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Mitochondria maintain maturation and secretion of lipoprotein lipase in the endoplasmic reticulum

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SYNOPSIS

Considering the physiological Ca^{2+} dynamics within the endoplasmic reticulum (ER), it remains unclear how efficient protein folding is maintained in living cells. Thus, utilizing the strictly folding dependent activity and secretion of lipoprotein lipase (LPL), we evaluated the impact of ER Ca^{2+} content and mitochondrial contribution to Ca^{2+}-dependent protein folding. Exhaustive ER Ca^{2+} depletion by inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPases caused strong but reversible reduction of cell associated and released activity of constitutive and adenovirus encoded human LPL in CHO-K1 and endothelial cells, respectively, which was not due to decline of mRNA or intracellular protein levels. In contrast, stimulation with the IP_3-generating agonist histamine only moderately and transiently affected LPL maturation in endothelial cells that paralleled a basically preserved ER Ca^{2+} content. However, in the absence of extracellular Ca^{2+} or upon prevention of transmitochondrial Ca^{2+} flux, LPL maturation discontinued upon histamine stimulation. Collectively, these data indicate that Ca^{2+}-dependent protein folding in the ER is predominantly controlled by intraluminal Ca^{2+} and largely maintained during physiological cell stimulation due to efficient ER Ca^{2+} refilling. Since Ca^{2+} entry and mitochondrial Ca^{2+} homeostasis are crucial for continuous Ca^{2+}-dependent protein maturation in the ER, their pathological alterations may result in dysfunctional protein folding.

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INTRODUCTION

The endoplasmic reticulum (ER) constitutes the main releasable Ca^{2+} store in most cell types and actively contributes to cellular Ca^{2+} homeostasis via the release and re-uptake of Ca^{2+} in the course of precisely controlled signaling cascades [1] and extensive communication with mitochondria and adjacent ion channels of the plasma membrane [2, 3].

Moreover, by its oxidizing milieu, resident molecular chaperones and folding catalysts, the lumen of the ER provides an optimized environment for the synthesis and posttranslational processing of most secretory and membrane proteins [4]. Among the most important molecular chaperones of the ER, calreticulin and its transmembrane homologue calnexin provide a highly efficient quality control system for immature glycoproteins [5].

Initially characterized as Ca^{2+}-binding protein of the sarcoplasmic reticulum [6], conformation [7], chaperone interactions [8], and thus, activity of calreticulin have been shown to be directly affected by endoplasmic reticulum free Ca^{2+} concentration ($[Ca^{2+}]_{ER}$). A decrease in ambient $[Ca^{2+}]$ renders the calreticulin cycle and associated folding catalysts, i.e. protein disulfide isomerase and ERp57, inactive [9]. However, these principles of Ca^{2+}-dependent protein folding have been assessed mainly on recombinant proteins [7, 8, 10] or artificially applying excessive ER stress [11-13] while a detailed analysis of its kinetics and correlation with physiological Ca^{2+} fluctuations are lacking. While there are reports that address this issue [14], it still remains questionable whether the ER protein folding machinery is influenced by spatial and temporal dynamics of $[Ca^{2+}]_{ER}$ in intact cells and if so, how efficient protein folding is maintained under physiological cell stimulation. Moreover, since recent studies revealed a crucial role of mitochondria in the process of rescuing the Ca^{2+} content of the ER during stimulation with IP_3-elevating agonists [15], the importance of mitochondrial for Ca^{2+}-dependent protein maturation in the ER awaits to be assessed.

Notably, protein misfolding is considered as cause and/or consequence of numerous diseases [16, 17] including diabetes mellitus [18]. The latter is known to be associated with

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mitochondrial dysfunction that becomes obvious by increased free radical production [19] and altered mitochondrial Ca^{2+} homeostasis [20]. However, since the link between mitochondrial Ca^{2+} homeostasis and ER protein folding has not been investigated so far, the consequences of pathological alterations in mitochondrial Ca^{2+} function on ER protein maturation cannot be estimated.

In order to elucidate the feasible impact of $[Ca^{2+}]_{ER}$ dynamics and the contribution of mitochondrial Ca^{2+} homeostasis on protein folding, the expression, secretion and enzyme activity of lipoprotein lipase (LPL) as a measure for the efficiency of protein processing machinery of the ER was investigated. LPL, the major lipolytic enzyme for the hydrolysis of circulating lipoproteins, is a secretory N-linked glycoprotein, which contains two conserved disulfide bridges, and that interacts with the Ca^{2+}-dependent protein folding machinery of the ER during its maturation [11, 13, 21]. As only the correctly folded protein is active and secreted [11, 21], LPL activity is dependent on proper maturation in the ER and, therefore, it was assumed to be a suitable sensor for ER chaperone-dependent folding processes.

MATERIALS AND METHODS

Materials

Cell culture media and substitutes, specific primers, DNA standards, Moloney murine leukaemia virus reverse transcriptase and first strand buffer were obtained from Invitrogen Life Technologies (Vienna, Austria). Fetal calf serum was from PAA (Linz, Austria). Cell culture plastic ware was purchased from Bertoni (Vienna, Austria). The RNeasy Mini kit was from Quiagen (VWR, Vienna, Austria). RQ1 RNase free DNase I and TransFast™ were obtained from Promega (Mannheim, Germany). ^{3}H-labeled and non-radioactive triolein, BHQ, histamine and BSA fraction V were from Sigma-Aldrich (Vienna, Austria). Fura-2/AM was from Molecular Probes Europe (Leiden, Netherlands). RNAguard and random hexameric

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primers were purchased from Amersham Biosciences (Freiburg, Germany). CGP 37157 was purchased from Tocris Cookson Ltd. (Northpoint, Avonmouth, Bristol, UK). Heparin was from Boehringer (Ingelheim, Germany). Hot Fire DNA Polymerase I was from Solis BioDyne (Tartu, Estonia). Luminol reagent was obtained from Santa Cruz (Szabo Scandic, Vienna Austria) and Ultima Gold scintillation cocktail was from Packard Biosciences (PerkinElmer, Vienna, Austria). X-ray films were from Siemens (Graz, Austria). Nitrocellulose, protein standards, dNTPs and all other chemicals were purchased from Roth (Lactan, Graz, Austria).

Cell culture and adenovirus infection

Chinese hamster ovary (CHO-K1) cells were cultured on 6 well dishes in Ham´s F12 containing 10 % FCS. Upon reaching confluence, cells were incubated with 1 % BSA in serum-free Dulbecco´s minimum essential medium for 24 h prior to experiment. The human umbilical vein derived endothelial cell line EA.hy926 [22] at passages higher than 44 was grown on 6 well dishes in culture medium containing 10 % fetal calf serum and 1 % HAT (5 mM hypoxanthine, 20 μM aminopterine, 0.8 mM thymidine). Subconfluent cells were infected with 100 MOI of adenovirus encoding human LPL [23] in culture medium containing 2 % fetal calf serum which was replaced for regular medium after app. 12 h.

Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR was performed as described previously [24]. Amplification was carried out with initial denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C and annealing at 55 °C for 30 seconds each and elongation at 72 °C for 60 seconds. The specific primers used for human LPL were 5´-GCA TTG CAG GAA GTC TGA CC-3´ and 5´-GAT GTT CTC ACT CTC GGC CA-3´ yielding a 632 bp product. As control a 537 bp fragment of glyceraldehyde-3-phosphate dehydrogenase was amplified using the primers 5´-

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ACC ACA GTC CAT GCC ATC AC-3´ and 5´-TCC ACC ACC CTG TTG CTG TA-3´. PCR products were separated on 2 % TAE-agarose gels.

