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The PI3K/Akt pathway is not a main driver in HDL-mediated cell protection

Adi Zheng, Gilles Dubuis, Carla Susana Mendes Ferreira, Jannick Pétremand, Güliz Vanli, Christian Widmann*

Department of Physiology, University of Lausanne, 1005 Lausanne, Switzerland

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ABSTRACT

High-density lipoproteins (HDLs) can protect cells against a variety of death-inducing stresses. This is often accompanied by activation of the anti-apoptotic Akt kinase but whether this activation mediates the protective functions of HDLs is still unclear. In this study, we evaluated the roles of PI3K/Akt signaling in endoplasmic reticulum (ER) stress- and starvation-induced cell death using pharmacological and genetic approaches to gain a better understanding of the relationship between Akt- and HDL-mediated protection. Three cell models were used for this purpose, a primary endothelial cell line, an insulinoma cell line and a colon adenocarcinoma cell line. Our results show that HDLs indeed elicited mild Akt activation in all the tested cellular models. PI3K is one of the main upstream proteins involved in Akt stimulation. In the three cellular models, LY294002, a PI3K inhibitor, only slightly blunted HDLs protection, indicating that HDLs induce PI3K-independent cell protection. Furthermore, genetic ablation or silencing of Akt did not abolish the protective effects of HDLs. This study demonstrates that the PI3K-Akt signaling pathway is not the main mediator of the cell protective functions of HDLs. Further investigation is therefore needed to identify the intrinsic mechanism of HDL-mediated cell protection.

1. Introduction

The classical functions of high-density lipoproteins (HDLs) are to regulate the dynamics of other lipoproteins, to mediate the transport of various bioactive molecules (proteins, lipids, siRNAs, hormones) in the circulation, and to facilitate the reverse transport of cholesterol from peripheral organs to the liver for excretion into the bile as well as to steroid hormone-producing organs [24]. HDLs are also now known to beneficially modulate the function, survival, proliferation, or differentiation of different cell types including endothelial cells, beta cells, and macrophages [1,2,5,10,16]. Many of these cytoprotective effects are not secondary to the modulation of cellular cholesterol homeostasis but rather believed to be elicited through specific agonists carried by HDLs [1,2,5,10,16,29]. However, the molecular characteristics of the HDL-mediated responses have been superficially investigated and only in a few cell types, in endothelial and pancreatic beta cells in particular [1,2,16,28]. Although the stimulatory effects of ApoA-I and HDLs on insulin secretion have been associated with their classical activity to stimulate cholesterol efflux via ATP binding cassette transporters ABCA1 and ABCG1 [9,15], not much is known on the signaling pathways by which HDLs improve function and survival of beta cells. The data suggesting the involvement of given signaling proteins in these beneficial effects are mostly of circumstantial nature. For example, Akt has been proposed to mediate the protective effect of HDLs against growth factor deprivation-induced cell apoptosis in human endothelial cells [19] but this was based on the use of PI3K inhibitors, the specificity of which being difficult to evaluate. In the case of beta cells, no HDL component has been identified to mediate the cell protective functions. For this cell type, the available information is mostly of negative nature: the SR-BI receptor is not required for HDL-mediated anti-apoptotic responses [21,25], over-expression of Akt paradoxically induces beta cell death [4,27] and this may be due to the ability of Akt to activate strong NF-kB signaling in these cells [4]. HDLs have also been shown to activate Akt in prostate cancer cells and a PI3K inhibitor could partially block HDL-induced cell proliferation [26] but here again the specificity of the inhibitor was not evaluated. The role of Akt in HDL-mediated anti-apoptotic functions remains therefore unclear. The aim of the present study was to assess pharmacologically and genetically if Akt is involved in HDL-induced cell protection and to revisit the importance of Akt signaling in this effect. Three different cellular systems were employed. The first one, the DLD-1 cancer cell line, was used because of the availability of Akt knock-out clones. The other two, the Min6 insulinoma cell line and the primary HUVEC endothelial cell line, correspond to classical cellular models used by many researchers

* Corresponding author at: Department of Physiology, Rue du Bugnon 7, 1005 Lausanne, Switzerland.
E-mail address: Christian.Widmann@unil.ch (C. Widmann).

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investigating the signaling and protective functions of HDLs [30].

2. Materials and methods

2.1. Chemicals

H_2O_2 solution, U0126, SB203580 and SP600125 were purchased from Sigma (ref. no. H1009, 662005, S8307, and S5567, respectively).

2.2. Lipoprotein isolation

HDLs were prepared from human serum by sequential density ultracentrifugation [11,12]. HDL concentration was determined based on cholesterol content using the CHOL kit (Roche; ref. 12015630).

2.3. Buffers

KREBS buffer (NaCl 127 mM, KCl 4.7 mM, $CaCl_2$ 1 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, HEPES 25 mM, $NaHCO_3$ 5 mM) was prepared from its individual components.

PBS (NaCl 0.68 g/100 ml, KH_2PO_4 0.04 g/100 ml, $NaH_2PO_4{\cdot}2\ H_2O$ 0.15 g/100 ml) was purchased from Bichsel (ref 100 0324; lot 161,616).

