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## R-(+)- $\alpha$ -lipoic acid inhibits endothelial cell apoptosis and proliferation: involvement of Akt and retinoblastoma protein/E2F-1

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**Artwohl M, Muth K, Kosulin K, de Martin R, Hölzenbein T, Rainer G, Freudenthaler A, Huttary N, Schmetterer L, Waldhäusl WK, Baumgartner-Parzer SM.** R-(+)- $\alpha$ -lipoic acid inhibits endothelial cell apoptosis and proliferation: involvement of Akt and retinoblastoma protein/E2F-1. *Am J Physiol Endocrinol Metab* 293: E681–E689, 2007. First published June 12, 2007; doi:10.1152/ajpendo.00584.2006.— Lipoic acid was recently demonstrated to improve endothelial dysfunction or retinopathy not only in rats but also in diabetic patients. We tested the hypothesis that R-(+)- $\alpha$ -lipoic acid (LA) directly affects human endothelial cell (EC) function (e.g., apoptosis, proliferation, and protein expression), independent of the cells' vascular origin. Macrovascular EC (macEC), isolated from umbilical (HUVEC) and adult saphenous veins and from aortae, as well as microvascular EC (micEC) from retinae, skin, and uterus, were exposed to LA (1  $\mu$ mol/l–1 mmol/l) with/without different stimuli (high glucose, TNF- $\alpha$ , VEGF, wortmannin, LY-294002). Apoptosis, proliferation, cell cycle distribution, and protein expression were determined by DNA fragmentation assays, [<sup>3</sup>H]thymidine incorporation, FACS, and Western blot analyses, respectively. In macro- and microvascular EC, LA (1 mmol/l) reduced ( $P < 0.05$ ) basal (macEC,  $-36 \pm 4\%$ ; micEC,  $-46 \pm 6\%$ ) and stimulus-induced (TNF- $\alpha$ : macEC,  $-75 \pm 11\%$ ; micEC,  $-68 \pm 13\%$ ) apoptosis. In HUVEC, inhibition of apoptosis by LA (500  $\mu$ mol/l) was paralleled by reduction of NF- $\kappa$ B. LA's antiapoptotic activity was reduced by PI 3-kinase inhibitors (wortmannin, LY-294002), being in line with LA-induced Akt phosphorylation (Ser<sup>437</sup>,  $+159 \pm 43\%$ ; Thr<sup>308</sup>,  $+98 \pm 25\%$ ;  $P < 0.01$ ). LA (500  $\mu$ mol/l) inhibited ( $P < 0.001$ ) proliferation of macEC ( $-29 \pm 3\%$ ) and micEC ( $-29 \pm 3\%$ ) by arresting the cells at the G<sub>1</sub>/S transition due to an increased ratio of cyclin E/p27<sup>Kip</sup> (4.2-fold), upregulation of p21<sup>WAF-1/Cip1</sup> ( $+104 \pm 21\%$ ), and reduction of cyclin A ( $-32 \pm 11\%$ ), of hyperphosphorylated retinoblastoma protein (macEC:  $-51 \pm 7\%$ ; micEC:  $-50 \pm 15\%$ ), and of E2F-1 (macEC:  $-48 \pm 3\%$ ; micEC:  $-31 \pm 10\%$ ). LA's ability to inhibit apoptosis and proliferation of ECs could beneficially affect endothelial dysfunction, which precedes manifestation of late diabetic vascular complications.

LONG-STANDING DIABETES frequently goes along with diabetic microangiopathy (diabetic retinopathy and nephropathy) and premature atherosclerosis. The latter represents the major cause of morbidity and mortality in diabetic patients, whereas

diabetic retinopathy is the leading cause of acquired blindness among young adults in Western societies.

One common crucial step in the progression of both atherosclerosis and diabetic microangiopathy is accelerated apoptosis of endothelial cells (EC), impairing endothelial barrier function and provoking increased EC turnover to maintain an intact endothelial lining (30, 40, 44). In the pathogenesis of diabetic retinopathy, loss of retinal microvascular EC via apoptosis is observed before other histopathology is detectable (40), and apoptotic EC seem to favor thrombus formation as well as vascular occlusion by their proadhesive and procoagulatory activity (11, 21). In diabetic nephropathy, tubular, interstitial, and endothelial apoptosis seem to contribute to a progressive reduction in nephron mass and to chronic kidney failure (35). In atherosclerosis, apoptotic EC are assumed to increase vascular permeability and to trigger smooth muscle cell migration, plaque erosion, and plaque rupture (29, 44).

$\alpha$ -Lipoic acid, an antioxidative nutritional supplement that is taken up after consumption, becomes intracellularly reduced to its dithiol form (DHLA). Both  $\alpha$ -lipoic acid and DHLA act as a potent redox pair in the organism by regeneration of endogenous antioxidants and oxidized proteins (10, 32).  $\alpha$ -Lipoic acid reduces oxidative stress (16, 30, 38), inhibits nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (10, 30, 38, 48), and prevents VEGF upregulation (38, 43). Recently,  $\alpha$ -lipoic acid was shown to inhibit linoleic acid-induced apoptosis in human aortic endothelial cells (37), and data obtained in rats suggest that  $\alpha$ -lipoic acid's beneficial effects on vascular dysfunction in the aorta and retina could relate to its antiapoptotic activity (30, 37). Yet it has to be elucidated whether  $\alpha$ -lipoic acid's antiapoptotic activity relates to endothelial cells of different vascular origin or is restricted to certain types of endothelial cells. In this context, it is of note that  $\alpha$ -lipoic acid's potential effects on endothelial proliferation have not been studied in detail (36). Similarly, it remains to be evaluated whether molecules potentially involved in vascular cells' death or survival pathways, as deduced from other models and experimental settings, are involved in  $\alpha$ -lipoic acid's antiapoptotic effects. Such molecules include phosphatidylinositol 3-kinase (PI 3-kinase)/Akt (1, 20, 27, 42), the pro- (bak, bax; 15, 46) and

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antiapoptotic (bcl-2; 15, 28, 39) members of the bcl-2 family, and the redox-sensitive transcription factor NF- $\kappa$ B, which has been implicated in both pro- and antiapoptotic pathways in EC (3, 13, 27). In addition, cell cycle regulatory molecules such as cyclins, inhibitors of cyclin-dependent kinases (cdks; e.g., p21<sup>WAF-1/Cip1</sup>), retinoblastoma protein (pRb), and the transcription factor E2F-1 are involved not only in cell cycle progression, but also in apoptosis (9, 17).