Western Blot analysis

Western blots were carried out according to our recent protocol [25]. Briefly, 50 μg total protein or 200 μl vacuum-concentrated supernatant were mixed with sample buffer yielding a final concentration of 4 % glycerol (v/v) 1 % SDS (w/v), 0.03 % brompehenol blue (w/v), 0.6 % 2-mercaptoethanol (v/v) in 12.6 mM Tris-HCl, pH adjusted to 6.8, and boiled at 95 °C for 10 minutes prior to separation on 12.5 % SDS-polyacrylamide gels. After electrotransfer of the proteins onto nitrocellulose membranes, unspecific binding sites were blocked by 5 % (w/v) non fat instant milk in PBS and the membranes probed at 4 °C overnight with rabbit polyclonal antibody against recombinant human LPL diluted 1:1000 in 5 % milk powder in PBS containing (in mM) 137 NaCl, 2.7 KCl, 8 Na_2HPO_4 x 2 H_2O, 1.5 KH_2PO_4; pH adjusted to 7.4. Subsequently, membranes were washed with PBS containing 0.05 % Tween-20 (v/v), incubated for 1 hour with horseradish-peroxidase conjugated secondary antibody and detected by enhanced chemiluminescence after additional washing steps.

Determination of LPL enzyme activity

Following incubation with the respective substances in culture medium containing 0.5 μg/ml heparin, supernatants were collected on ice and the cells lysed by sonification in chilled 50 mM NH_3/NH_4Cl solution (pH 8.1). Protein concentration of cell lysates was determined according to the established method of Lowry [26]. LPL activity was determined by the hydrolysis of ^{3}H-labeled triglycerides as described previously [27]. 1 μCi ^{3}H-triolein and 920 μg non-radioactive triolein per sample were evaporated under a stream of nitrogen and mixed with 20 μl 1 M Tris-HCl, pH adjusted to 8.6, 20 μl 1% Triton X-100 and 80 μl H_2O by extensive sonication on ice-cooled water. Following addition of 40 μl heat-inactivated (50°C,

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1 h) human serum containing apoCII as activator and 40 μl 10% BSA, 100 μl of cell lysates or supernatant were mixed with 200 μl of the resultant chilled substrate and incubated for 30 min at 37 °C under continuous shaking. The reaction was stopped by 3.25 ml of a organic solvent mixture consisting of methanol : chloroform : n-heptane (1.41:1.25:1, v/v/v). By addition of 1 ml 0.1 M K_2CO_3/H_3BO_3, pH adjusted to 10.5, free fatty acids became extractable from a two phase system by vigorous shaking for 20 s and subsequent centrifugation at 3000 rpm for 20 min. 1 ml of the upper phase was mixed with 8 ml Ultima Gold scintillation cocktail and measured in a β-counter (Beckmann, Munich, Germany). LPL activity was expressed as amount of free fatty acids, hydrolised per minute by lipolytic enzyme contained within or in the supernatant of 1 mg of total cellular protein as described previously [27]. This assay was not directly affect by BHQ, histamine or CGP 37187.

Determination of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$)

Cytosolic free Ca^{2+} concentration was measured as previously described [28]. Briefly, endothelial cells grown on glass cover slips or culture dishes of 3 cm diameter were loaded for 45 min at room temperature in the dark in LB containing 2 μM fura-2/AM. Prior experiments, cells were washed twice with LB and equilibrated for further 15 min in EB in the dark. Cells were illuminated alternatively at 340 and 380 nm (340HT15 and 380HT15; Omega Optical, Brattleboro, VT, USA) and emission was monitored at 510 nm (510WB40; Omega Optical). $[Ca^{2+}]_{cyto}$ was expressed as $(F_{340}/F_{380})/F_0$.

Measurement of $[Ca^{2+}]_{ER}$

Intraluminal free Ca^{2+} concentration was monitored as described previously [3, 29]. In brief, at a confluency of app. 80%, endothelial cells grown on glass cover slips were transiently transfected with 2 μg of purified pcDNA3 encoding the improved versions of ER-targeted $D1_{ER}$ [30] using TransFast™ Transfection Reagent according to the manufacturer´s protocol.

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Between 36 and 48 h after transfection, cells were used for experiment. $D1_{ER}$ was excited at 440 nm (440AF21, Omega Optical) and emission was collected simultaneously at 535 and 480 nm using an optical beam splitter (Dual-View MicroImagerTM, Optical Insights, Visitron Systems, Puchheim, Germany). Changes of $[Ca^{2+}]_{ER}$ were expressed as $(F_{535}/F_{480})/F_0$. Each data point represents the mean ± SEM of 3 to 4 cells over a measurement period of 3 min.

Statistics

Data represent the mean ± SEM. Error bars are shown for every data point presented. If no bar is visible, the S.E.M. was smaller than the symbol size. Analysis of variance (ANOVA) and Scheffe´s *post hoc* F test were used for evaluation of the statistical significance. $P<0.05$ was defined to be significant.

RESULTS

To elucidate the general mechanisms of the regulation of LPL maturation by ER Ca^{2+} content, we initially investigated the maturation and secretion of constitutive LPL in CHO-K1 cells [31] under control conditions and upon exhaustive Ca^{2+} depletion of the ER.

Inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) yielded efficient Ca^{2+} depletion of the ER

As a consequence of an inhibition of SERCA with 2,5-di-(tert-butyl)-1,4-hydroquinone (BHQ; 15 µM), ER Ca^{2+} content exhaustively decreased long lastingly (Fig.1A) [3, 15] despite continuous presence of extracellular Ca^{2+}. This effect was fully reversible upon removal of BHQ. Concomitantly, $[Ca^{2+}]_{cyto}$ increased and remained elevated as long as BHQ was present (Fig.1B).

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SERCA inhibition attenuated activity and secretion, but not expression of endogenous LPL

To induce endogenous expression of LPL, CHO-K1 cells were propagated in serum free medium for 24 h prior experiment. Ca^{2+} depletion of the ER with 15 μM BHQ resulted in gradual decline of LPL activity in cell lysates (Fig.1C). Subsequent removal of BHQ yielded complete recovery of enzyme activity within 1 h (Fig.1C). LPL activity released into the supernatant of CHO-K1 cells rose constantly (Fig.1D). Application of 15 μM BHQ retained extracellular LPL activity at low level, while immediately after washout of BHQ, the secretion of active LPL increased with identical kinetics as in control (slope: 3.0 ± 0.7 with vs. 3.0 ± 0.2 (nmol/mg x min) x h^{-1} without BHQ pre-treatment) (Fig.1D). By RT-PCR, the mRNA levels of LPL and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were found to be unaffected by 3 h treatment with BHQ (Fig.1E).

These data suggest, that endogenous transcription of LPL in wild-type CHO cells is not altered by depletion of $[Ca^{2+}]_{ER}$, whereas its enzymatic activity and secretion are reversibly reduced.

Due to the lack of a suitable antibody against LPL of hamster origin, possible alterations on the protein level could not be verified. Consequently, in order to correlate ER Ca^{2+} content further with protein transcription, expression as well as maturation and secretion, human LPL was over-expressed in endothelial cells utilizing adenovirus [23].

Depletion of $[Ca^{2+}]_{ER}$ attenuated activity and secretion of adenovirus-encoded LPL

In endothelial cells, adenovirus-mediated expression of human LPL yielded a steady enzyme activity in cell lysates and continuous LPL secretion into the medium (Fig.2). In the presence of 15 μM BHQ, which achieved exhaustive ER Ca^{2+} depletion similar to that shown in CHO-

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K1 cells [3], LPL activity in cell lysates decreased and partially recovered upon removal of BHQ (Fig.2A).

