-dextran (150 kDa of dextran, 100 $\mu\text{g}/\text{ml}$ in culture medium; Sigma-Aldrich) were assayed as described (29) and the influence of the addition of 2×10^5 monocytes onto the endothelial layer was tested. At various time points after addition of monocytes, samples were collected from the acceptor chambers for measurement of fluorescence intensity using a FluoStar Galaxy microplate reader (BMG Labtechnologies), excitation 485 nm, emission 520 nm.

Construction of a brain EC line overexpressing enhanced GFP (EGFP) F-actin

To enable live cell imaging of actin cytoskeleton behavior, a brain EC line overexpressing EGFP-labeled actin (EGFP-actin) was established. The retroviral construct LZRS-EGFP-actin-IRES-zeocin (30) (a gift from Dr. P. L. Hordijk, Sanquin Research, Amsterdam, The Netherlands) was transfected using calcium phosphate into amphotropic Phoenix retrovirus producer cells (31) for the generation of helper-free amphotropic retroviruses. Virus-containing supernatant was used to transduce the rat brain EC line GP8.3 as previously described (26). Transduced GP8.3 cells were selected with 25 $\mu\text{g}/\text{ml}$ zeocin (Invitrogen Life Technologies) for 2 wk. Expression and localization of filamentous EGFP-actin was determined by phalloidin-rhodamine staining. GP8.3 cells expressing EGFP-actin were grown on collagen-coated Lab-Tek chamber slides (Nalge Nunc International) at 10% confluence. Cells were cultured for 48 h in endothelial serum-free medium basal growth medium (Invitrogen Life Technologies) and treated for 10 min with 5 μM lysophosphatidic acid (LPA; Sigma-Aldrich) to induce the formation of F-actin fibers (32). Cells were stained with phalloidin-rhodamine (Molecular Probes) (see *Three-dimensional live cell digital imaging microscopic analysis and quantification of F-actin fiber formation*) and images were taken using the 3i Marianas multidimensional digital imaging microscopy workstation (see *Detection of ROS production*). EGFP-actin-expressing cells were validated in our in vitro migration and adhesion models. Expression of ICAM-1, VCAM-1, PECAM-1, and MHC class II was validated using flow cytometric analysis as previously described (21).

Detection of ROS production

ROS production in cocultures of monocytes and brain ECs was followed online using dihydrorhodamine (DHR). DHR reacts with ROS in a peroxidase-like reaction to yield fluorescent rhodamine 123 (33, 34). EGFP-actin-expressing GP8.3 cells were cultured on collagen-coated 12-mm diameter Menzel coverslips (Fisher Scientific). Monocytes (1×10^6) were incubated for 30 min with 0.5 μM DHR, loaded for 10 min with Hoechst 1399 (Molecular Probes), and added to the brain endothelial layer. Three-dimensional live cell microscopy was performed with a Zeiss Axiovert 200 Marianas inverted microscope (3i Marianas digital imaging microscopy workstation) equipped with a charge-coupled device camera (Cooke Sensicam, 1280×1024 pixels) and a temperature-controlled heat block. The microscope, camera, and data viewing process were controlled by Slide-Book software (version 4.0; Intelligent Imaging Innovations). Recordings were made with a custom $\times 20$ lens (Zeiss). ROS production in a coculture of primary monocytes and brain ECs was quantified using Amplex Red (Molecular Probes), a probe that, in the presence of HRP, reacts with ROS to produce highly fluorescent resorufin (35). Monocytes and GP8.3 cells were preincubated for 1 h with 0.8 mM EDTA (Merck) or 100 $\mu\text{g}/\text{ml}$ heparin in a buffer containing 132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1 mM MgSO_4 , 1.2 mM K_2PO_4 , 1 mM CaCl_2 , and 0.5% (w/v) BSA. Monocytes (1×10^5) and Amplex Red reaction mixture were added to the GP8.3 cells and ROS formation was detected using a FluoStar Galaxy microplate reader (BMG Labtechnologies) at 37°C , excitation 550 nm, emission 590 nm. Fluorescence intensity was measured every minute during 60 min, and relative ROS formation was deduced from the increase of fluorescence intensity in time.