2.4. Cells and cell culture

Wild-type or Akt 1/2 double knock-out DLD-1 (gift from Prof. Bert Volgenstein at the Core Cell center Baltimore) cells were maintained in RPMI 1640 (Gibco; ref. 61,870–010; lot 1,880,320) supplemented with 10% FBS (Gibco; ref. 10, 270–106; lot 42G5062K) at a temperature of 37 °C with 5% CO_2.

MIN6 clone B1 mouse insulinoma cells (kindly provided by Dr. P. Halban, University Medical Center, Geneva, Switzerland) were cultured in high-glucose DMEM (Gibco; ref. 61,965–026) supplemented with 15% FBS, 1 mM of sodium pyruvate (Gibco; ref. 11, 360–070) and 70 µM freshly added beta-mercaptoethanol (Gibco; ref. 31, 350–010) at a temperature of 37 °C with 5% CO_2.

HUVEC cells (kindly provided by Dr. Florent Allagnat, University of Lausanne, Lausanne, Switzerland) were cultured in EGM-2 BulletKit (Lonza; CC-3156 + CC-4176), at a temperature of 37 °C with 5% CO_2.

2.5. Cell lysis

Cells were washed with 1 ml PBS, harvested and lysed for 20 min on ice in 50 µl mono Q-c lysis buffer (70 mM β-glycerophosphate, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 100 µM Na_3VO_4, 1 mM dithiothreitol, 20 µg of aprotinin per ml; 10 mM NaF, 1 tablet of protease inhibitor per 50 ml). After a 1-min centrifugation (16′000 *g* at 5 °C) the pellet was discarded and the cell lysate was kept at −20 °C until use.

2.6. Akt1/2 knockdown

The pLKO.1-puro vectors encoding the shRNAs targeting mouse Akt1 (#1028) and Akt2 (#1029) were purchased from Sigma (TRCN0000304735 and TRCN0000310882). These constructs were used to generate lentiviruses bearing a puromycin resistant gene and shRNAs against either Akt1 or Akt2. Min6 cells were first infected with the shAkt1-encoding lentivirus for three days followed by a one week selection in the presence of 2 µg/ml puromycin. Then, these cells were infected with the shAkt2-encoding lentivirus for three days.

2.7. Protein quantitation (Bradford assay)

Two µl of the protein-containing samples were incubated for 20 min in 200 µl of 0.2 M NaOH and 1.8 ml of Bradford solution (10% orthophosphoric acid, 100 µM comassie blue, 5% ethanol) and absorbance was measured at 595 nm.

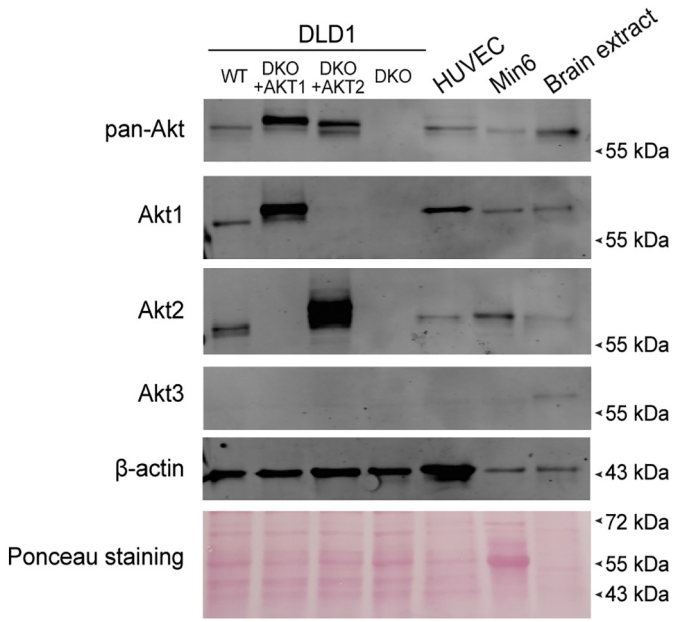

Fig. 1. Akt isoform expression in different cell lines.
Akt1, Akt2 and Akt3 levels in the cell lines used in this study were assessed by western blotting using the indicated antibody. For DLD-1 cells, wild-type (WT) and Akt1/2 double knock-out (DKO) cells were tested. Akt1 and Akt2 were re-expressed separately in DKO cells to assess the specificity of the corresponding antibodies. Mouse brain extract was used as a positive control for the detection of Akt isoforms (Akt3 in particular). Loading was assessed by Ponceau staining.