In agreement with the results obtained on the constitutive LPL in CHO-K1 cells, activity of adenovirus-coded human LPL increased gradually in the medium of untreated endothelial cells with a slope of 6.3 ± 0.7 (nmol/mg x min) x h^{-1} in the first 3 h. Addition of 15 μM BHQ arrested the enzyme activity at low level. In consequence of BHQ washout, in the first 3 h LPL activity in the supernatant started to rise with a slope comparable to that initially seen in untreated cells (6.8 ± 1.2 (nmol/mg x min) x h^{-1}) (Fig.2B).

Adenovirus-mediated expression of LPL was unaffected by depletion of $[Ca^{2+}]_{ER}$

As shown in figure 3A, mRNA levels of human LPL in relation to GAPDH remained unchanged in endothelial cells by up to 4 h of BHQ treatment and during the subsequent washout period. Moreover, the content of LPL protein within equal amounts of cell lysate was unaffected by BHQ (Fig.3B, upper panel). However, the additional band of lower molecular weight that was detectable with the antibody increased slightly within the first hour after BHQ application and disappeared during the washout period. In the supernatant of adenovirus-infected endothelial cells, LPL protein content paralleled the raise in enzymatic activity found in the medium. Consecutively, BHQ abolished LPL secretion and LPL protein in the medium did not reach a detectable level up to 3 h after removal of the SERCA inhibitor (Fig.3B, middle panel). In comparison, the expression level of α-actin remained unaffected under each condition (Fig3B, lower panel).

These data indicate, that strong depletion of the ER by BHQ diminished the activity and secretion of adenovirus-encoded human LPL in endothelial cells in a partially reversible manner, while mRNA levels and intracellular protein content remained unaffected by BHQ treatment.

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Histamine caused moderate ER Ca^{2+} depletion despite strong elevation of cytoplasmic Ca^{2+}

In order to analyse the impact of agonist-triggered ER Ca^{2+} depletion on LPL maturation and secretion, the effects of the IP_3-generating autacoid histamine on $[Ca^{2+}]_{ER}$ and the activity of adenovirus-encoded human LPL were investigated in endothelial cells.

Stimulation of endothelial cells expressing LPL with 100 μM histamine in the presence of extracellular Ca^{2+} resulted in immediate and reversible reduction of $[Ca^{2+}]_{ER}$ (Fig.4A). This effect was comparable with that achieved with BHQ but represented an transient phenomenon as ER Ca^{2+} content fully recovered within 1h even in the presence of the agonist. This histamine-initiated ER Ca^{2+} depletion was accompanied by a rapid initial cytosolic Ca^{2+} elevation and a subsequent elevated Ca^{2+} plateau until washout of the agonist (Fig.4B).

Agonist-induced ER Ca^{2+} lowering transiently attenuated activity and secretion of human LPL

Upon application of 100 μM histamine, intracellular LPL activity was reduced after 1 h and subsequently reestablished slowly to reach complete recovery after 4 h despite the presence of the agonist (Fig.4C). In the supernatant histamine reduced LPL activity in the medium for 2 h while further progression of LPL secretion was only slightly diminished (Fig.4D).

However, in the absence of extracellular Ca^{2+} (i.e. EGTA-containing solution), histamine (100 μM) yielded complete ER Ca^{2+} depletion (Fig.4A) that was accompanied by a transient cytosolic Ca^{2+} elevation (Fig.4B). Under these conditions, intracellular LPL activity upon histamine did not recover and continuously decreased over time (Fig.4C). Consistently, in the absence of extracellular Ca^{2+}, secretion of LPL was permanently prevented by stimulation with histamine (Fig.4D). In agreement with our findings that point to a tight correlation between ER Ca^{2+} content and LPL folding, EGTA *per se* (i.e. without histamine

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stimulation), which has been previously shown to result in a slowly ER depletion [32] and yielded app. 50% ER depletion within 4h (data not shown) gradually diminished intracellular activity and secretion of LPL over time (Fig. 6A & B).

Thus, in contrast to BHQ that yielded exhaustive and long lasting ER Ca^{2+} depletion and, consequently, prevented posttranslational processing of LPL, cell stimulation with an IP_3-generating agonist under physiological conditions (i.e. in the presence of extracellular Ca^{2+}) only weakly and transiently (within 4h) attenuated the maturation and secretion of LPL, most likely due to the moderate ER Ca^{2+} depletion under these conditions.

To investigate the contribution of mitochondrial Ca^{2+} flux to the maintenance of the Ca^{2+}-dependent protein folding machinery of the ER during cell stimulation with the physiologically relevant agonist histamine, mitochondrial Ca^{2+} extrusion was prevented by CGP 37157, an inhibitor of mitochondrial Na^+/Ca^{2+} exchanger, that represents the main mechanism of Ca^{2+} extrusion from mitochondria in endothelial cells [3, 33].

Inhibition of mitochondrial Ca^{2+} flux prevented LPL maturation and secretion

CGP 37157 was recently demonstrated to inhibit mitochondrial Ca^{2+} flux and, thus, ER Ca^{2+} refilling upon stimulation in endothelial cells very efficiently [3]. As shown in figure 5A, in the presence of CGP 37157 (20 μM) histamine-induced ER Ca^{2+} depletion was more pronounced than in control while cytosolic free Ca^{2+} remained elevated (Fig.5B).

In the presence of CGP 37157, cytosolic LPL activity was largely decreased by stimulation with histamine and did not recover with time (Fig.5C). Accordingly, LPL secretion was prevented upon histamine stimulation in the presence of CGP 37157 (Fig.5D). In contrast, application of CGP 37157 without any further treatment did not affect ER Ca^{2+} content within 4h (data not shown) and also had no effect on intracellular or secreted LPL activity (Fig.6A & B).

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These data suggest, that besides extracellular Ca^{2+}, mitochondrial Ca^{2+} transfer essentially contributes to Ca^{2+}-dependent maturation of LPL in the ER and its subsequent secretion during cell stimulation with an IP_3-generating agonist.

DISCUSSION

In virtually all non-excitable cells, the ER serves as main releasable Ca^{2+} store and actively contributes to cellular Ca^{2+} homeostasis and posttranslational protein processing [1]. Notably, the activity of some of the most important ER-resident chaperones, such as calreticulin, calnexin and BiP, was recognized to be sensitive to ambient Ca^{2+} [9]. However, it remains still unclear whether and, if so, how eminent Ca^{2+} fluctuations faced by the ER under physiological conditions due to regular cell stimulation [2, 3, 15] influence ER chaperone activity [8]. Additionally, since mitochondria are of pivotal importance to direct entering Ca^{2+} towards the ER for its refilling [15], the contribution of such mitochondrial Ca^{2+} transfer to Ca^{2+}-dependent protein maturation in the ER urges to be elucidated. Therefore, the efficiency of Ca^{2+}-dependent protein maturation in the ER was determined utilizing LPL, the activity and secretion of which strictly depends on proper folding by Ca^{2+}-sensitive chaperons and folding catalysts in the ER [11, 13, 21]. Consequently, LPL expression was correlated with enzyme activity as a measure of correctly folded LPL.

Initially, the activity of endogenous LPL in serum-starved CHO-K1 cells [31] was assessed. Under resting conditions, intracellular LPL activity remained constant over the whole experiment, while in the supernatant a linear accumulation of LPL activity occurred. These findings are in agreement with the general concept of a continuous LPL synthesis, maturation and its subsequent secretion [31]. However, if the ER was exhaustively depleted from Ca^{2+} by the SERCA inhibitor BHQ [3], intracellular LPL activity disappeared and fully recovered 1 h

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after BHQ washout. The decline of cellular LPL activity due to BHQ was associated with retention of LPL secretion that was completely restored 2 h after BHQ removal. Since the changes in LPL activity and secretion were not accompanied by alterations in the mRNA level, these data suggest that ER Ca^{2+} depletion prevents protein maturation and, thus, its subsequent secretion.