Therefore, this study aims to test the hypothesis that R(+)- $\alpha$ -lipoic acid (LA) affects both apoptosis and proliferation in human micro- (micEC) and macrovascular endothelial cells (macEC), independent of the cells' vascular origin. To characterize the respective underlying mechanisms, the present study also evaluates molecules previously related to death/survival pathways in other models and experimental settings, including bcl-2 family members, NF- $\kappa$ B, PI 3-kinase/Akt, and cell cycle regulators.

## MATERIALS AND METHODS

If not stated otherwise, chemicals were from Sigma Chemical (St. Louis, MO). LA [(R)-1,2-dithiolane-3-pentanoic acid as trometamol sodium] was from Viatrix Pharma (Vienna, Austria), EGM-2 bullet medium was from BioWhittaker (Verviers, Belgium), and VEGF and TNF- $\alpha$  were from BioSource (Nivelles, Belgium). Fetal calf serum (FCS) was from Hyclone (Logan, UT), EC growth supplement was from Technoclone (Vienna, Austria), and heparin was from Biochrom (Berlin, Germany). Penicillin/streptomycin and fungizone were from Cambrex (Walkersville, MD). Methyl-<sup>3</sup>H]thymidine, Megaprime DNA labeling system, and horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG were from Amersham Pharmacia (Buckinghamshire, UK). DNase I and RNase A were from Boehringer (Mannheim, Germany). The TdT-FragEL kit was from Oncogene (Boston, MA), Tween-20 was from Bio-Rad (Hercules, CA), and the BCA protein assay and Super Signal Substrate were from Pierce (Rockford, IL). The p53 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), that against E2F-1 was from NeoMarkers (Fremont, CA), those against Akt and P-Akt at Ser<sup>437</sup> were from BioVision (Mountain View, CA), and that against P-Akt at Thr<sup>308</sup> was from Cell Signaling Technology (Beverly, MA). Antibodies against I $\kappa$ B $\alpha$ , phospho-I $\kappa$ B $\alpha$ , p27<sup>Kip</sup>, p21<sup>WAF-1/Cip1</sup>, cyclin-dependent kinase 2 (cdk2), and cyclin D3 were from Transduction Laboratories (Lexington, KY), those against cyclins A and E were from BD Pharmingen (San Diego, CA), and those against pRb, bcl-2, bak, and bax were from Calbiochem (Cambridge, MA).

### Isolation and Culture of Human Vascular Cells

Umbilical cords were obtained from the Departments of Obstetrics and Gynecology, saphenous veins from the Division of Vascular Surgery, retinae from the Department of Ophthalmology, and aortic endothelial cells from the Clinical Institute of Pathology. All cell types used were obtained in accordance with local law and approved by the local ethics committee.

Human umbilical vein (HUVEC), adult vein (HAVEC), aortic (HAEC), and retinal EC (HREC), as well as human retinal pericytes (HRPYC), were prepared and cultured as described (4, 5, 7, 8) and used as individual isolates. Human uterus microvascular EC (HUMEC) and human skin micEC (HSMEC) were from Technoclone (Vienna, Austria) and were used as pools derived from at least five different donors and cultured according to the supplier's instructions (7).

HUVEC were used in first subculture, whereas, due to their limited availability, HAVEC, HAEC, micEC, and HRPYC were used in passages 2–4.

Cell cultures were maintained at 37°C and 5% CO<sub>2</sub>, and subcultures were performed using trypsin-EDTA. Cultures were purified using Dynabeads (Dyna, Hamburg, Germany) coated with CD31 (HAVEC, HAEC, HREC) or 3G5 cell surface ganglioside (HRPYC) (4). EC were identified by typical phase contrast "cobblestone" morphology and by expression of von Willebrand factor antigen. Pericytes were characterized by presence of  $\alpha$ - and F-actin and by staining with monoclonal antibody 3G5.

### Incubation of Vascular Cells With LA

EC were incubated (48 h) with 1  $\mu$ mol/l–1 mmol/l LA (dissolved in double-distilled water) vs. intraindividual control cells (without addition of LA) in medium 199 supplemented with 20% FCS, 40 mg/l EC growth supplement, 5 U/ml heparin, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 500 ng/ml fungizone. Some experiments were performed in the presence of different stimuli (50 ng/ml VEGF, 30 mmol/l glucose, 1,333 U/ml TNF- $\alpha$ , 5–200 nmol/l wortmannin, 0.1–1  $\mu$ mol LY-294002). The underlying mechanisms of LA's effects were studied primarily in HUVEC due to the other cell types' limited availability.

### [<sup>3</sup>H]Thymidine Apoptosis Assays

[<sup>3</sup>H]Thymidine apoptosis assays were performed as described (5, 6, 8). In brief, semiconfluent plates were labeled (48 h) with [<sup>3</sup>H]thymidine (37 kBq/ml, 36 h) and subsequently replated into 24-well culture plates (5  $\times$  10<sup>4</sup> cells/well). After adherence (4 h), cells were incubated (48 h) with/without 1  $\mu$ mol/l–1 mmol/l LA. Some experiments were performed in the presence of 30 mmol/l glucose, 1,333 U/ml TNF- $\alpha$ , 5–200 nmol/l wortmannin, or 0.1–1  $\mu$ mol/l LY-294002. Cells were then lysed, and fragmented (apoptotic) vs. total radiolabeled DNA was counted using a 1900 TR liquid scintillation analyzer (Canberra Packard, Meriden, CT). Experiments were performed in triplicates. Results of experimental cultures exposed to LA in the absence or presence of different stimuli are presented in relation to intraindividual control cultures (without LA and other stimuli). Control cultures were set to 100%.

### In Situ Apoptosis Staining

After exposure (48 h) of the cells to LA, apoptosis was measured using the TdT-FragEL kit according to the manufacturer's instructions (8). Briefly, fixed and permeabilized cells were labeled with biotin-dNTP/streptavidin-horseradish peroxidase at sites of apoptotic DNA damage. Counterstaining with methyl green helped to quantify viable (green) vs. apoptotic (brown) cells by counting  $\geq$ 1,000 nuclei in multiple fields in a blinded fashion under the microscope. Apoptosis is given as percentage of positively stained nuclei (brown) to total nuclei in relation to intraindividual control cultures (set to 100%).