Three-dimensional live cell digital imaging microscopic analysis and quantification of F-actin fiber formation

To study real-time changes in cytoskeletal F-actin, EGFP-actin expressing GP8.3 cells were cultured on collagen-coated 42-mm diameter glass coverslips (Leica Microsystems) at 10% confluence. Cells were cultured for

48 h in endothelial serum-free medium basal growth medium (Invitrogen Life Technologies) and treated with 300 μM LA, or with superoxide, generated with 0.02 U/ml xanthine oxidase and 100 μM hypoxanthine as previously described (4). Live cell microscopy was performed with a Leica TCS SP2 AOBS confocal laser scanning microscope equipped with a temperature-controlled heat block.

For quantification of cytoskeletal rearrangements, GP8.3 cells were grown on collagen-coated Lab-Tek chamber slides (Nalge Nunc International) at 10% confluence. Cells were cultured for 48 h in endothelial serum-free medium basal growth medium (Invitrogen Life Technologies) and treated with 300 μM LA, or with superoxide, generated with 0.02 U/ml xanthine oxidase and 100 μM hypoxanthine as previously described (4). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), blocked with 10% FCS in PBS, and stained for 1 h with phalloidin-rhodamine (Molecular Probes). Images were taken using the 3i Marianas digital imaging microscopy workstation. For each acquisition session, photographs were captured using a standardized procedure. To quantify F-actin stress fibers, the digital image analysis program

AnalysisS was used. A region of interest was defined for each cell inside the ring of F-actin fibers at the cell borders. For each region of interest, the percentage of immunopositive area was calculated. The threshold was kept constant throughout the analysis of all cells.

Statistical analysis

Data were analyzed statistically by means of ANOVA and Student's *t* test, or by means of Mann-Whitney *U* test nonparametric statistics. Statistical significance was defined as $p < 0.05$.

Results

LA reduces clinical signs and cellular infiltration in acute EAE

Acute EAE is characterized by a monophasic course with a mean peak of disease at day 15 and spontaneous recovery after 20 days. The antioxidant LA dose-dependently protected animals from developing acute EAE. From day 6 after induction, EAE animals