However, at this point it was conceivable that ER Ca^{2+} depletion harms protein translation resulting in attenuation of active LPL protein despite unchanged mRNA levels. Unfortunately, potential effects of a reduction of ER Ca^{2+} content on LPL protein levels could not be verified due to the lack of a suitable antibody against hamster LPL. In order to provide information on the protein content for the correlation of LPL expression/maturation/secretion with ER Ca^{2+} concentration, we used adenovirus-mediated over-expression of human LPL [23], against which a polyclonal antibody was available. Furthermore, any modulation of the gene expression of constitutive LPL by Ca^{2+}-sensitive transcription factors could be excluded due to the stable LPL transcription driven by the virus promotor. In addition, to avoid contamination by constitutively expressed LPL, cells lacking endogenous LPL [34, 35] were infected. Accordingly, due to our extensive experience on the Ca^{2+} homeostasis in the human umbilical vein derived endothelial cell line EA.hy926 [2, 3, 15, 29, 36], these cells were chosen as a suitable model system.

In agreement with previous reports [3, 15], SERCA inhibition by BHQ efficiently emptied the ER and permanently elevated cytosolic free Ca^{2+} concentration also in endothelial cells that were infected with adenovirus-encoding human LPL (data not shown). Concurrently, activity of ectopically expressed human LPL in cell lysates decreased upon BHQ addition, which was in line with a blunted LPL accumulation in the medium. However, in contrast to the complete recovery of LPL activity in CHO-K1 cells upon BHQ washout, in infected endothelial cells LPL activity only partially recovered despite total refilling of ER Ca^{2+} stores [3, 15]. The slower onset of LPL appearance in the supernatant under these

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conditions might be either related to the delay in intracellular LPL recovery after BHQ or to the change in the cell type used.

Expression of functional recombinant proteins, especially at high concentrations as achieved by viral infection, requires an efficient and undisturbed operation of the protein processing machinery of the ER [37], which can easily be overloaded [38]. Thus, it is reasonable to speculate that delay of LPL recovery following washout of BHQ is due to attenuation of LPL synthesis and/or maturation by ER stress [39]. Nevertheless, these data are in line with that obtained in CHO-K1 cells and point to a strong correlation between LPL maturation/secretion and ER Ca^{2+} content. Importantly, the effect of ER Ca^{2+} depletion on the activity of LPL was not due to alterations in mRNA levels and intracellular protein content. Albeit, there was no intracellular accumulation of LPL despite prevented secretion, pointing to mobilization of ER-associated degradation under these conditions [40]. Consistently, efficient degradation of LPL by ER-associated degradation mechanisms has been shown for maturation-defective variants of that protein in the context of inherited primary lipase deficiency [41] and cld/cld mice [42].

Thus, the maturation and secretion of both ectopically over-expressed and endogenous LPL proved to be sensitive to depletion of $[Ca^{2+}]_{ER}$.

Nevertheless, it remains still unclear whether the ER folding machinery is also susceptible to fluctuations in ER Ca^{2+} content in response to physiological stimuli. Therefore, the impact of the physiological mediator histamine on LPL maturation/secretion was elucidated in endothelial cells expressing adenovirus-encoded human LPL. Remarkably, even stimulation with supramaximal concentrations of this IP_3-generating agonist (i.e. 100 μM histamine, [36]), was found to initiate limited ER Ca^{2+} depletion due to counteracting refilling processes as described recently [3, 15]. In line with these previous reports, the reduction of ER Ca^{2+} content as well as cytosolic Ca^{2+} elevations in response to histamine were comparable to that

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achieved by BHQ. Moreover, ER Ca^{2+} content recovered within 1h even in the presence of the agonist. In agreement with such transient ER Ca^{2+} depletion and the subsequent ER Ca^{2+} refilling in the presence of histamine, secretion of adenovirus-encoded LPL was delayed after 2h histamine stimulation but recovered with the same slope as in resting cells thereafter. In contrast, the recovery of the intracellular activity of LPL in histamine stimulated cells was much slower and full recovery was found after 5h histamine stimulation. These data indicate that upon ER Ca^{2+} refilling, the recovery of intracellular LPL maturation is masked by the rapid secretion of newly folding protein. Overall, these data point to a strict dependency between LPL maturation, secretion and $[Ca^{2+}]_{ER}$. However, our data, that LPL maturation and secretion were prevented if the cells were stimulated with histamine in the absence of extracellular Ca^{2+} (i.e. conditions were no ER Ca^{2+} refilling occurs, [3]), suggest that for the maintenance of Ca^{2+}-dependent protein maturation in the ER requires Ca^{2+} entry to preserve ER Ca^{2+} content during physiological cell stimulation. Notably, attenuation of Ca^{2+} entry pathways has been frequently observed under pathological conditions (e.g. diabetes mellitus, [43]) and, thus, pathologically altered Ca^{2+} channel activity might contribute to protein misfolding in diseases.

Similar to the removal of extracellular Ca^{2+}, inhibition of trans-mitochondrial Ca^{2+} flux by CGP 37157 [3, 15] prevented Ca^{2+}-dependent protein maturation in the ER. These findings are in line with our previous report that mitochondrial Ca^{2+} flux is prerequisite for ER Ca^{2+} refilling during agonist stimulation and points to the maintenance of the Ca^{2+}-dependent protein folding machinery of the ER in a physiological environment as new, so far unknown function of mitochondria. In view of these findings and the increasing evidence of mitochondrial dysfunction in various metabolic diseases (e.g. diabetes mellitus, [20, 44] it is tempting to speculate that disease-associated alterations in mitochondrial (Ca^{2+}) function may

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subsequently affect Ca^{2+}-dependent protein maturation in the ER and, thus, facilitate cellular dysfunction by incorrectly folded or incompletely assembled proteins.

In conclusion, these data suggest that the protein folding machinery of the ER is weakly affected by agonist-mediated $[Ca^{2+}]_{ER}$ fluctuations, while displaying strong sensitivity to pronounced and/or long-lasting attenuation of $[Ca^{2+}]_{ER}$. Thus, under physiological conditions ER protein maturation is largely preserved regardless of cell stimulation with IP_3-generating agonists due to strong ER Ca^{2+} refilling processes that depend on Ca^{2+} entry and trans-mitochondrial flux and efficiently counteract ER Ca^{2+} depletion. Based on the strong evidences that cellular/mitochondrial Ca^{2+} homeostasis represents an early target of cell dysfunction in numerous diseases (e.g. diabetes mellitus [45-47] or atherosclerosis [48]), the impact of the reported alterations in intracellular Ca^{2+} handling on protein maturation in the ER urges to be explored in more detail.