### [<sup>3</sup>H]Thymidine Proliferation Assay

Growing EC were left untreated (control cells) or exposed to LA with/without addition of 50 ng/ml VEGF in the presence of [<sup>3</sup>H]thymidine (final concentration: 37 kBq/ml) in medium 199 supplemented with 5% FCS, heparin, and antibiotics. After 40 h, cells were trypsinized, lysed, and harvested, and incorporated [<sup>3</sup>H]thymidine was counted as described (4, 5, 8). Samples were tested in quadruplicates. Results of experimental cultures exposed to LA in the absence or presence of VEGF are presented in relation to intraindividual control cultures (without LA or VEGF). Control cultures were set to 100%.

### Propidium Iodide Cell Cycle Analysis

Exponentially growing cells, incubated (48 h) with/without LA, were scraped off the dishes, washed, and fixed, and total DNA stained with propidium iodide (1  $\mu$ g/ml) was analyzed with a fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson, Heidelberg,

Germany), as described previously (4, 8). Data are presented as percentage of cells remaining in the respective cell cycle phase.

#### Western Blot Analyses

**Protein expression.** After incubation (48 h) with/without LA, EC were lysed in cold Weinberg buffer (4–6, 8), and total protein was measured using the BCA protein assay according to the manufacturer's instructions. Aliquots, containing exactly 10  $\mu\text{g}$  of total protein, were loaded in every lane and subsequently subjected to SDS polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose membranes (Schleicher & Schüll, Kassel, Germany). After controlling for homogenous sample loading by staining the blots with Ponceau S, blocking of unspecific binding sites with nonfat dry milk (5% in Tris-buffered saline) containing 0.05% Tween-20 was followed by incubation with primary antibodies and detection with horseradish peroxidase-conjugated anti-mouse- or anti-rabbit IgG using Super Signal substrate. Results are expressed in relation to intraindividual control cells (set to 100%).

**Protein phosphorylation.** Phospho-I $\kappa$ B and total I $\kappa$ B expression were evaluated after exposure (overnight) of HUVEC to 1 mmol/l LA and subsequent coincubation (2 h) with LA and TNF- $\alpha$ . Phospho-Akt (Ser<sup>473</sup> and Thr<sup>308</sup>) and total Akt expression were determined after exposure (1 h) of HUVEC to 50–500  $\mu\text{mol/l}$  LA. Results of protein phosphorylation are expressed as phosphorylated Akt and phosphorylated I $\kappa$ B to total Akt and total I $\kappa$ B, respectively. The ratio of phosphorylated to total protein in experimental cells (exposed to different stimuli) was related to the phosphorylated/total protein ratio of Akt and I $\kappa$ B in intraindividual control cells (set to 100%, without the respective stimuli).

Western blots were exposed to Kodak XAR5-Omat films, followed by densitometry (gel documentation system; MWG Biotech, Ebersberg, Germany) using Gene Profiler 3.56 for Windows (Scanlytics, Fairfax, VA).

#### Statistics

Since results obtained in micEC and macEC were comparable, data obtained from microvascular HREC, HUVEC, and HSMEC and from macrovascular HUVEC, HAVEC, and HAEC were pooled for statistical analysis. Data are expressed as means  $\pm$  SE. Statistical analysis was performed by paired Student's *t*-test (SPSS for Windows 7.5.1), using Bonferroni correction for multiple comparisons. A *P* value of 0.05 was considered significant.

## RESULTS

### Apoptosis

**Determination of apoptosis.** Whereas LA had no effect on cell death of HRPYC [at 500  $\mu\text{mol/l}$ :  $106.5 \pm 2.3\%$  vs. control cells set to 100%;  $n = 6$ , not significant (NS)], LA concentration dependently reduced basal apoptosis in macEC (HUVEC, HAVEC, and HAEC; Fig. 1A) and micEC (HREC and HUVEC; Fig. 1B), as measured by [<sup>3</sup>H]thymidine apoptosis assays and in situ apoptosis staining showing comparable results. In addition, LA reduced stimulus-induced apoptosis (Fig. 1, A and B).

LA's antiapoptotic activity was reduced by addition of the PI 3-kinase inhibitors LY-294002 (HUVEC; Fig. 2A) and wortmannin (HUVEC and HAEC; Fig. 2B, and HREC; Fig. 2C).

**Apoptosis-related proteins.** LA slightly reduced protein expression of bcl-2 (at 500  $\mu\text{mol/l}$ :  $-22 \pm 9\%$ ,  $P < 0.05$ ) in HUVEC ( $n = 6$ ), whereas neither bak ( $+11 \pm 19\%$ , NS) nor bax ( $+12 \pm 18\%$ , NS) was affected. LA concentration dependently increased phosphorylation of Akt at Ser<sup>437</sup> (Fig. 3A) and at Thr<sup>308</sup> (Fig. 3B), and it reduced basal as well as