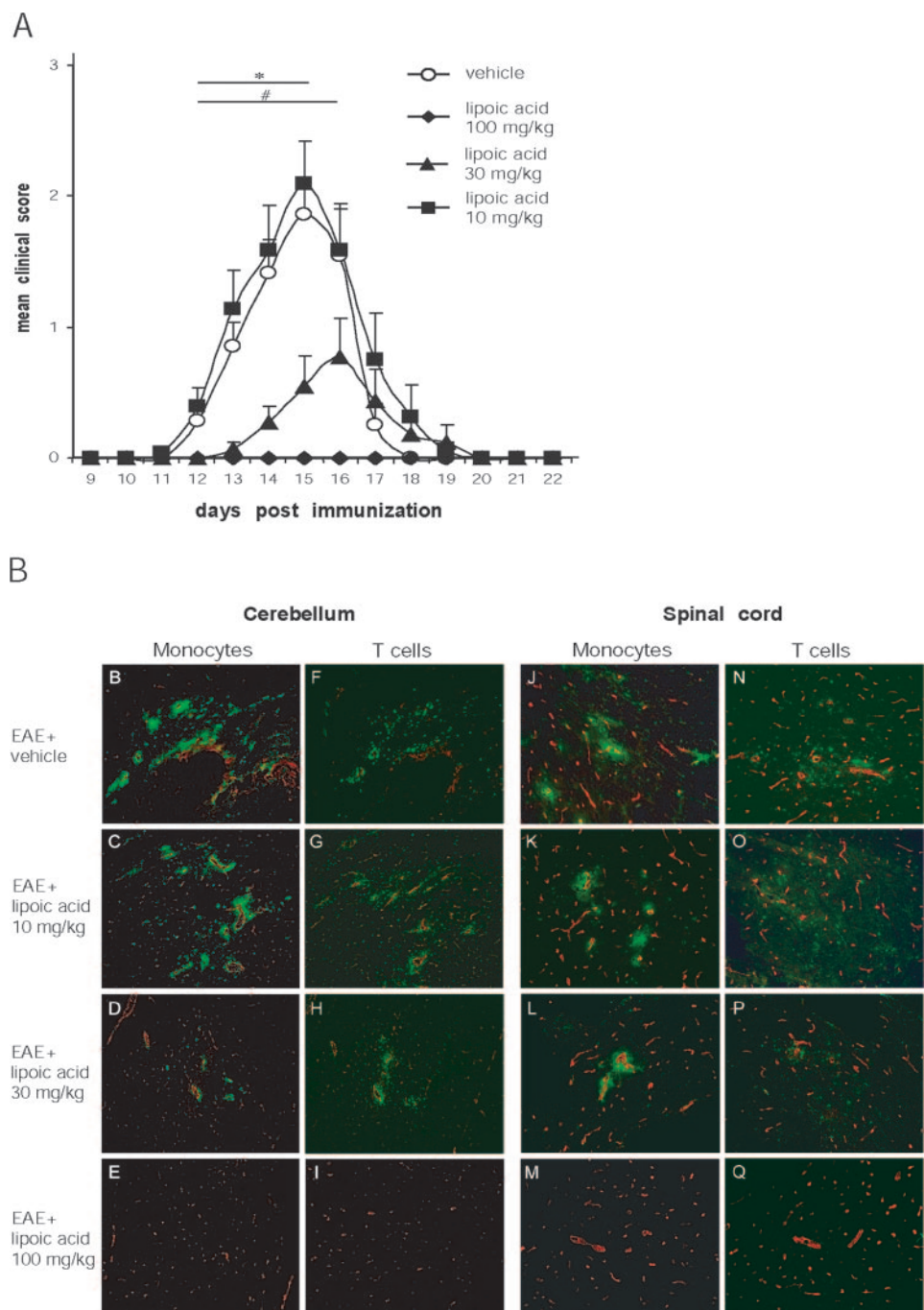


FIGURE 1. LA dose-dependently suppresses acute EAE and prevents monocyte infiltration into the CNS. **A**, Lewis rats sensitized for EAE ($n = 14$) were injected s.c. with three different doses of LA (100, 30, and 10 mg/kg) or with vehicle from day 6 after immunization. Both the 30 and 100 mg/kg treatment significantly reduced (*, $p < 0.05$ and #, $p < 0.05$, respectively, as determined by Mann-Whitney *U* test) clinical symptoms of EAE. Data represent means of clinical score \pm SEM. **B–Q**, Immunofluorescent analysis of ED1-positive monocyte-derived macrophages (**B–E** and **J–M**, in green) and R7.3-positive T cells (**F–I** and **N–Q**, in green), in the cerebellum (**B–I**) and spinal cord (**J–Q**) of vehicle or LA-treated EAE animals sacrificed at day 16 of EAE. Von Willebrand Factor-positive blood vessels are shown in red. Magnification, $\times 100$.

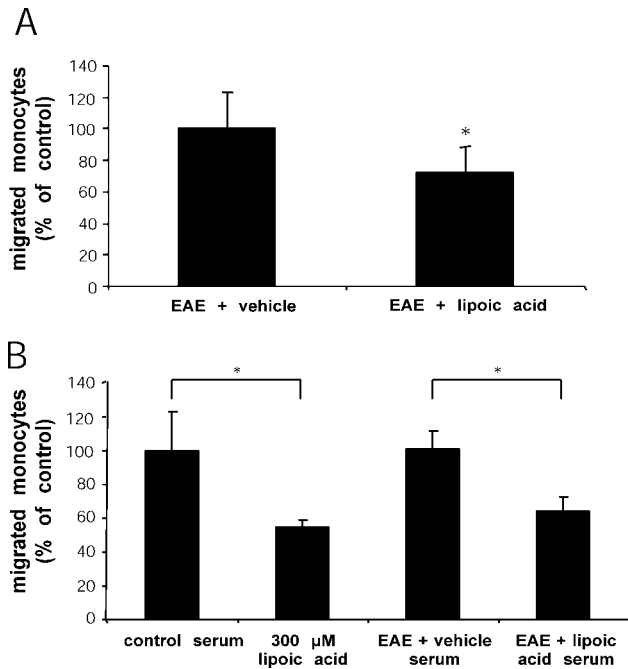


FIGURE 2. LA treatment of acute EAE reduces the migratory capacity of monocytes. *A*, Peripheral blood monocytes isolated at day 13 of LA-treated (100 mg/kg) EAE animals showed reduced capacity to migrate across a monolayer of brain ECs *in vitro* compared with monocytes isolated from vehicle-treated EAE animals. Experiments were performed in the presence of 50% serum derived from the same animals. Data are presented as the mean of 10 wells \pm SEM. *, $p < 0.05$, determined by Student's *t* test. *B*, The presence of 50% serum from LA-treated EAE animals (100 mg/kg) or 300 μ M LA reduced the migration of peripheral blood monocytes from untreated control animals across a monolayer of brain ECs compared with serum from vehicle-treated EAE animals. Serum of healthy control rats had no effect. Data are presented as the mean \pm SEM of three animals of three wells per animal. *, $p < 0.05$, by Student's *t* test.