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FIGURE LEGENDS

Figure 1. Inhibition of SERCA is a proper tool for an exhaustive ER Ca^{2+} depletion and consequently attenuates activity and secretion, but not transcription of endogenous LPL

Cells were transiently transfected with $D1_{ER}$ or loaded with fura-2/am in order to monitor Ca^{2+} signals in the ER (**A**) and cytoplasm (**B**), respectively. Cells were kept in the continuous presence of 2 mM extracellular Ca^{2+} under control conditions (continuous line, open circles; A, n=3; B, n=8) or were stimulated with 15 μM BHQ as indicated (dotted line, filled circles; A, n=3; B, n=8). Tracings show the average curve, points and error bars represent the mean ± SEM from all experiments. *$P<0.05$ vs. control. *Panels C, D and E:* Serum-starved CHO-K1 cells were propagated in culture medium under control condition (continuous line, open circles) or with application of 15 μM BHQ as indicated (dotted line, filled circles). LPL activity in cell lysates (**C**) and supernatants (**D**) was measured in hourly taken samples as described in the Methods section. One representative experiment out of 3 is shown. *$P<0.05$ vs. control. (**E**) Using RT-PCR, the constitutive expression of GAPDH and LPL in untreated CHO-K1 cells ("**-**") and after 3 h exposure to 15 μM BHQ ("**+**") were validated.

Figure 2. ER Ca^{2+} depletion reduces activity and secretion of adenovirus-encoded LPL in endothelial cells

Cells were infected with adenovirus encoding human LPL (100 MOI) and incubated in serum-free culture medium without any further treatment (continuous line, open circles) or an inhibition of SERCA by addition of 15 μM BHQ as indicated (dotted line, filled circles). Every hour, samples were taken and LPL activity in cell lysates (**A**) and aliquots of the supernatant (**B**) was acquired as described in the Methods section. One representative experiment out of 3 is shown. Tracings show the average curve, points and error bars represent the mean ± SEM from all experiments. *$P<0.05$ vs. control.

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Figure 3. Adenovirus-mediated expression of LPL is not altered by BHQ treatment

By RT-PCR (**A**), the adenovirus-mediated transcription of LPL (left panel) was assessed in endothelial cells and normalized for endogenous GAPDH (right panel) after 1, 2 and 3 h in the absence (control), presence (BHQ) or following washout (BHQ washout) of 15 μM BHQ. Tracings show the average curve, points and error bars represent the mean ± SEM of three independent experiments. *$P<0.05$ vs. control. For Western Blot analysis (**B**), identical amounts of protein of cell lysates (upper panel) and identical volumes of the corresponding supernatants (lower panel) were separated on 12.5% SDS-polyacryamide gels and blotted onto nitrocellulose. Lanes show the expression of LPL and α-actin in adenovirus-infected endothelial cells after 1, 2, 3 and 4 h in the absence (control) or after 1, 2 and 3 h in presence (BHQ) or following washout (BHQ washout) of 15 μM BHQ. Positions of standard proteins are marked.

Figure 4. Stimulation of endothelial cells with histamine yields temporary ER Ca^{2+} depletion despite large elevation in the cytoplasmic Ca^{2+} concentration and transiently lowers activity and secretion of LPL whereas removal of extracellular Ca^{2+} yields pronounced Ca^{2+} depletion and attenuation of LPL maturation

Ca^{2+} signals in the ER (**A**) and cytoplasm (**B**) of endothelial cells were monitored using $D1_{ER}$ and fura-2, respectively. The respective Ca^{2+} concentration was monitored under control conditions (continuous line, open circles A, n=3; B, n=8) or upon stimulation with 100 μM histamine in the presence of 2 mM extracellular Ca^{2+} (dotted line, filled circles A, n=3; B, n=10) or 1 mM EGTA (i.e. nominal Ca^{2+}-free buffer; discontinuous line, filled squares; A, n=3; B, n=10) as indicated. Tracings show the average curve and points represent the mean ± SEM from all experiments. *$P<0.05$ vs. control. *Panels C and D:* Adenovirus-infected endothelial cells were incubated in serum-free culture medium without (continuous line, open circles) or with addition of 100 μM histamine (dotted line, filled circles) or stimulated with

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histamine in in nominal Ca^{2+}-free solution (i.e. serum-free culture medium containing 2.5 mM EGTA; dotted line, filled squares. Hourly, LPL activity in cell lysates (**C**) and aliquots of the supernatant (**D**) was validated as described in the Methods section. *$P<0.05$ vs. control (n=6).

Figure 5. The inhibition of trans-mitochondrial Ca^{2+} flux yields pronounced Ca^{2+} depletion of the ER, prevents its refilling and decreases activity and secretion of LPL during physiological cell stimulation

Ca^{2+} signals in the ER (**A**) and cytoplasm (**B**) of endothelial cells were monitored using $D1_{ER}$ and fura-2, respectively. As indicated, cells were either kept under control conditions (Control, continuous line, open circles; A, n=3; B, n=8) or stimulated with 100 μM histamine in Ca^{2+} containing buffer (Histamine, discontinuous line, filled circles; A, n=3; B, n=8) or in the presence of 20 μM CGP 37157 (dotted line, open squares; A, n=4; B, n=9) and the respective Ca^{2+} concentration was monitored as described under Methods. *$P<0.05$ vs. control. *Panels C and D:* Adenovirus-infected endothelial cells were incubated in serum-free culture medium (continuous line, open circles) or stimulated with 100 μM histamine without (dotted line, filled circles) or with 20 μM CGP 37187 (dotted line, open squares). LPL activity was measured hourly in cell lysates (**C**) and aliquots of the supernatant (**D**). $^{\#}P<0.05$ vs. control (n=6) *and* *$P<0.05$ vs. histamine (n=3-6).

Figure 6. Activity and secretion of LPL is marginally attenuated by removal of extracellular Ca^{2+} but not affected by CGP 37157 per se.

Adenovirus-infected endothelial cells were incubated in serum-free culture medium without (continuous line, open circles) and with 20 μM CGP 37187 (dotted line, open squares) or 2.5 mM EGTA (discontinuous line, filled squares). LPL activity was measured hourly in cell lysates (**A**) and aliquots of the supernatant (**B**). $^{\#}P<0.05$ vs. control (n=6) *and* *$P<0.05$ vs. histamine (n=3-6).

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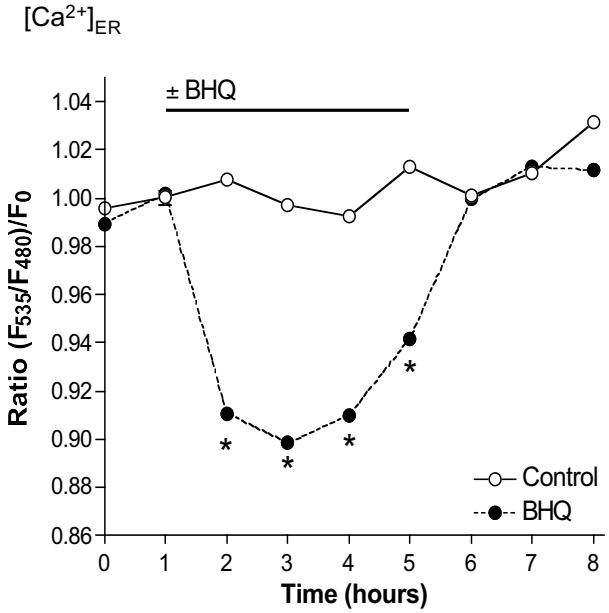
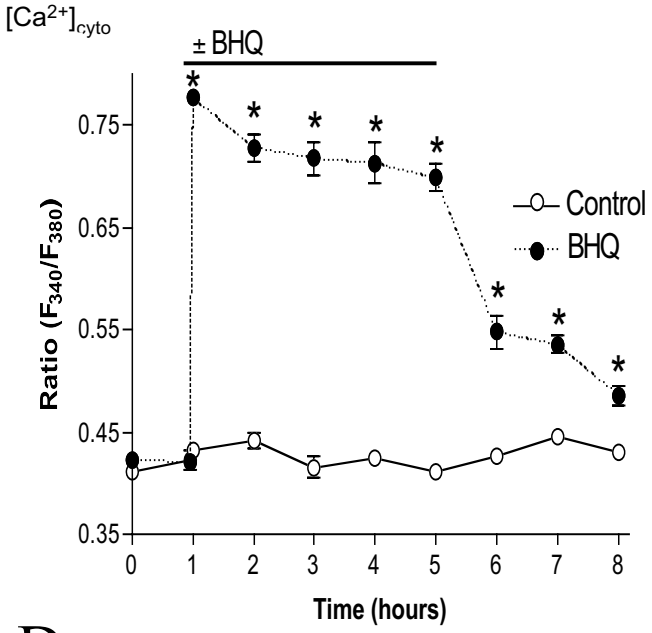
Figure 1