2.8. Western blotting

Fifty µg of protein lysates were loaded in a 10% polyacrylamide gel. Proteins were migrated for 2 h at 100 V (running buffer: 312 mM Tris, 1.92 M glycine, 0.2% SDS). Proteins were then transferred to a nitrocellulose membrane (0.45 µm; BIO-RAD; ref. 1,620,115) for 1 h using a voltage of 100 V (transfer buffer: 2.5 mM Tris, 1.92 M glycine, 2% SDS, 20% methanol). After Ponceau (0.1% Ponceau, 50% acetic acid) staining to confirm proper protein transfer, the membrane was blocked using 5% BSA in TBS-tween 20% for 30 min. Membranes were subsequently incubated with a phospho(serine 473)-Akt antibody (Cell signaling; ref. 9271 s; lot 14; 1:1000 in 5% BSA) overnight at 4 °C, subjected to three 10 min sTBS-tween 20% washes, then incubated with a secondary antibody (Alexa Fluor 680 goat anti-rabbit; Thermofisher ref. A21109, lot 1,816,534; diluted 1:10000 in 5% BSA) for 1 h at 4 °C and subjected to three 10 min washes in TBS-Tween 20%. The same procedure was repeated for the detection of β-actin, with the membrane being incubated with a β-actin antibody (Cell Signaling Technology; ref. 4970 s, lot 14; diluted 1:1000 in 5% BSA) for 1 h at 4 °C and then submitted to the same washes and incubation with secondary antibody as mentioned for phospho-Akt. Concerning the western blot evaluation of MAPK pathway activation, the following antibodies were used: phospho(T^{180}/Y^{182})-p38 (rabbit monoclonal IgG), phospho(T^{202}/Y^{204})-p44/42 (rabbit monoclonal IgG), phospho(T^{183}/Y^{185}) p-JNK (rabbit polyclonal), and phospho(S73)-c-Jun (rabbit polyclonal), all obtained from Cell Signaling Technology (ref. 4631/lot 2, 4376/lot 9, 9251/lot 10, 9164/lot 11, respectively). These antibodies were used at a 1/1000 dilution in 5% BSA in TBS-tween 20% at 4°C overnight. The membranes were next subjected to three 5 min TBS-tween 20% washes and then incubated with an anti-rabbit secondary Alexa Fluor 680-coupled antibody (Life Technology; ref. A21109/lot 1584296) at a 1/5000 dilution in 5% milk in TBS-tween 20% for 1 h at room temperature. Ponceau staining was quantified by Image J and used to normalize the expression of the proteins of interest.