were treated daily with different doses of LA (10, 30, 100 mg/kg) or vehicle by s.c. injection. At day 12 after immunization, vehicle-treated EAE animals started to lose tail tonus and at day 15 all animals were clinically ill (mean clinical score of 1.9 ± 0.19 at day 15) (Fig. 1). Treatment of EAE animals with 100 mg/kg LA completely prevented the onset of EAE. EAE animals treated with 30 mg/kg LA had a delayed onset of disease, starting at day 13 ($p < 0.05$), and their clinical scores were significantly reduced (mean clinical score of 0.8 ± 0.30 at day 16, $p < 0.05$). At a lower dose (10 mg/kg), EAE animals were not protected against the development of clinical signs, and clinical scores were comparable ($2.1 \pm$

0.33 at day 15) to those of vehicle-treated EAE animals. All animals were recovered at day 20. The number of infiltrating monocytes and T cells in the CNS in the course of EAE was studied in the cerebellum, brain stem, and spinal cord. LA was found to dose-dependently reduce cerebral entry of leukocytes compared with vehicle-treated EAE controls (Fig. 1, *B–Q*).

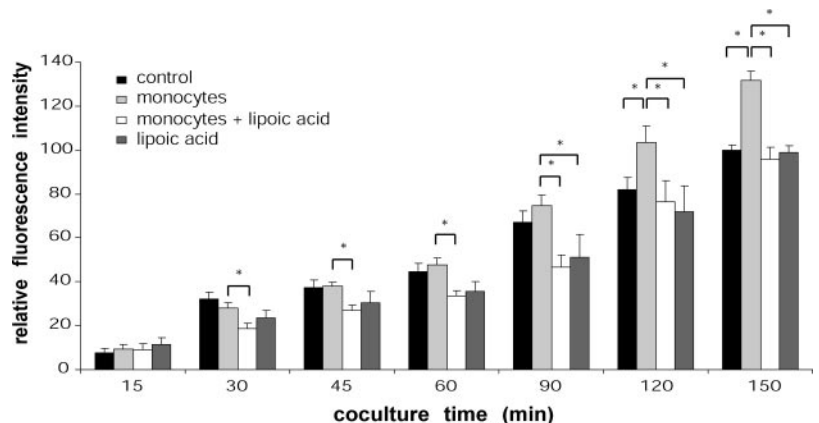
LA decreases monocyte migration in vitro

The migratory capacity of monocytes isolated from EAE animals to cross a monolayer of brain EC was studied *in vitro*. Monocytes isolated from LA-treated EAE animals at day 13 had an impaired migratory capacity compared with monocytes isolated from vehicle-treated EAE animals in the presence of serum of identical animals ($28 \pm 16.5\%$ reduction, $p < 0.01$) (Fig. 2*A*). Similarly, monocytes isolated from healthy control animals revealed decreased migration in the presence of serum of LA-treated EAE animals, compared with serum of vehicle-treated EAE animals ($36 \pm 8.1\%$ reduced migration, $p < 0.05$) (Fig. 2*B*). Furthermore, monocytes isolated from control animals showed reduced migration levels in the presence of 300 μ M LA in the medium ($46 \pm 4.1\%$ reduced migration, $p < 0.05$) (Fig. 2*B*). Incubation of monocytes with LA (300 μ M) 24 h before the migration assay (performed in the absence of LA) also reduced monocyte migration by $58 \pm 12.3\%$, whereas incubation of brain ECs before the migration assay did not affect cellular migration (data not shown). LA influenced the migratory capacity of monocytes without affecting their adhesive properties or their cellular expression of adhesion molecules known to be involved in the migration process (21), such as VLA-4, LFA-1, and PECAM-1 on monocytes and ICAM-1, VCAM-1, and PECAM-1 on brain ECs (data not shown).

LA reduces monocyte-induced BBB permeability in vitro

To assess the effect of monocytes on the permeability of the BBB *in vitro*, primary rat brain ECs and astrocytes were cocultured on Transwell filters. Adhesion of monocytes to the endothelial layer time-dependently enhanced leakage of FITC-dextran (150 kDa, $21 \pm 7.8\%$ increase compared with control after 120 min, increasing to $32 \pm 4.2\%$ after 150 min, $p < 0.05$), indicating that monocytes affect BBB permeability *in vitro* (Fig. 3). In the presence of LA (300 μ M) monocyte-induced leakage of the endothelial monolayer was significantly reduced ($10 \pm 2.4\%$ reduction after 30 min, further reducing to $36 \pm 5.3\%$ after 150 min, $p < 0.05$). Our results demonstrate that the interaction of monocytes with brain ECs affects the integrity of the endothelial monolayer, which is prevented by the presence of LA.

