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

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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).

α-Lipoic acid, an antioxidative nutritional supplement that is taken up after consumption, becomes intracellularly reduced to a potent redox pair in the organism by regeneration of endogenous antioxidants and oxidized proteins (10, 32). It acts as a potent redox partner for NADPH to regenerate NAD+. α-Lipoic acid reduces oxidative stress (16, 30, 38), inhibits nuclear factor-κB (NF-κB) activation (10, 30, 38, 48), and prevents VEGF upregulation (38, 43). Recently, α-lipoic acid was shown to inhibit linoic acid-induced apoptosis in human aortic endothelial cells (37), and data obtained in rats suggest that α-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 α-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 α-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 α-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 antiapoptotic molecules potentially involved in vascular cells’ death or survival pathways, as deduced from other models and experimental settings, are involved in α-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 antiapoptotic Akt and retinoblastoma protein/E2F-1

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antiapoptotic (bcl-2; 15, 28, 39) members of the bcl-2 family, and the redox-sensitive transcription factor NF-κB, 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\(^{WAF-1/Cip1}\)), 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(+-)α-lipoic acid (LA) affects both apoptosis and proliferation in human micro- (mEC) 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-κB, 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 Viatris Pharma (Venna, Austria), EGM-2 bullet medium was from BioWhittaker (Verviers, Belgium), and VEGF and TNF-α were from BioSource (Nivelles, Belgium). Fetal calf serum (FCS) was from Hyclone (Logan, UT), EC growth supplement was from Technoclone (Venna, Austria), and heparin was from Biochrom (Berlin, Germany). Penicillin/streptomycin and fungizone were from Cambrex (Walkersville, MD). Methyl-[\(^3\)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/Thr\(^{37/47}\) were from BioVision (Mountain View, CA), and that against P-Akt at Thr\(^{308}\) was from Cell Signaling Technology (Beverly, MA). Antibodies against IκBα, phospho-IκBα, p27\(^{Kip1}\), p21\(^{WAF-1/Cip1}\), 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 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\(_2\), and subcultures were performed using trypsin-EDTA. Cultures were purified using Dynabeads (Dynal, 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 α- and F-actin and by staining with monoclonal antibody 3G5.

**Incubation of Vascular Cells With LA**

EC were incubated (48 h) with 1 μmol/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 μg/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-α, 5–200 nmol/l wortmannin, 0.1–1 μmol/l LY-294002). The underlying mechanisms of LA’s effects were studied primarily in HUVEC due to the other cell types’ limited availability.

**\(^{3}\)HThymidine Apoptosis Assays**

\(^{3}\)HThymidine apoptosis assays were performed as described (5, 6, 8). In brief, semiconfluent plates were labeled (48 h) with \(^{3}\)Hthymidine (37 kBq/ml, 36 h) and subsequently replated into 24-well culture plates (5 × 10\(^4\) cells/well). After adherence (4 h), cells were incubated (48 h) with/without 1 μmol/l–1 mmol/l LA. Some experiments were performed in the presence of 30 mmol/l glucose, 1,333 U/ml TNF-α, 5–200 nmol/l wortmannin, or 0.1–1 μmol/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 ≥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%).

**\(^{3}\)HThymidine 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 \(^{3}\)Hthymidine (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 \(^{3}\)Hthymidine 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 μg/ml) was analyzed with a fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson, Heidelberg,
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 μg 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-IκB and total IκB expression were evaluated after exposure (overnight) of HUVEC to 1 mmol/l LA and subsequent coincubation (2 h) with LA and TNF-α. Phospho-Akt (Ser473 and Thr308) and total Akt expression were determined after exposure (1 h) of HUVEC to 50–500 μmol/l LA. Results of protein phosphorylation are expressed as phosphorylated Akt and phosphor-ylated IκB, 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 IκB 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 (Scan-lytics, Fairfax, VA).

**Statistics**

Since results obtained in micEC and macEC were comparable, data obtained from microvascular HREC, HUMEC, and HSMEC and from macrovascular HUVEC, HAVEC, and HAEC were pooled for statistical analysis. Data are expressed as means ± 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 μmol/l: 106.5 ± 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 HUMEC; Fig. 1B), as measured by [³H]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 (HREC and HAEC; Fig. 2B, and HREC; Fig. 2C).