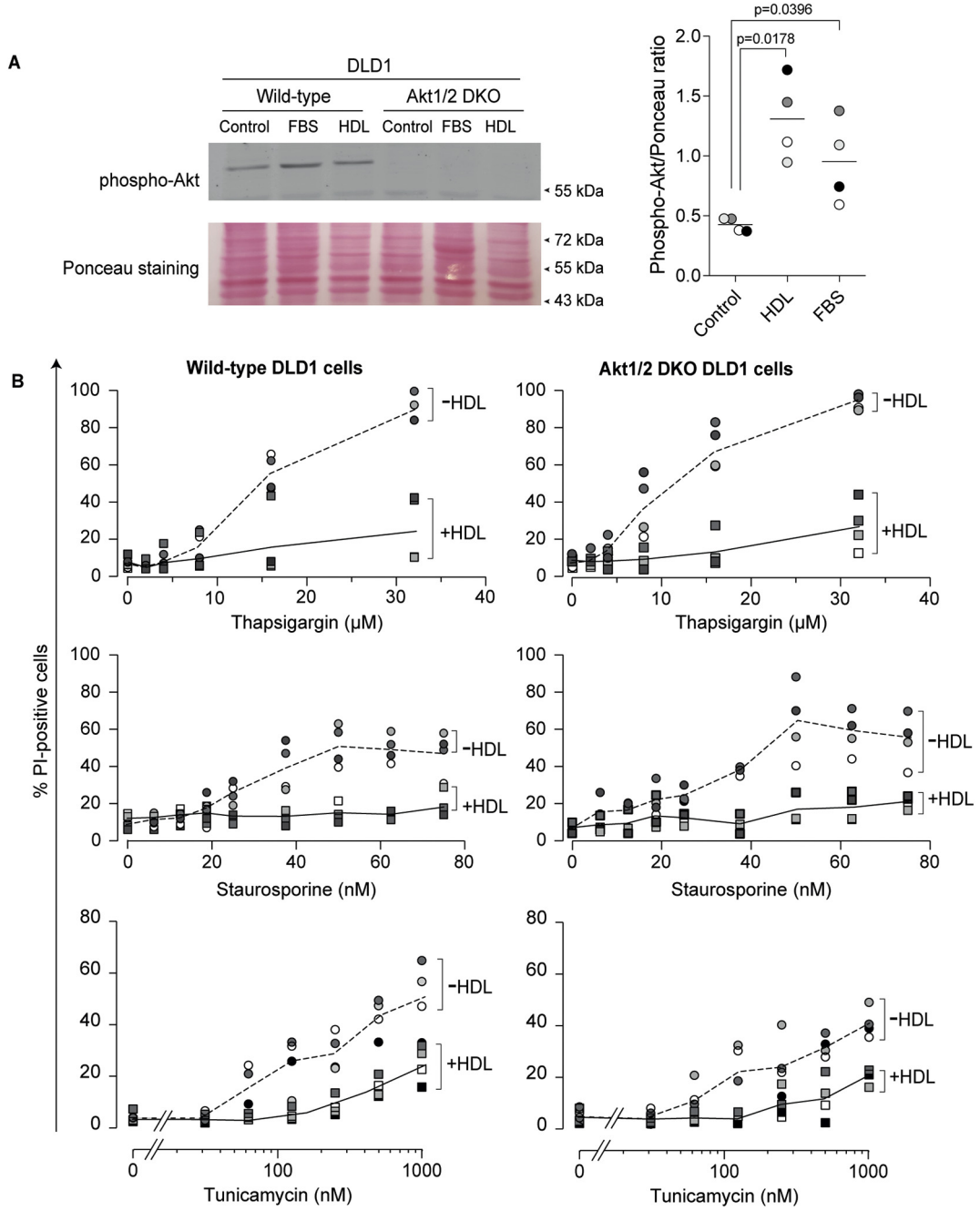

Fig. 2. Akt is not required for HDL-mediated DLD-1 protection against ER stress.
A. Wild-type or Akt1/2 double knock-out (DKO) DLD-1 cells (500′000 cells per well of 6 well-plates) were starved for 30 min and then either treated with 20% FBS or 1 mM HDL for 20 min or maintained in the starving medium for 20 min (control). The cells were then lysed to determine the expression levels of phospho-Akt (serine 473) by western blot analysis (left panel). Phospho-Akt values were then normalized to the corresponding Ponceau staining values (right panel). B. Wild-type or Akt1/2 double knock-out cells (100′000 cells per well of 6 well-plates) were treated for 72 h with the indicated drugs in the presence [+HDL] or in the absence [-HDL] of 1 mM HDL-cholesterol. Cell death was measured by PI assay. Symbols with different shades of grey correspond to independent experiments. The curves go through the average values of the different experiments.

2.9. Cell viability (propidium iodide assay)

After the indicated treatment, cells (including the floating cells in the medium) were collected and then incubated with eight μg/ml propidium iodide (PI) in PBS for five minutes at room temperature. PI incorporation into cells was then analyzed by flow cytometry.

3. Pycnosis assessment

After the indicated treatment, HUVEC cells were incubated three minutes at 37 °C with 5 μg/ml PI and 5 μg/ml Hoechst 33342. The percentage of pycnotic/necrotic cells was then determined visually using fluorescence microscopy. At least 500 cells were counted for each condition.

3.1. Statistics

The results were analyzed using the GraphPad Prism software. Unless otherwise stated, the straight horizontal lines in the graphs correspond to the median. Two-way analysis of variance (ANOVA) were

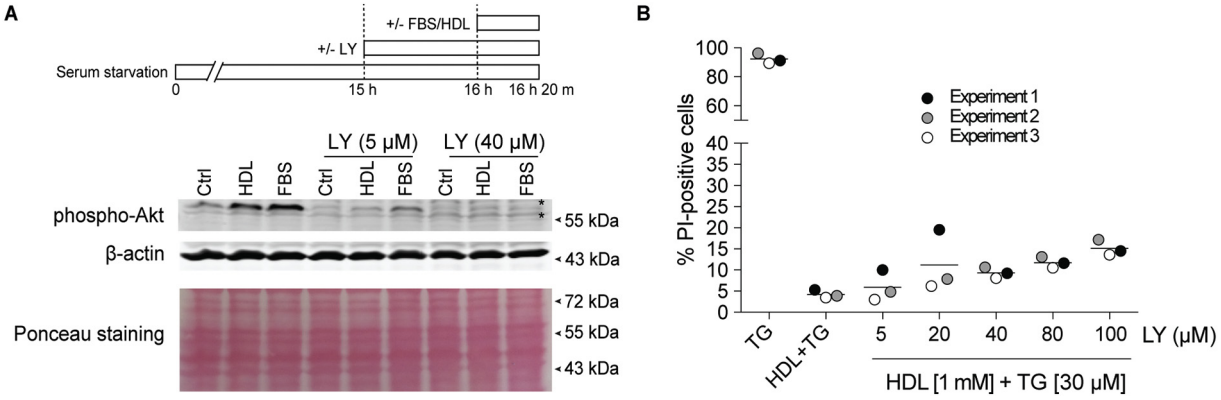

Fig. 3. PI3K is not required for HDL-mediated DLD-1 protection against thapsigargin.
A. Wild-type DLD-1 cells were starved overnight with serum-free RPMI medium and then preincubated for 1 h with the indicated concentrations of LY and finally with 20% FBS or 1 mM HDL for 20 min (see scheme above the figure). The expression levels of actin and phospho-Akt (serine 473) were monitored by western blotting. *, non-specific bands. B. Wild-type DLD-1 cells were incubated with the indicated treatment for 24 h. Cell death was assessed by PI assay.