FIGURE 3. LA reduces monocyte-induced permeability of brain ECs. Confluent monolayers of primary rat brain EC were cocultured with astrocytes on Transwell filters (0.4 μ M) and leakage of FITC-conjugated dextran (150 kDa) was measured. Addition of primary rat monocytes increased leakage of FITC-dextran to the acceptor compartment. LA reduced both basal and monocyte-induced leakage of FITC-dextran. Data are presented as the percentage of control fluorescence at $t = 150$ min. Data are expressed as the mean \pm SEM of three independent experiments of three individual Transwells. *, $p < 0.05$, by Student's *t* test.



ROS are produced upon the interaction of monocytes with brain EC

Our results strongly suggest that cellular interaction of monocytes with brain ECs may induce ROS production and may subsequently affect the integrity of the endothelial monolayer. To demonstrate ROS production in a coculture of monocytes and brain ECs, the ROS-sensitive probe DHR was used. Live observation showed that ROS are generated in time upon the interaction of monocytes with a monolayer of brain ECs (Fig. 4A). Quantification of ROS production using the fluorescent probe Amplex Red reveals that in a coculture of monocytes and brain ECs, significantly enhanced levels of ROS are produced compared with being in a culture of brain ECs alone (Fig. 4B). The calcium chelator EDTA and heparin, both known to reduce monocyte adhesion to brain ECs (27), inhibited ROS production, indicating that this process was dependent on the interaction of monocytes with brain ECs. Using this set-up, no production of NO was detected within 4 h after adhesion of monocytes to brain ECs (data not shown).

Superoxide directly induces cytoskeleton changes in brain EC

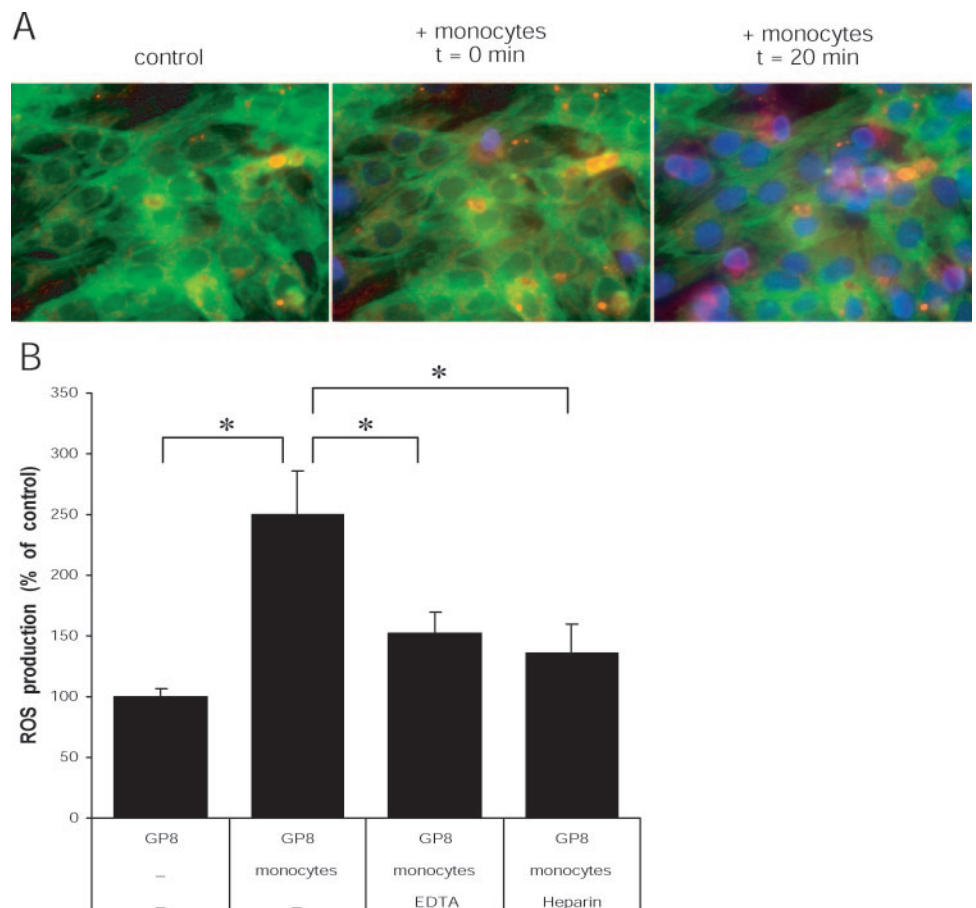
A brain EC line expressing EGFP-actin was generated to study the effect of superoxide on the cytoskeletal rearrangements using real-time confocal microscopy. Monocyte adhesion, transendothelial migration, and expression of adhesion molecules after cytokine stimulation were unaffected by EGFP-actin expression compared with GP8.3 brain ECs (data not shown) (21). Expression and localization of filamentous EGFP-actin in transfected GP8.3 cells was validated via F-actin staining with phalloidin on control cells and cells stimulated with LPA, which is a well-known inducer of stress fibers (Fig. 5A). Comparison of F-actin and EGFP-actin (Fig.