A

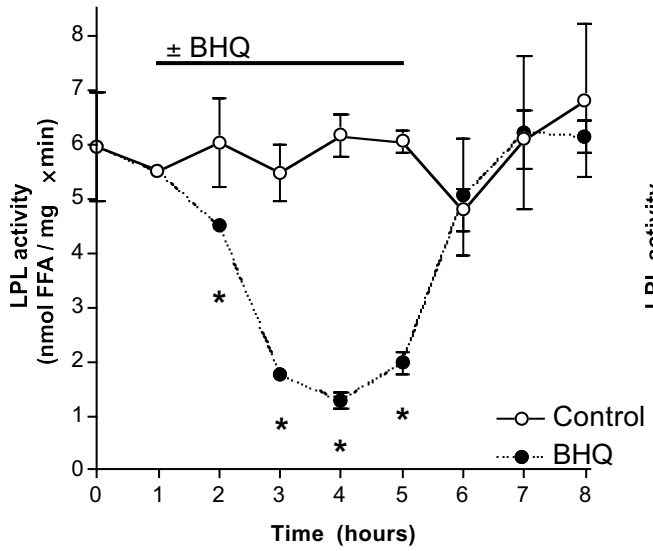


B



C

intracellular LPL



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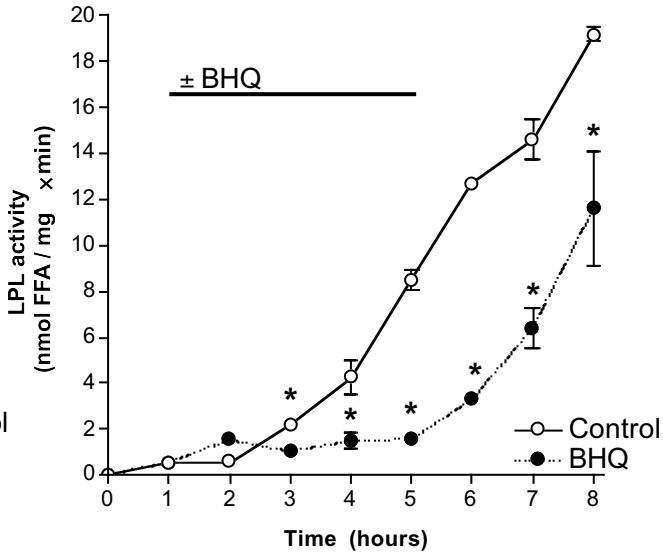
secreted LPL



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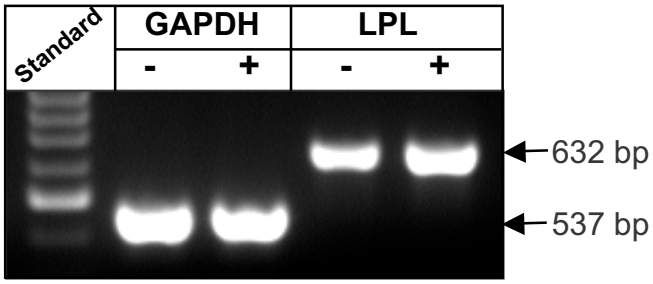