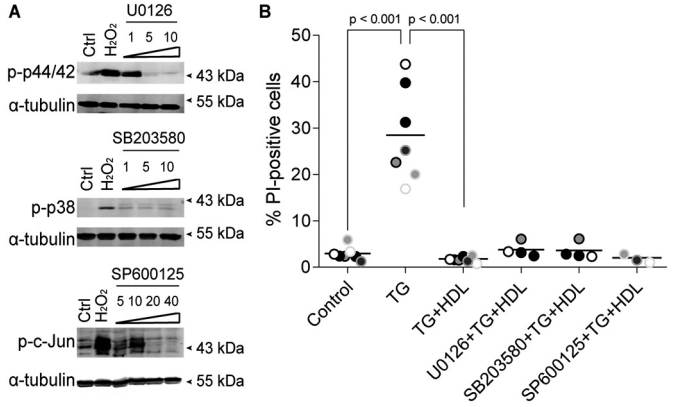

Fig. 4. MAPK pathways are not involved in HDL-mediated cell protection.
A. Wild-type DLD-1 cells were starved overnight with serum-free RPMI medium and then preincubated for 1 h with increasing concentrations (μM) of the indicated MAPK pathway inhibitors and finally with 5 mM H_2O_2 for 20 min. The expression levels of tubulin, phospho-p38 (Thr^{180}/Tyr^{182}), phospho-p44/42 (Thr^{202}/Tyr^{204}), and phospho-c-Jun were monitored by western blotting. B. Wild-type DLD-1 cells were incubated with 20 μM thapsigargin with or without 1 mM HDL in the presence of 10 μM SB203580, 10 μM U0126, or 20 μM SP600125 for 24 h. Cell death was assessed by PI assay. The black lines represent the mean. The results are derived from 3 to 7 independent experiments indicated by different shades of grey.

used for comparing dose-response profile between two groups. Other comparisons between two groups were performed using Student's *t*-test.

4. Results

To assess the role of Akt in the cellular protection mediated by HDLs, we first analyzed the protein expression of the three Akt isoforms (Akt1, Akt2, Akt3) by western blot in the different cell models used in this study: the DLD-1 human colon cancer cell line, the HUVEC human normal umbilical vein endothelial cells and the MIN6 mouse immortalized pancreatic beta cell line. A brain extract containing all three Akt isoforms was used as a positive control. As shown in Fig. 1, Akt1 and Akt2 are expressed in DLD-1, HUVEC and MIN6, but not Akt3, which is mainly involved in brain development [6]. Fig. 1 also validates the specificity of the antibodies used in the study.

To first determine whether HDLs could activate Akt, we used the DLD-1 colorectal adenocarcinoma cell line that expresses Akt1 and Akt2 but not Akt3 [7] and the corresponding Akt1 and Akt2 double knockout cells (Fig. 1). As a positive control, starved cells were incubated with 20% FBS that leads to Akt phosphorylation on serine 473 (Fig. 2A). Similarly to FBS, HDLs stimulated Akt phosphorylation in starved cells (Fig. 2A). Phosphorylated Akt was not detected in knockout cells, as expected (Fig. 2A).

Thapsigargin and staurosporine are two different apoptosis inducers. Thapsigargin is a SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor that induces ER (endoplasmic reticulum) calcium depletion and then ER stress [21] while staurosporine is a broad and non-specific kinase inhibitor [13]. To assess the role of Akt in HDL-mediated protection, wild-type DLD-1 and Akt1/2 double knock-out DLD-1 cells were exposed to either of these drugs (Fig. 2B). Thapsigargin and staurosporine induced cell death in a dose-dependent manner in wild-type DLD-1 cells and this was counteracted by HDL treatment. However, there was no difference in HDL-mediated protection against these drugs in cells lacking Akt1 and Akt2. Similar results were found when tunicamycin, another ER stressor that blocks protein glycosylation, was used [22]. LY294002 (LY) is an inhibitor targeting phosphatidylinositol 3-kinase (PI3K), one of the main upstream activators of Akt. LY suppressed Akt phosphorylation induced by HDLs (Fig. 3A) but this did not or only minimally affect the ability of HDLs to protect cells against TG (Fig. 3B). Akt activation by HDLs plays therefore little or no role in the manner by which HDLs prevent TG-induced DLD-1 cell death.

In addition to PI3K/AKT signaling, MAPK pathways are also known to play important roles in the regulation of cell survival [31,32]. In this context, it is worth mentioning that HDLs can stimulate the phosphorylation and activation of the p44/42 and p38 MAPKs [8]. To specifically test if the ERK, p38 and JNK MAPK pathways influence the manner by which HDLs protect DLD-1 cells, specific MAPK pathway inhibitors were used (Fig. 4A). Fig. 4B shows that inhibition of the three MAPK pathways did not alter the protective effects of HDLs against thapsigargin in DLD-1 cells.

HDLs have been shown previously to promote beta cell function and survival. For example, HDLs protect beta cells against ER stressors such as TG, cyclopiazonic acid or tunicamyin [21, 22]. The earlier observation that TG induces a reduction in Akt activity in the Min6 mouse insulinoma cell line and that this is partially blunted by HDLs may be an indication that Akt plays a role in the ability of HDLs to protect beta cells [22]. We therefore evaluated in more details the role of Akt in the HDL-mediated protective functions in Min6 cells. In these cells, HDLs induced a transient phosphorylation of Akt on serine 473 site with a peak stimulation after about 20 min (Fig. 5A). Akt stimulation by HDLs and insulin in Min6 cells was greatly reduced by the LY PI3K inhibitor (Fig. 5B). However, the protection against thapsigargin conferred by HDLs was unaffected by LY or by Wortmannin, another chemically-unrelated PI3K inhibitor (Fig. 5C). Additionally, LY did not alter the