5A, left and middle, respectively) demonstrates that bundles of EGFP-actin colocalize completely with F-actin stress fibers. The histograms (Fig. 5A, far right) represent the fluorescence intensity of each pixel on a line scan (Fig. 5A, line scans are shown in the left and middle panels). Peaks in the histogram correspond with F-actin bundles in the cells and histograms confirm colocalization of EGFP-actin and F-actin (phalloidin). LPA-stimulated cells (Fig. 5A, bottom) contained a higher number of stress fibers compared with untreated control cells (Fig. 5A, top). As shown in Fig. 5B, newly formed F-actin stress fibers could be detected as early as 30 min after exposure to superoxide (generated with xanthine oxidase and hypoxanthine), which continued up to 60 min after superoxide exposure. In the presence of 300 μ M LA, no induction of stress fibers formation by superoxide was observed. Quantification of the number of F-actin fibers demonstrated a significant increase after 60 min superoxide exposure, which was inhibited by LA (Fig. 5C). Superoxide did not affect viability or adhesion molecule expression of brain ECs in the concentration used in these experiments (data not shown).

Discussion

The present study demonstrates that the antioxidant LA potently inhibits monocyte migration across the BBB in vivo as well as in vitro by affecting monocyte migration and stabilizing BBB integrity. We show that the clinical signs of rats suffering from acute EAE are dose-dependently reduced by LA, which was associated with a decrease in the number of perivascular infiltrates, in line with previous publications in various murine models of EAE (15, 16). We demonstrate that LA dose-dependently reduces monocyte

FIGURE 4. ROS are produced upon interaction of monocytes with brain ECs. *A*, ROS production during coculture of monocytes with EGFP-actin expressing GP8.3 cells was followed online using live cell microscopy. ROS production was visualized by DHR oxidation (EGFP-actin in green, oxidized DHR in red, nuclear staining of monocytes in blue). *B*, ROS production in a coculture of primary monocytes with GP8.3 cells was quantified using Amplex Red. ROS production was defined as increase of fluorescence intensity in the wells after addition of monocytes. Blocking adhesion with EDTA or heparin served as negative control. Data are expressed as the percentage of ROS production in untreated cocultures and presented as the mean \pm SEM of three independent experiments performed in triplicate. *, $p < 0.05$, by Student's t test.