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Figure 2

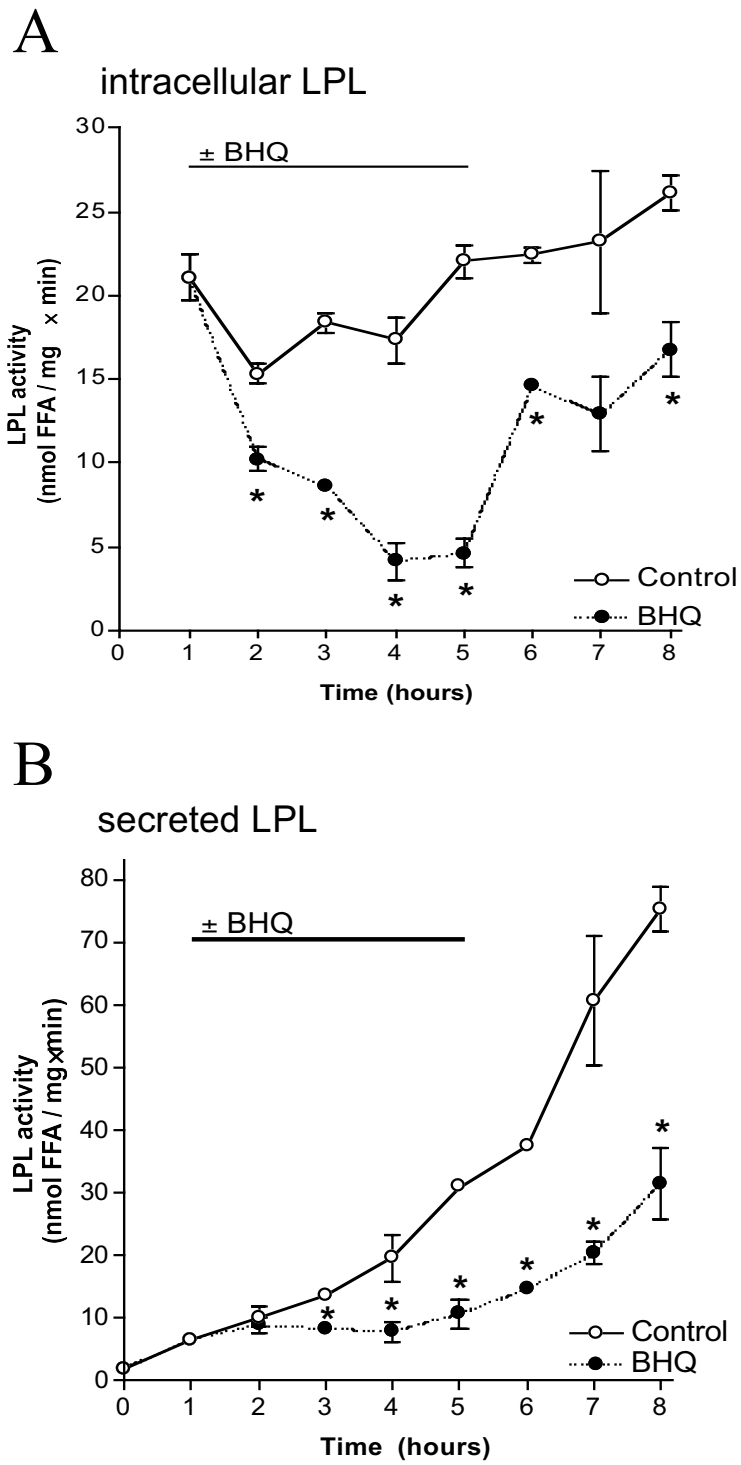

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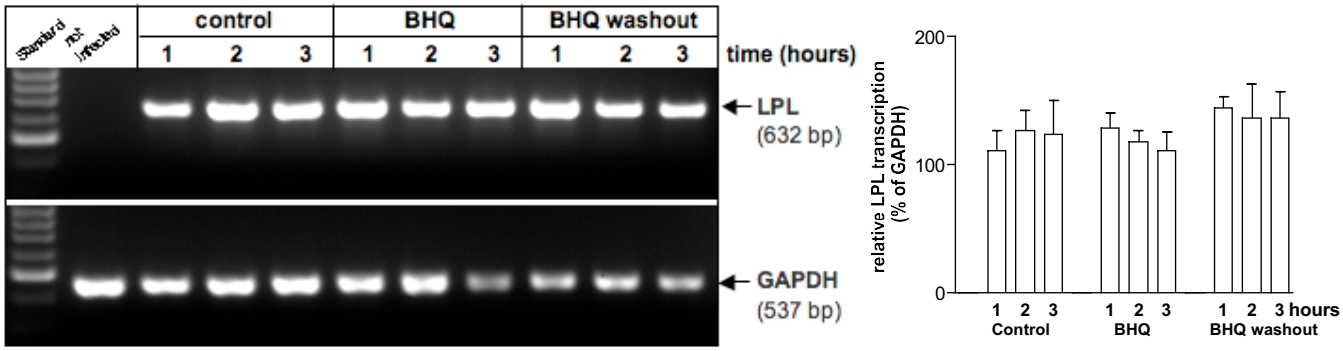
Figure 3

A



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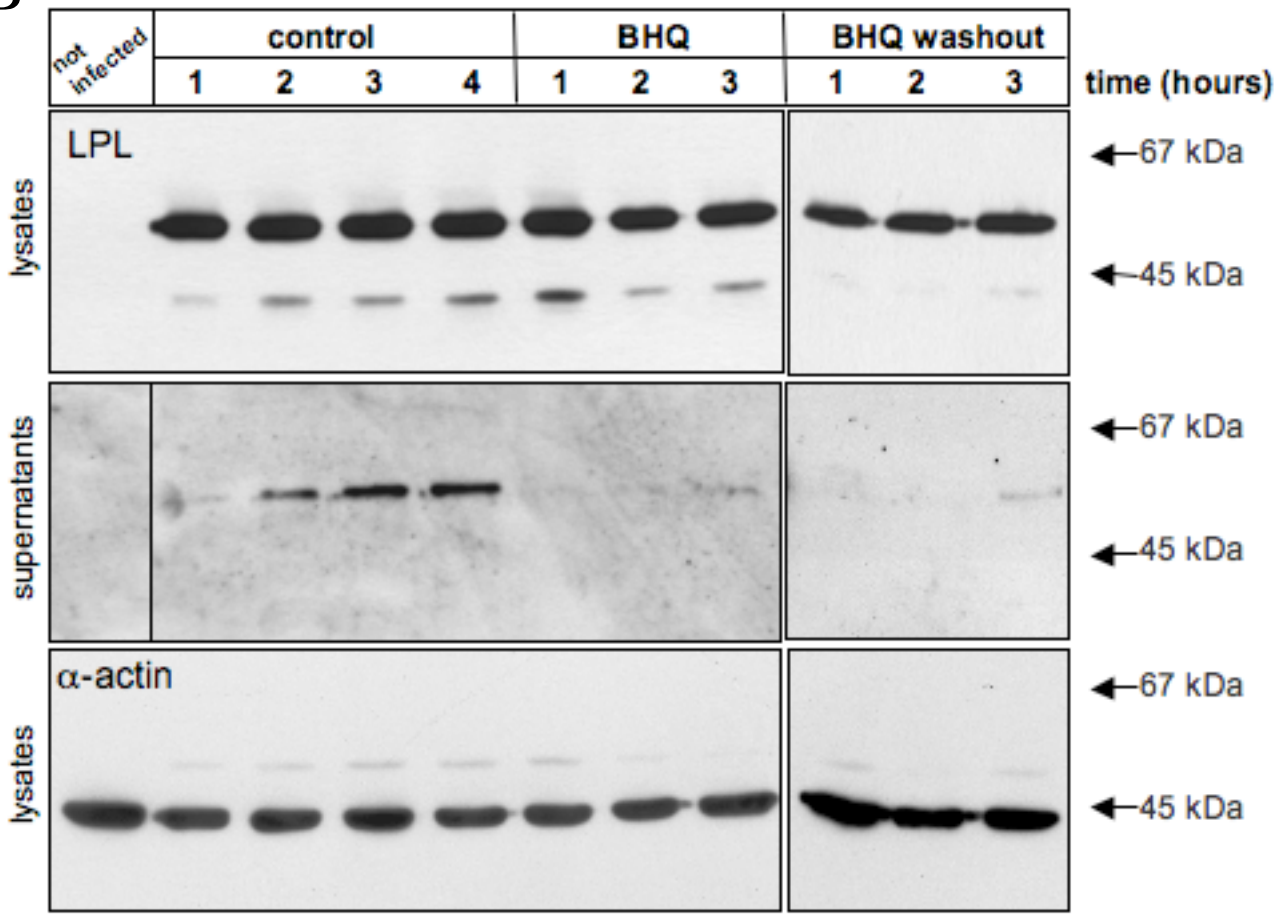