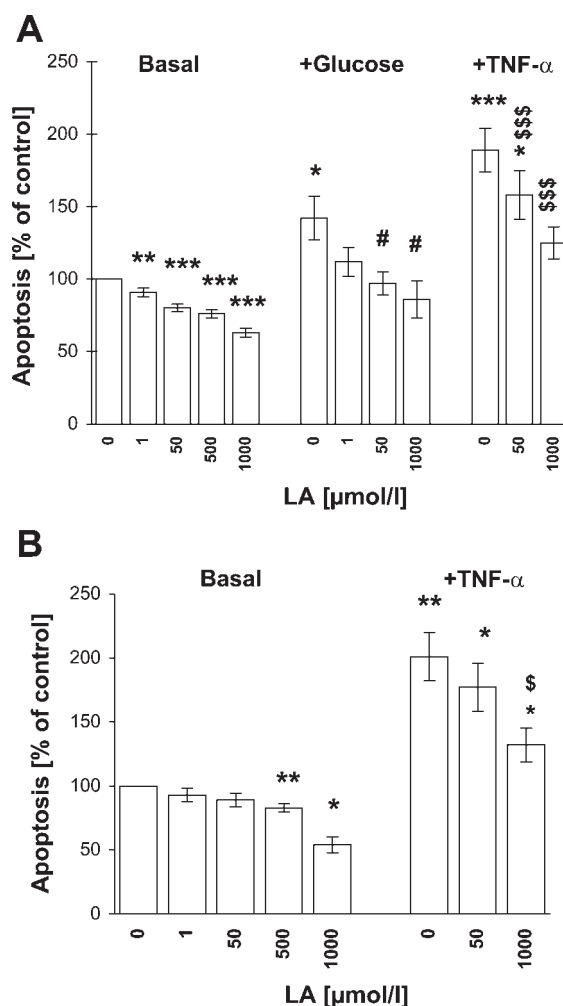


Fig. 1. Inhibition by R(+)- $\alpha$ -lipoic acid (LA) of apoptosis. A: inhibition by LA (48 h) of basal [5.5 mmol/l glucose; human umbilical vein (HUVEC), human aortic (HAEC), and human adult endothelial cells (HAVEC);  $n = 16$ ], high glucose (30 mmol/l, HUVEC;  $n = 4$ ), and TNF- $\alpha$  (1,333 U/ml, HUVEC and HAEC;  $n = 7$ ) induced apoptosis in macrovascular endothelial cells. B: inhibition of basal and TNF- $\alpha$ -induced apoptosis in microvascular endothelial cells [human retinal (HREC) and human uterus microvascular endothelial cells (HUVEC);  $n = 7$ ]. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%); # $P < 0.05$  vs. high glucose-treated cells; \$ $P < 0.05$ ; \$\$\$ $P < 0.001$  vs. TNF- $\alpha$ -treated cells.

TNF- $\alpha$ -stimulated phosphorylation of NF- $\kappa$ B's inhibitory subunit, I $\kappa$ B $\alpha$  (Fig. 3C).

### Proliferation and Cell Cycle

**Proliferation.** LA concentration dependently reduced basal and VEGF-induced proliferation in macEC (Fig. 4A) and micEC (Fig. 4B). LA's antiproliferative activity could also be shown in HRPYC (at 500  $\mu\text{mol/l}$ :  $-29.6 \pm 6\%$ ,  $P < 0.01$ ,  $n = 5$ ).

**Cell cycle distribution.** LA increased the number of HUVEC in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle, whereas the share of cells in S and G<sub>2</sub>/M phase was proportionally reduced (Fig. 5).

**Cell cycle-related proteins.** In HUVEC ( $n = 6$ ), LA had no effect on expression of cyclin D3 (G<sub>1</sub> phase:  $+12 \pm 23\%$ , NS) but increased that of cyclin E (G<sub>1</sub>/S transition; Fig. 6A) and reduced that of cyclin A (S phase; Fig. 6B). Protein expression of p53 ( $-8 \pm 10\%$ , NS) and of cdk2 ( $+10 \pm 7\%$ , NS), the

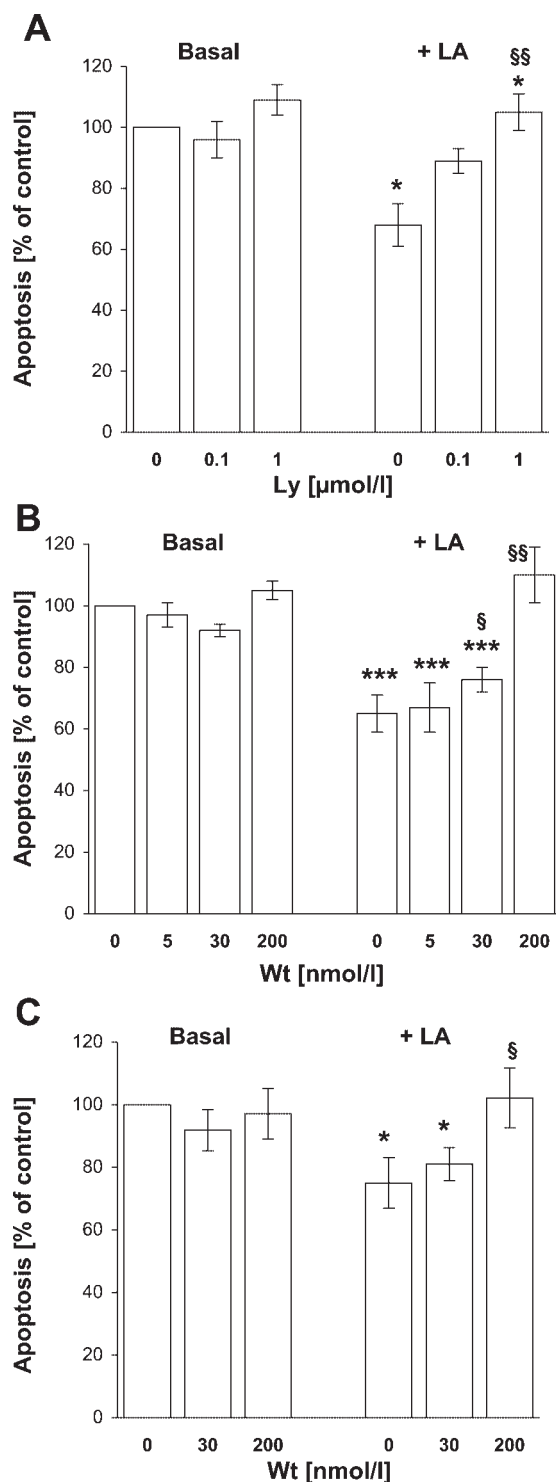


Fig. 2. Abrogation of LA's antiapoptotic activity. Abrogation of LA's (500  $\mu\text{mol/l}$ ) antiapoptotic effect by LY-294002 (LY; A) in HUVEC ( $n = 4$ ) and by wortmannin (Wt; B) in macrovascular endothelial cells (HUVEC and HAEC;  $n = 8$ ). C: counteraction of LA's (500  $\mu\text{mol/l}$ ) antiapoptotic activity by Wt in microvascular HREC ( $n = 4$ ). \* $P < 0.05$ ; \*\*\* $P < 0.001$  vs. untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%); § $P < 0.05$ ; §§ $P < 0.01$  vs. LA-treated cells.

latter forming complexes with cyclins D3, E, and A, were not affected by LA. LA, however, concentration dependently reduced the expression of p27<sup>Kip</sup> (Fig. 7A) but increased that of p21<sup>WAF-1/Cip1</sup> (Fig. 7B), with each molecule regulating activi-

ties of different cyclin/cdk complexes. In parallel, LA concentration dependently reduced hypo- (at 500  $\mu\text{mol/l}$ :  $-33 \pm 8\%$ ,  $P < 0.01$ ,  $n = 10$  macEC;  $-38 \pm 18$ ,  $P < 0.05$ ,  $n = 4$  micEC) and hyperphosphorylated pRb (Fig. 8, A and B) as well as the transcription factor E2F-1 (Fig. 8, C and D) in HUVEC, HAEC, and HREC.