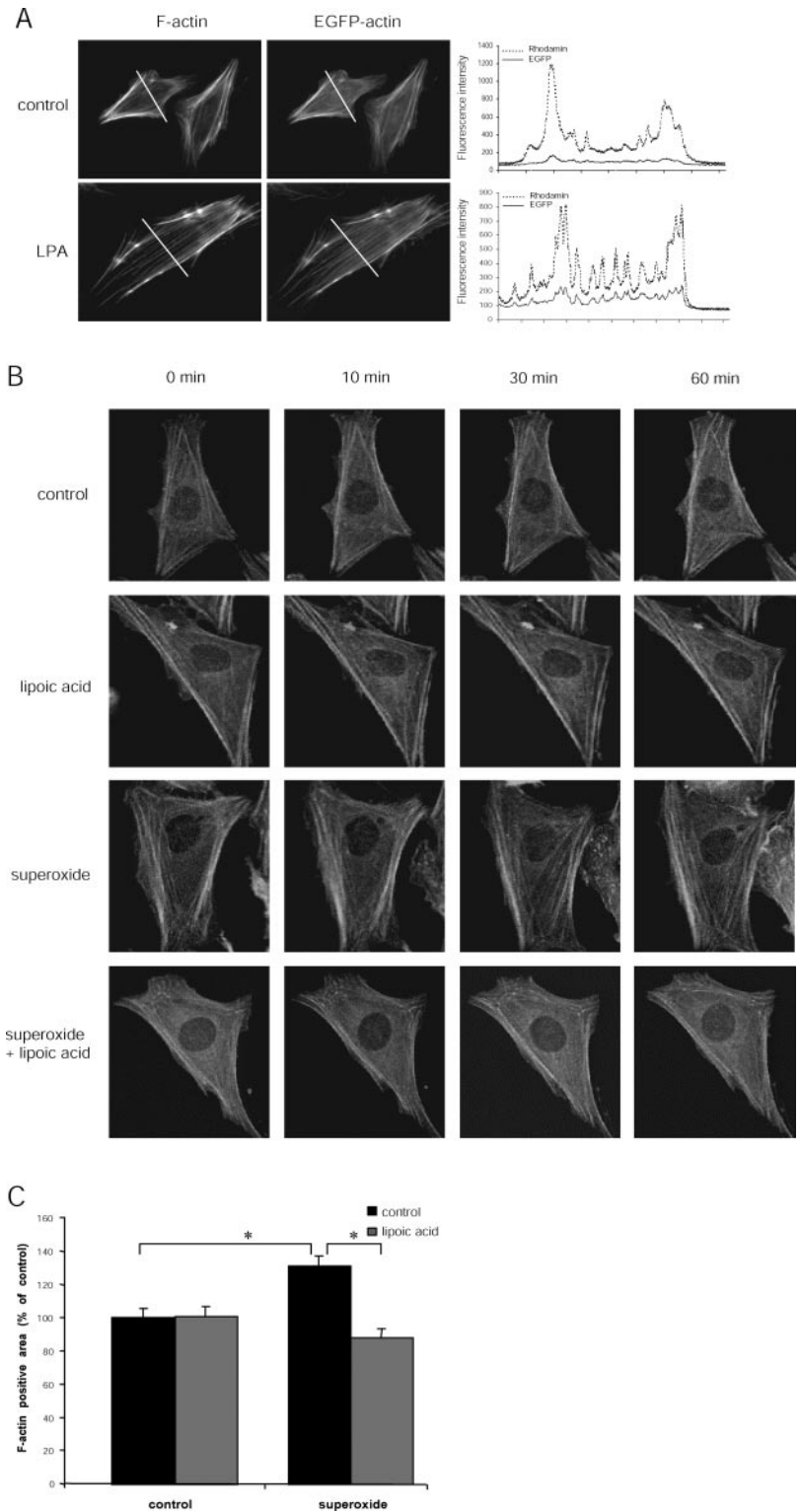


FIGURE 5. LA reduces superoxide-induced cytoskeletal rearrangements in brain ECs. *A*, EGFP-actin expression in EGFP-actin-transfected GP8 cells was validated with phalloidin-rhodamine staining in control cells (*top*) and LPA-stimulated cells (*bottom*). EGFP expression (*right*) and phalloidin staining (*left*) are shown. The histograms, representing fluorescence intensity of each pixel on the line scans through the cells, demonstrate complete colocalization of EGFP-actin (solid line histogram) and F-actin (dotted line histogram) in both control cells and LPA-stimulated cells. *B*, The formation of actin stress fibers in EGFP-transfected brain ECs was followed online using live cell confocal microscopy. Thirty minutes after exposure to superoxide, actin stress fibers were formed. LA prevented superoxide-induced stress fiber formation. Per condition, one representative cell of 30 cells is shown. *C*, Quantification of stress fiber formation in phalloidin-rhodamine stained GP8.3 cells after 1 h of exposure to superoxide and/or LA. Stress fiber formation was quantified by measuring the F-actin-positive area inside the ring of actin fibers at the cell borders. LA significantly reduced superoxide-induced stress fiber formation. Data are shown as mean \pm SEM of at least 150 cells per condition. *, $p < 0.05$, by Student's *t* test.

infiltration into the CNS in the course of acute EAE. Other antioxidants, such as catalase, bilirubin, *N*-acetyl-L-cysteine, and the flavonoid luteolin, have also been shown to suppress clinical and histological manifestation of EAE (11–14). Monocytes derived from LA-treated animals possessed a reduced migratory capacity. Identically, in the presence of serum of LA-treated EAE animals monocyte migration was decreased, suggesting that metabolic derivatives of LA, possibly dehydrolipoic acid, 3-ketolipoic acid, or bisnorlipoic acid, are present in the serum and are biologically

active (17). Both bisnorlipoic acid and 3-ketolipoic acid have antioxidant activity and are detectable in human plasma up to 5 h after oral administration of LA (36).