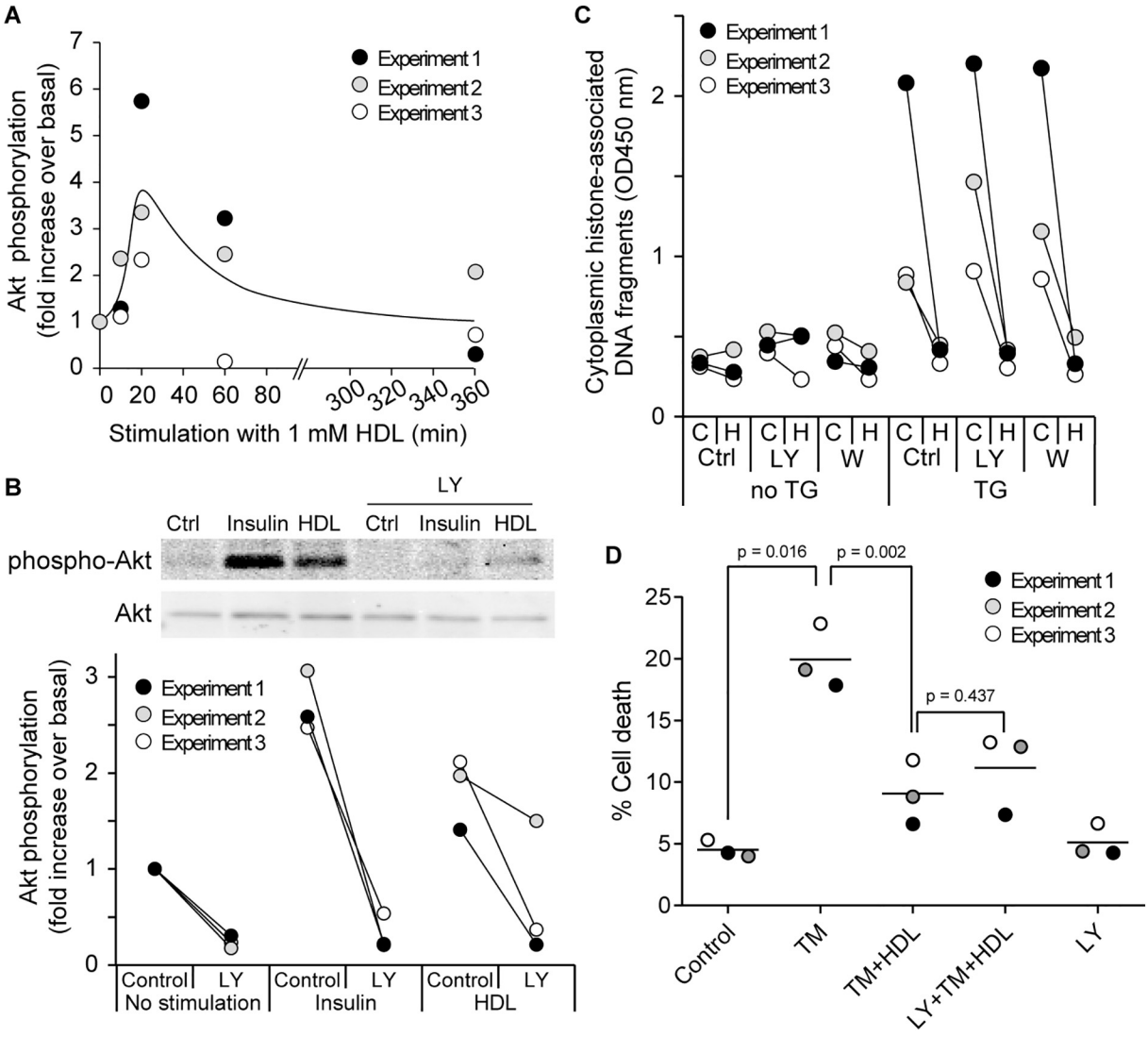

Fig. 5. The PI3K/Akt signaling pathway is not playing a significant role in HDL-mediated protection against ER stress in Min6 cells.
A. Min6 cells were stimulated for the indicated periods of time with 1 mM HDL. Akt stimulation was assessed by Western blotting using an anti-S473 phospho Akt antibody. Results were derived from three independent experiments (indicated by circles with different shades of grey). The curve goes through the average values of the three experiments. B. Min6 cells were incubated 2 h in KREBS medium containing 2 mM glucose, then preincubated or not with 10 μM LY294002 (LY) for 30 min, and finally stimulated or not for 20 min with 500 nM insulin or 2 mM HDL. Akt stimulation was assessed by western blotting using a phospho Akt antibody. Results were derived from 3 independent experiments. Data points from a given experiment are linked. C. Min6 cells were left untreated or treated by 0.5 μM thapsigargin with or without 1 mM HDL in the absence or in the presence of PI3K inhibitors [0.5 μM Wortmannin (W); 10 μM LY294002 (LY)]. Cell death was measured 24 h later by assessing the release of cytoplasmic DNA-bound histones. The results are derived from three independent experiments. D. Min6 cells were left untreated or treated with 2 μg/ml tunicamycin (TM), with or without 1 mM HDL, and in the absence or in the presence of a PI3K inhibitor [10 μM LY294002 (LY)]. Cell death was measured 24 h later by scoring pycnotic cells.

ability of HDLs to protect Min6 cells against tunicamycin-induced death (Fig. 5D).