## DISCUSSION

This study shows LA to inhibit apoptosis in different types of human macro- and microvascular endothelial cells. This also relates to stimulus-induced (30 mmol/l glucose or 1,333 U/ml TNF- $\alpha$ ) apoptosis. Similar results have recently been described

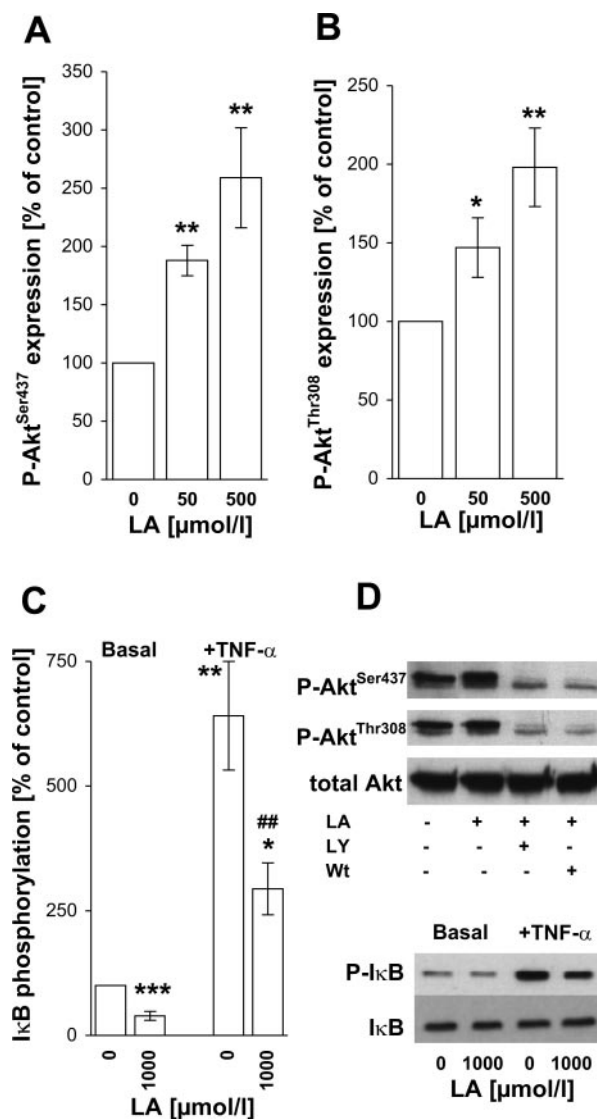


Fig. 3. Modulation by LA of apoptosis-related proteins. Concentration-dependent increase of Akt phosphorylation (P-Akt) at Ser<sup>437</sup> (A) and Thr<sup>308</sup> (B) in HUVEC ( $n = 6$ ) exposed (1 h) to LA. C: inhibition of basal and TNF- $\alpha$  (for the last 2 h) stimulated phospho-I $\kappa$ B $\alpha$  protein expression in HUVEC ( $n = 6$ ) after exposure (18 h) to LA. D: representative Western blots of 1 experiment showing 1) Akt phosphorylation at Ser<sup>437</sup> and Thr<sup>308</sup> (60 kDa) being increased by 500  $\mu\text{mol/l}$  LA and decreased by the phosphatidylinositol 3-kinase inhibitors LY (1  $\mu\text{mol/l}$ ) or Wt (200 nmol/l) in relation to corresponding total Akt (60 kDa) and 2) phospho-I $\kappa$ B (41 kDa) with corresponding total I $\kappa$ B (41 kDa) expression in HUVEC. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%); ## $P < 0.01$  vs. TNF- $\alpha$ -treated cells.

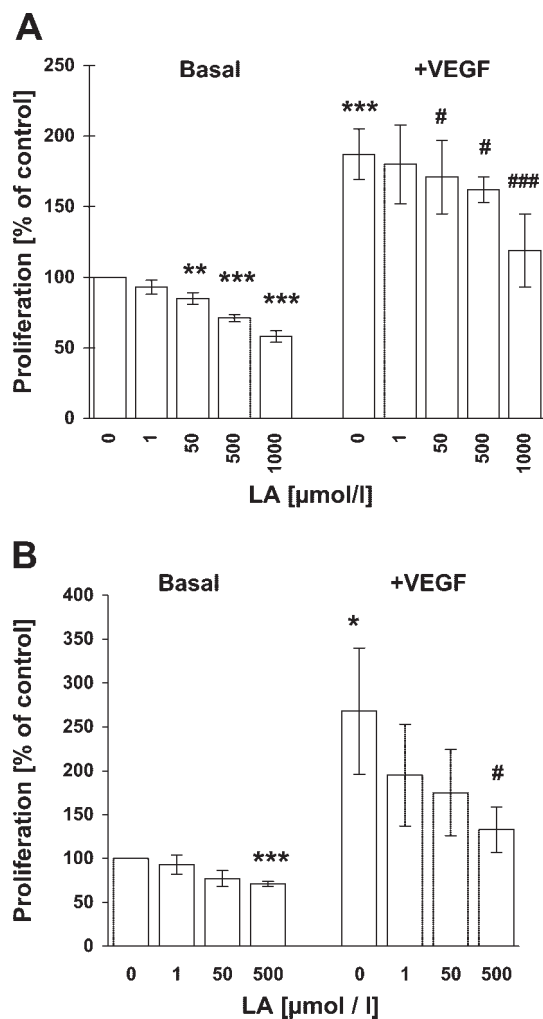


Fig. 4. Inhibition by LA of proliferation. *A*: inhibition by LA of macrovascular endothelial cells' basal (HUVEC, HAEC, and HAVEC;  $n = 10$ ) and VEGF-induced (50 ng/ml) proliferation (HUVEC;  $n = 6$ ). *B*: inhibition by LA of microvascular endothelial cells' basal and VEGF-induced (50 ng/ml) proliferation (HREC;  $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%); # $P < 0.05$ ; ### $P < 0.001$  vs. VEGF-stimulated cells.