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Figure 4

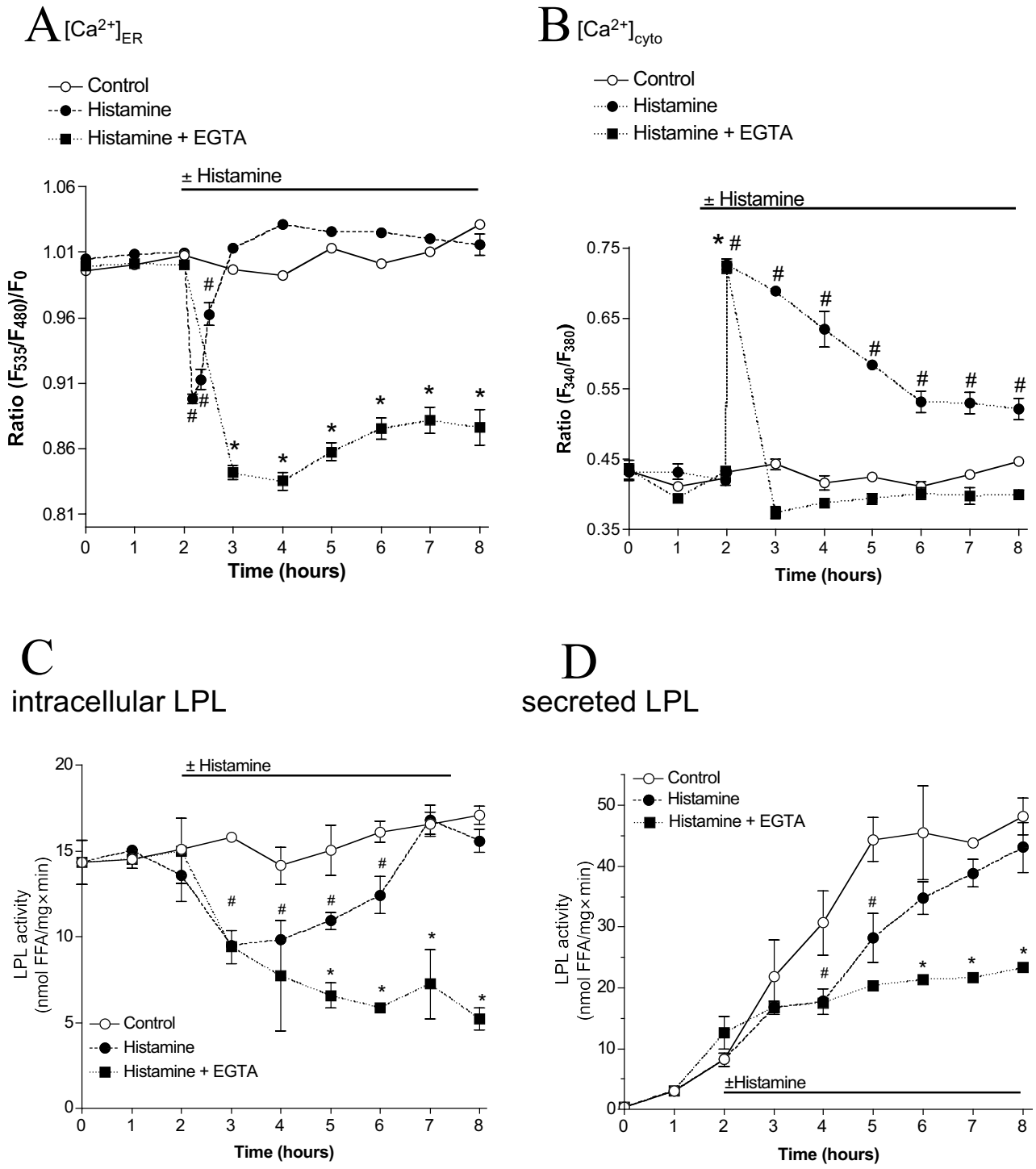

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Figure 5

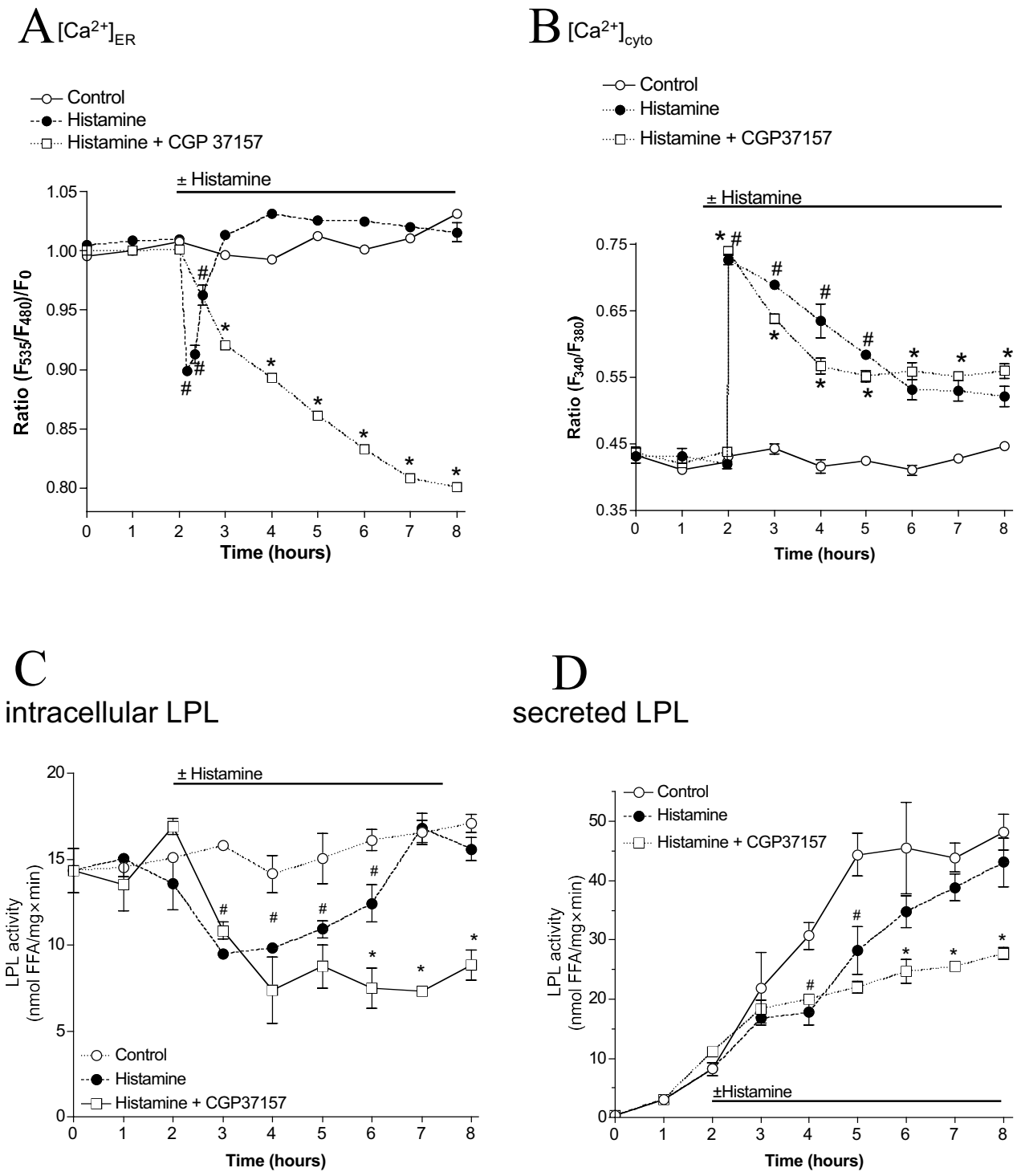

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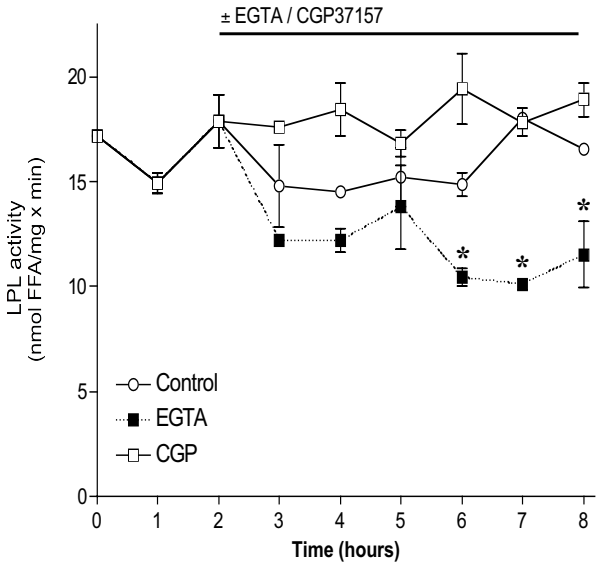
Figure 6

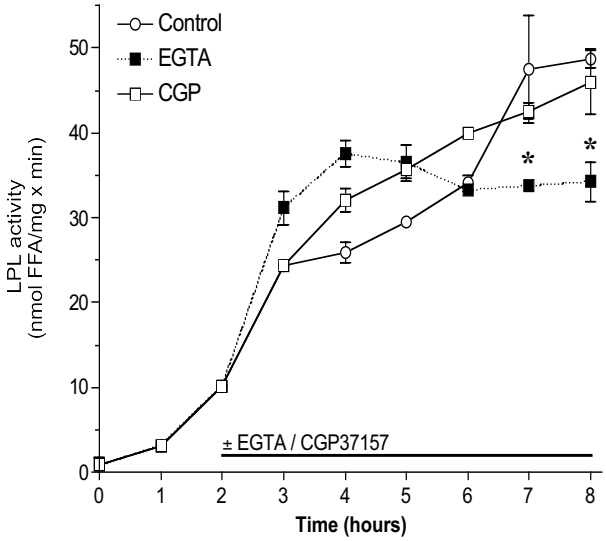
A

intracellular LPL



B

secreted LPL



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