LA may influence cellular antioxidant activity by various mechanisms. Besides its direct scavenging of ROS and other radicals, LA may also exert its effect via induction of phase II detoxification enzymes NAD(P)H:quinone oxidoreductase and GST (20). In addition, LA induces elevated levels of glutathione, possibly through enhancement of the availability of the precursor cysteine (18). Our

in vitro data indicate that LA increased cellular levels of glutathione in both monocytes and brain EC (data not shown). However, the presence of LA in the medium is required to reduce cellular migration, suggesting a direct scavenging effect on ROS.

LA is known to reduce the migration of human T cells in vitro across a fibronectin barrier via down-regulation of VLA-4 and reducing matrix metalloproteinase-9 activity (37). In our experiments, using similar LA concentrations, adhesion molecule expression of both monocytes and brain ECs was not affected, nor was the adhesion of monocytes to brain ECs (data not shown), suggesting that the protective effect of LA is not mediated by modulation of adhesion molecule expression in our experimental setting. Future studies are needed to demonstrate whether LA affects the affinity of integrins expressed on monocytes or influences downstream signaling pathways of endothelial adhesion molecules such as members of the Ig superfamily. For instance, it has been shown that inhibition of the activation of small GTPases, especially RhoA, in the brain endothelium by various agents resulted in a decreased cellular migration in vitro as well as in vivo (2, 3, 38, 39).

The interaction of monocytes with brain ECs was shown to trigger ROS production in monocytes in these cocultures. In turn, released ROS may influence BBB integrity (4) and induce BBB permeability (40–42), which we quantitatively assessed. The interaction of monocytes with the BBB in vitro (primary brain EC cocultured with astrocytes) induced a dysfunction by enhancing the permeability of the endothelial monolayer for a fluorescent dye, as was shown previously (43). We are the first to show that the induced leakage is prevented by the presence of the ROS scavenger LA, suggesting that ROS are directly involved in monocyte-induced BBB permeability. Remodeling of the endothelial actin cytoskeleton is necessary for transendothelial migration of monocytes and associated with permeability changes. In this study, we quantitatively assessed that LA in time prevents ROS-induced changes in the brain endothelial cytoskeleton, thus preventing monocyte migration. The formation of stress fibers in brain ECs due to the exposure to superoxide occurred in a similar time interval as the enhanced permeability of the BBB in vitro induced by the interaction of monocytes with brain EC, indicating that LA may act directly on the integrity of the BBB. For rearrangement of the actin cytoskeleton, activation of the member of the small GTPase family RhoA is required (2). Recently, it was shown that exogenous superoxide is able to induce Rho activation in aortic smooth muscle cells (44). In addition, observations from our group demonstrated that antioxidants are able to reduce RhoA activity in monocytes (11).

Both LA and dehydrolipoic acid are powerful scavengers of peroxynitrite (45), and therefore LA may have an additional protective effect via scavenging of NO and peroxynitrite, a product of the interaction of superoxide and NO. Scavenging peroxynitrite or NO with uric acid has been shown to be beneficial in EAE (46). In vitro, increased NO levels are detectable after 24 h coculture of monocytes and brain ECs (our unpublished observation). In the present study, we cocultured monocytes and brain ECs for maximally 4 h and in this period NO production was not detected. Furthermore, inhibition of NO production by *N*^G-nitro-L-arginine methyl ester had no significant effect on monocyte migration across brain ECs (data not shown). It is therefore likely that LA predominantly exerted its protective effect on BBB stability and monocyte migration via scavenging of ROS.

Therapeutic administration of LA has been effective in diseases such as diabetic polyneuropathy (47, 48), diabetic nephropathy (49), and burning mouth syndrome (50). Recently, a pilot study in patients who have MS demonstrated that daily oral administration of LA for 2 wk was well tolerated and resulted in measurable

serum levels of LA (51). Serum levels of matrix metalloproteinase-9 and soluble ICAM-1, markers of inflammatory activity in MS (52, 53), were decreased in LA-treated patients; however, the duration of this study was too short to demonstrate significant effects on clinical symptoms (51).

In conclusion, we show that the antioxidant LA is an effective reducer of monocyte migration across the BBB and stabilizes the BBB in vitro, making it a potent inhibitor of cerebral inflammation and a promising therapy for the treatment of MS.

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Disclosures

The authors have no financial conflict of interest.

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