(37) for fatty acid-induced apoptosis in human aortic endothelial cells. Since accelerated apoptosis of vascular endothelial cells relates to the development and progression of microangiopathy (11, 40) and to premature atherosclerosis (29, 44), LA's antiapoptotic activity hints at vasoprotective action of this antioxidant. This contention is supported by the finding that  $\alpha$ -lipoic acid administration beneficially affects apoptosis in aorta, cardiac tissue, and retina of rat models (12, 30, 37). Inhibition of retinal capillary cell death by  $\alpha$ -lipoic acid has, however, been shown without further discrimination between endothelial cells and pericytes (30), whereas our study clearly shows LA to inhibit apoptosis in retinal endothelial cells, but not in pericytes.

In our model, LA's antiapoptotic action relates neither to upregulation of antiapoptotic bcl-2 nor to downregulation of proapoptotic bak/bax. A lack of effect on the bcl-2 family of proteins was similarly reported (39) after short-term administration of  $\alpha$ -lipoic acid in bovine aortic endothelial cells.  $\alpha$ -Lipoic acid supplementation for several weeks, however,

increases bcl-2 and decreases bak/bax in diabetic rats' cardiac tissue and in normal rats' left ventricular endothelial cells (12, 39), suggesting that LA's effects on the bcl-2 family of proteins could depend on tissue type and/or experimental setting.

This study shows inhibition of basal and TNF- $\alpha$ -induced NF- $\kappa$ B activation by LA in HUVEC, as indirectly measured by decreased phosphorylation of NF- $\kappa$ B's inhibitory subunit, I $\kappa$ B $\alpha$ . Although these data are in line with earlier studies (10, 14, 30, 38, 48), and inhibition of NF- $\kappa$ B activation parallels reduction by LA of basal and TNF- $\alpha$ -induced apoptosis, a regulatory role of NF- $\kappa$ B in LA's antiapoptotic effects remains speculative.

The PI-3 kinase inhibitors wortmannin and LY-294002 reduced LA's antiapoptotic effect as well as LA-induced Akt phosphorylation at Ser<sup>437</sup> and Thr<sup>308</sup>, suggesting that reduction of endothelial cell death by LA depends on activation of PI 3-kinase and its downstream target, the serine/threonine kinase Akt (protein kinase B). LA's antiapoptotic effect was already detectable at the lowest concentration tested (1  $\mu\text{mol/l}$ ) and dose-dependently sustained until the highest concentration applied (1 mmol/l). Since the dose-response curves are smooth, there is no doubt that PI 3-kinase-dependent inhibition of apoptosis, although proven for 500  $\mu\text{mol/l}$ , should also be valid for lower LA concentrations. This assumption is confirmed in that the mechanism deduced from these experiments, i.e., Akt phosphorylation, was subsequently verified using lower LA concentrations (50  $\mu\text{mol/l}$ ).

Moreover, our findings are in line with previous reports (19, 24, 25, 42) postulating that PI 3-kinase and Akt could have a key role in endothelial cell survival pathways. Similar results have only recently been found in hepatocytes, where inhibition of apoptosis by  $\alpha$ -lipoic acid as well is associated with activation of the PI 3-kinase/Akt pathway (18).

In contrast to other activators of Akt (VEGF, insulin), which promote endothelial proliferation and/or modify angiogenesis or maturation/stabilization of blood vessels (20), we found that LA dose-dependently inhibits basal and VEGF-stimulated proliferation in micro- and macrovascular endothelial cells. Although the role of Akt in endothelial proliferation and angiogenesis is still a matter of debate, it has been clearly shown that Akt-activating agents/molecules do not necessarily trigger endothelial proliferation, as excellently reviewed and discussed by Dimmeler and Zeiher (20). Whereas LA's antiapoptotic action is restricted to endothelial cells, inhibition by LA of proliferation is also observed in HRPYC.

Proliferation is driven by binding of cyclins (periodically synthesized cell cycle proteins) to cyclin-dependent kinases (cdks). These activated, i.e., "cyclin-bound" cdks subsequently phosphorylate pRb, which in its unphosphorylated form sequesters the transcription factor E2F. Due to its phosphorylation, pRb changes its conformation and liberates E2F, which, in its free form, induces transcription of cyclins as well as of several DNA replication enzymes and thereby promotes cell cycle progression (17).

In LA-treated HUVEC, cdk2 (forming complexes with cyclins D3, E, and A) and cyclin D3 ( $G_1$  phase) remained unaffected, cyclin E ( $G_1/S$  transition) was increased, and cyclin A (S phase) was decreased, suggesting LA's antiproliferative activity to relate to inhibition of the cells' transition from the  $G_1$  to the S phase of the cell cycle. This is also confirmed by accumulation of HUVEC in the  $G_0/G_1$  phase and a propor-