To genetically validate the pharmacological results obtained in Min6 cells, we silenced Akt1 and Akt2 in these cells using shRNA-encoding lentiviruses. As shown in Fig. 6, knocking-down Akt1 and Akt2 did not alter the ability of HDLs to protect Min6 cells against thapsigargin. Altogether these results indicate that, even though HDLs can activate Akt, this stimulation does not appear to be required for the protective activities of HDLs.

HDLs have also been shown to protect endothelial cells against cell death. Confirming earlier results [19], Fig. 7A shows that HDLs induced Akt phosphorylation in the primary endothelial HUVEC cell line but to a much reduced extent compared to serum. HDLs antagonized starvation-induced HUVEC cell death, but also thapsigargin-induced death (Fig. 7C-D). However, blocking Akt phosphorylation with LY (Fig. 7B-D) did not prevent HDL-mediated protection against starvation or thapsigargin, indicating that in primary cells, Akt does not play a significant role in HDL-mediated protection.

5. Discussion

Accumulating evidence shows that HDLs exert a significant effect on diverse diseases including diabetes [30], cardiovascular disease [3] and cancer [26], through its anti-oxidative, anti-inflammatory properties, as well as anti-apoptotic responses. Nevertheless, the intracellular signal targets are poorly understood, if at all. It has been reported that HDLs binds to the ABCA1 and ABCG1 transporters and to the scavenger receptor class B, type I (SR-BI) and that these bindings initiate signaling events in some models [14,17]. HDLs have been shown to activate the PI3K-Akt pathway in a number of experimental systems (this study and

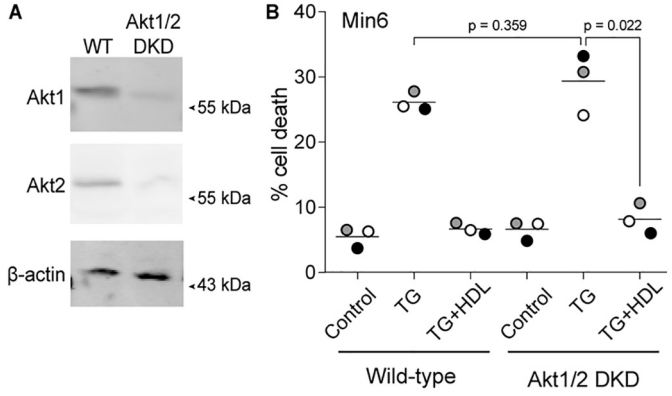

Fig. 6. Effect of Akt silencing in Min6 cells on the protective function of HDLs. A. Akt1/2 knock-down in Min6 cells was performed through infection with lentiviruses bearing Akt isoform-specific shRNAs. The downregulation of Akt1 and Akt2 was assessed by western blot analysis. B. Wild-type Min6 cells and Akt1/2 double knockdown (DKD) Min6 cells were treated with 1 μM thapsigargin with or without 1 mM HDL for 24 h. Cell death was then measured by scoring pycnotic cells. The results are derived from three independent experiments indicated by different shades of grey.

[18,20]). Our aim was to test whether Akt is a key downstream signaling regulator conveying the protective functions of HDLs. Our data demonstrate that PI3K is at best only minimally involved in HDL-mediated protection against starvation or ER stress-induced cell death and that Akt is dispensable for the protective functions of HDLs.

Akt was found to be activated, to rather marginal extent, by HDLs in all the three models tested in the present work. We initially expected that Akt disruption could at least partially prevent HDLs to inhibit cell death induced by these stimuli. However, genetic disruption of both Akt1 and Akt2 did not affect the ability of HDLs to protect cells. We did observe a slight reduction in the protective functions of HDLs when PI3K, the upstream regulator of Akt, was inhibited. There might therefore be a PI3K-dependent factor (or an off-target of the PI3K inhibitors used) contributing marginally to the manner by which HDLs protect cells (or the basal resistance capacity of the cells). But if this is the case, our data clearly indicate that these factors are distinct from Akt.

Akt has been suggested to mediate the protective effects of HDLs against growth factor deprivation in the human endothelial HUVEC cells in a short time experiment assay by using the PI3K inhibitor LY294002 [19]. In this earlier publication, starvation induced apoptosis after only 3 h of starvation, something we could not reproduce here (3 days of starvation were required to achieve more than 40% HUVEC death in the present study). In our hands however, we could find no evidence that PI3K-mediated Akt activation contributed to HDL-stimulated HUVEC protection against starvation.

The evidence presented in this work indicate that signaling through Akt or MAPK pathways is not participating in the protection conferred by HDLs. A distinct pro-survival signaling route might therefore be