**Apoptosis-related proteins.** LA slightly reduced protein expression of bcl-2 (at 500 μmol/l: −22 ± 9%, P < 0.05) in HUVEC (n = 6), whereas neither bak (+11 ± 19%, NS) nor bax (+12 ± 18%, NS) was affected. LA concentration dependently increased phosphorylation of Akt at Ser473 (Fig. 3A) and at Thr308 (Fig. 3B), and it reduced basal as well as TNF-α-stimulated phosphorylation of NF-κB’s inhibitory subunit, IκBα (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 μmol/l: −29.6 ± 6%, P < 0.01, n = 5).

**Cell cycle distribution.** LA increased the number of HUVEC in G1/G0 phase of the cell cycle, whereas the share of cells in S and G2/M phase was proportionally reduced (Fig. 5).

**Cell cycle-related proteins.** In HUVEC (n = 6), LA had no effect on expression of cyclin D3 (G1 phase: +12 ± 23%, NS) but increased that of cyclin E (G1/S transition; Fig. 6A) and reduced that of cyclin A (S phase; Fig. 6B). Protein expression of p53 (−8 ± 10%, NS) and of cdk2 (+10 ± 7%, NS), the
latter forming complexes with cyclins D3, E, and A, were not affected by LA. LA, however, concentration dependently reduced hypophosphorylated pRb (at 500 µmol/l: 33 ± 8%, P < 0.01, n = 10 macEC; 38 ± 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-α) apoptosis. Similar results have recently been described by 10.220.33.4 on September 21, 2017 http://ajpendo.physiology.org/ Downloaded from
LA inhibits apoptosis by reducing the levels of proapoptotic proteins while increasing the levels of antiapoptotic proteins. This effect is observed in both macrovascular and microvascular endothelial cells, as well as in pericytes. The mechanism behind this effect involves the PI3-kinase/Akt pathway, which is activated by LA.

LA also inhibits the phosphorylation of NF-κB, another key regulator of apoptosis. This is mediated by the reduction of p65 and p50 subunits, which are essential for NF-κB activation.

Inhibition of retinal capillary cell death by LA shows the potential of this compound in the treatment of retinal diseases. LA's antiapoptotic activity hints at vasoprotective action of LA's effects.

The PI-3 kinase inhibitors wortmannin and LY-294002 reduced LA's antiapoptotic effect as well as LA-induced Akt phosphorylation at Ser473 and Thr308, suggesting that reduction of endothelial cell death by LA depends on activation of PI3-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 μmol/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 PI3-kinase-dependent inhibition of apoptosis, although proven for 500 μmol/l, should also be valid for lower LA concentrations.

Moreover, our findings are in line with previous reports (19, 24, 25, 42) postulating that PI3-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 α-lipoic acid as well is associated with activation of the PI3-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 (G1 phase) remained unaffected, cyclin E (G1/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 G1 to the S phase of the cell cycle. This is also confirmed by accumulation of HUVEC in the G0/G1 phase and a propor-
tional reduction of the share of cells residing in S and G2/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 p27Kip and p21WAF-1/Cip1 (22). p27Kip 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 p27Kip, however, indicates that LA-exposed HUVEC have already entered the cell cycle (45) and suggests that p27Kip does not account for the observed cell cycle arrest in HUVEC.

LA, however, upregulates p21WAF-1/Cip1 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 p21WAF-1/Cip1 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 p21WAF-1/Cip1-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).

Fig. 6. Modulation by LA of cyclins. Protein expression of cyclins, i.e., cyclin E (G1/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 mmol/l LA) cells (set to 100%).
Of importance, LA exerts many of its effects in human endothelial cells already at 1 μmol/l (inhibition of apoptosis and modulation of p27kip, hyper-pRb, and E2F-1). At 50 μmol/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 p27kip, p21WAF-1/Cip1, hyper-pRb, and E2F-1.

Peak plasma concentrations observed in humans after oral administration of 200 mg or infusion of 600 mg of α-lipoic acid’s racemic mixture (26, 41) or after oral administration of 600 mg LA (33) clearly lie within 1 and 50 μmol/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 μmol/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).

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 α-lipoic acid’s effects observed in vivo.

**GRANTS**

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