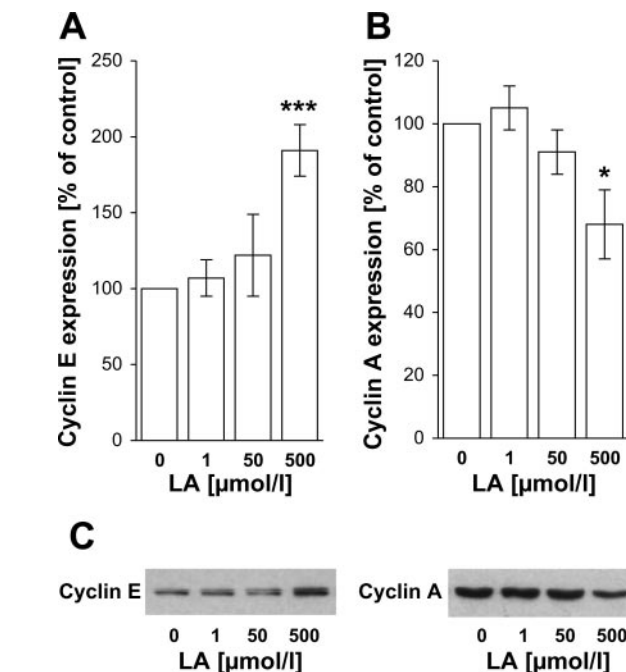
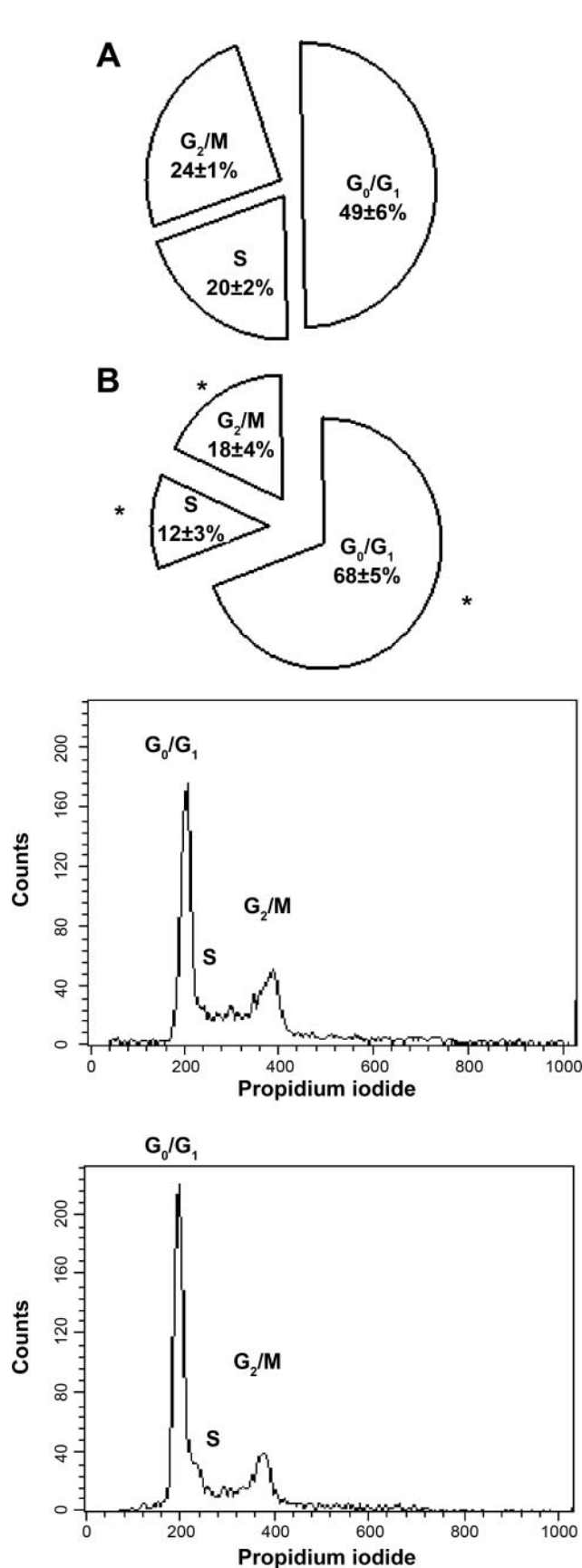


Fig. 6. Modulation by LA of cyclins. Protein expression of cyclins, i.e., cyclin E (G<sub>1</sub>/S transition; A) and cyclin A (S-phase cyclin; B), in HUVEC ( $n = 6$ ) exposed (48 h) to LA. C: representative Western blots of 1 experiment showing cyclin E (50 kDa) as well as cyclin A (60 kDa) expression in HUVEC. \* $P < 0.05$ ; \*\*\* $P < 0.001$  vs. intraindividual untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%).

tional reduction of the share of cells residing in S and G<sub>2</sub>/M phases, as shown by FACS analyses.

The activity of certain cyclin/cdk complexes not only depends on their subunits' expression levels but additionally is controlled by cdk inhibitors like p27<sup>Kip</sup> and p21<sup>WAF-1/Cip1</sup> (22).

p27<sup>Kip</sup> accumulates during cell quiescence and inhibits the activity of cyclin E-cdk2 and cyclin A-cdk2. The LA-mediated increase of cyclin E with concomitant reduction of p27<sup>Kip</sup>, however, indicates that LA-exposed HUVEC have already entered the cell cycle (45) and suggests that p27<sup>Kip</sup> does not account for the observed cell cycle arrest in HUVEC.

LA, however, upregulates p21<sup>WAF-1/Cip1</sup> expression and probably thereby inhibits cyclin-cdk activities, a mechanism already shown for inhibition of proliferation by laminar shear stress (2). LA-induced increase in p21<sup>WAF-1/Cip1</sup> and the observed inhibition of cell cycle progression are, however, independent of p53 upregulation. Similar findings have already been reported (5, 7, 8) for other models of stimulus-induced cell cycle arrest, particularly in endothelial cells. The p21<sup>WAF-1/Cip1</sup>-mediated cell cycle arrest occurs upstream of pRb and apparently prevents both phosphorylation of pRb and the release of transcriptionally active E2F, which is mirrored by downregulation of cyclin A (S phase), representing a target of E2F-1.

Fig. 5. Modulation by LA of cell cycle distribution. Cell cycle distribution in HUVEC ( $n = 4$ ) exposed (48 h) to untreated intraindividual control cells (A) compared with 1 mmol/l LA (B). Representative histograms of cell cycle distribution in untreated control cells (C) and HUVEC exposed to 1 mmol/l LA (D). \* $P < 0.05$  vs. the corresponding cell cycle phase of control cells (i.e., in relation to A).

Of importance, LA exerts many of its effects in human endothelial cells already at 1  $\mu\text{mol/l}$  (inhibition of apoptosis and modulation of p27<sup>Kip</sup>, hyper-pRb, and E2F-1). At 50  $\mu\text{mol/l}$ , LA modulates nearly all parameters evaluated in our study, including inhibition of apoptosis, Akt phosphorylation at both sites, basal and VEGF-stimulated inhibition of proliferation, and modulation of p27<sup>Kip</sup>, p21<sup>WAF-1/Cip1</sup>, hyper-pRb, and E2F-1.