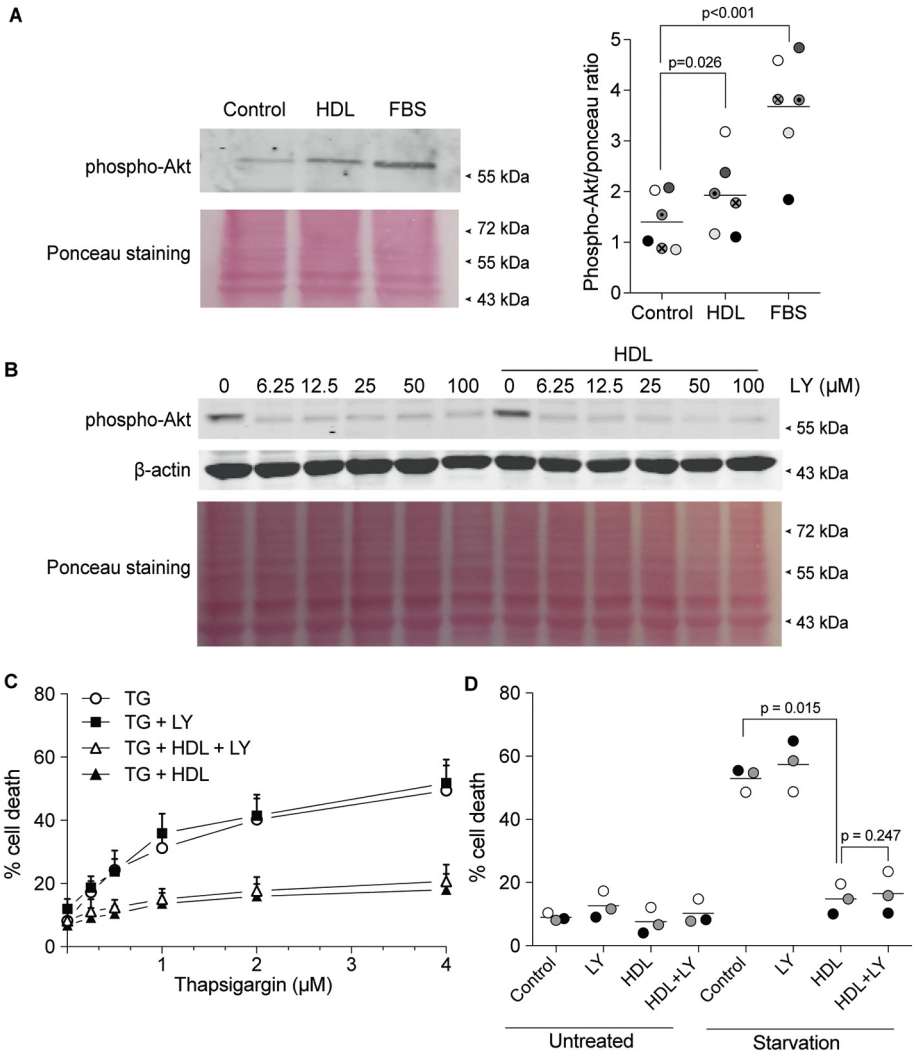

Fig. 7. The PI3K/Akt signaling pathway is not required for HDL-mediated cell protection in HUVEC cells.
A. HUVEC cells (500′000 cells per well of 6 well-plates seeded 24 h before treatment) were starved for 18 h using EGM-2 media and then either treated with 20% FBS or 1 mM HDLs for 20 min or were maintained in the starving medium for this 20 min period of time (Control). The cells were then lysed to determine the expression levels of phospho-Akt (serine 473) by Western blot analysis (left panel). Phospho-Akt values were then normalized to the corresponding Ponceau staining values. Differently-labelled symbols correspond to different independent experiments. B. HUVEC cells were starved with EGM-2 medium without serum and supplements for 18 h and then treated with 20% FBS or 1 mM HDL for 20 min with the indicated concentrations of LY. The expression levels of phospho-Akt (serine 473) was detected by Western blot. C. HUVEC cells were incubated with the indicated treatment for 72 h. Cell death was assessed by Hoechst assay. (mean ± SEM, three independent experiments). D. HUVEC cells (35′000 cells per well of 96 well-plates seeded 24 h before treatment) were treated for 72 h with EGM-2 media with or without supplements in the presence or in the absence of 1 mM HDL-cholesterol and 12.5 μM LY. Cell death was measured by scoring pycnotic cells.

stimulated by HDLs. The activation of NF-κB could be such an alternative protective signaling pathway activated by HDLs. However, even though NF-κB is known to be protective in a number of experimental settings, it appears to be detrimental in some cells [23]. For example, sustained NF-κB stimulation can lead to pancreatic beta cell death [4], indicating that NF-κB is unlikely to mediate the protective abilities of HDLs in this cell type. One might also envision that HDLs favor cell survival, not by stimulating a given signaling pathway, but by preventing the stressors from exerting their detrimental actions in cells, either through sequestration of the stressors or by interfering with the interaction of the stressors with their cellular targets.

In conclusion, our study investigated the role of Akt in HDL protective function using both pharmacological and genetic approaches in several cellular models. It demonstrates unequivocally that Akt is not a critical player for HDLs to inhibit cell death, at least for the apoptotic inducers used in this study (thapsigargin, staurosporine, tunicamycin, and starvation). Additional research efforts are required to understand the protection conferred by HDLs as this may help finding new strategies to treat diseases such as diabetes or atherosclerosis.

Conflict of interests

The authors confirm that this article content has no conflict of interest.

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Contribution

Conception and design of study: AZ, GV, JP, CW.
Acquisition of data: AZ, GD, CSMF, GV.
Analysis and/or interpretation of data: AZ, CW.
Drafting the manuscript: AZ, CW.
Revising the manuscript and approval of the submitted version: AZ, GD, CSMF, GV, JP, CW.

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