Peak plasma concentrations observed in humans after oral administration of 200 mg or infusion of 600 mg of  $\alpha$ -lipoic acid's racemic mixture (26, 41) or after oral administration of 600 mg LA (33) clearly lie within 1 and 50  $\mu\text{mol/l}$ . As outlined above, these physiologically relevant concentrations of LA have been used in the present study.

In addition, our data show that the effects remain stable up to 500  $\mu\text{mol/l}$ –1 mmol/l LA. With respect to these pharmacological LA concentrations, it should be mentioned that, although usually taken as a measure for physiological concentrations, plasma levels do not necessarily reflect intracellular or tissue concentrations. The latter could be clearly higher due to cell or tissue specific accumulation. So, although not proven in humans, LA could accumulate in certain tissues (e.g., aorta), as deduced from experiments with rats (33).

Moreover, presentation of data performed with a broader concentration range including both physiologically relevant and pharmacological concentrations is of advantage to evaluate LA's effects and to compare the presented data with other in vitro studies having used similar or higher LA concentrations (10, 37, 48).

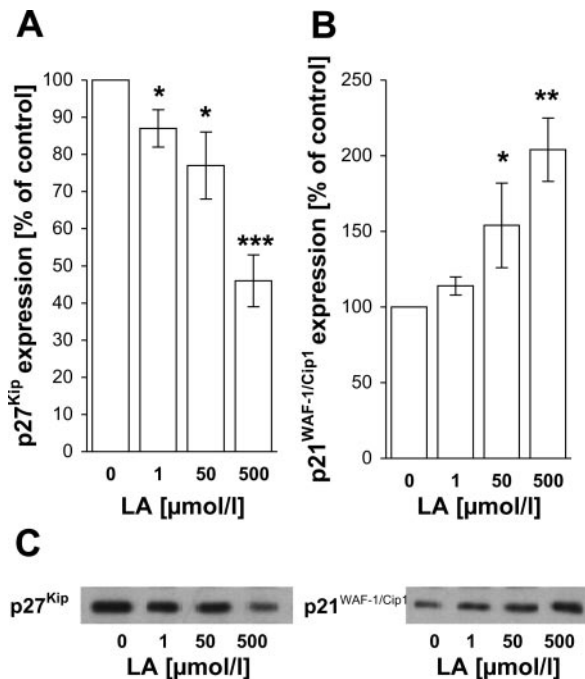


Fig. 7. Modulation by LA of cyclin-dependent kinase (cdk) inhibitors. Protein expression of inhibitors of cdk, i.e., p27<sup>Kip</sup> (A) and p21<sup>WAF-1/Cip1</sup> (B), in HUVEC ( $n = 6$ ) exposed (48 h) to LA. C: representative Western blots of 1 experiment showing p27<sup>Kip</sup> (27 kDa) as well as p21<sup>WAF-1/Cip1</sup> (21 kDa) expression in HUVEC. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. intraindividual untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%).

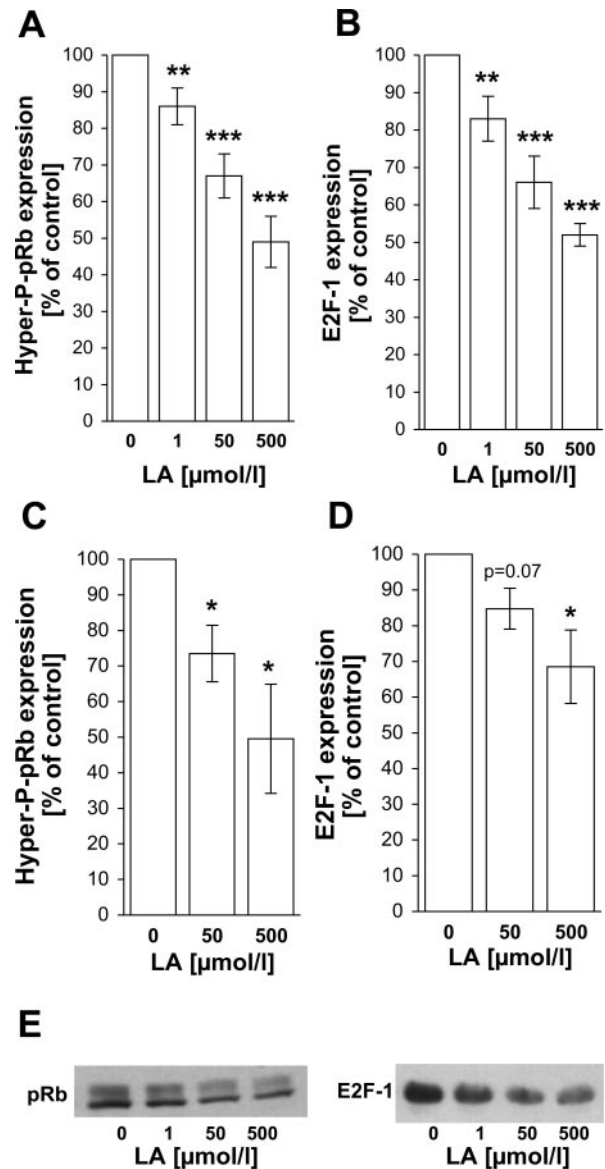


Fig. 8. Modulation by LA of retinoblastoma protein (pRb) and E2F-1. Inhibition of hyperphosphorylated pRb (hyper-P-pRb; A) and E2F-1 expression (B) in macrovascular endothelial cells (HUVEC and HAEC;  $n = 10$ ) exposed (48 h) to LA. Inhibition of hyper-P-pRb (C) and E2F-1 expression (D) in microvascular endothelial cells (HREC;  $n = 4$ ) exposed (48 h) to LA. E: representative Western blots of 1 experiment showing pRb (110–116 kDa) as well as E2F-1 (46 kDa) expression in HUVEC. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. intraindividual untreated (0  $\mu\text{mol/l}$  LA) cells (set to 100%).

In conclusion, this is the first study showing LA to reduce both apoptosis (via activation of Akt) and proliferation (via pRb/E2F) in human vascular endothelial cells independent of their vascular origin. Therefore, LA could have a role in prevention or amelioration of diabetes-related vascular disease. Further studies will, however, be necessary to evaluate whether the pathways identified in our human endothelial cell culture model also apply to  $\alpha$ -lipoic acid's effects observed in vivo.

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