Lithocholic acid induces endoplasmic reticulum stress, autophagy and mitochondrial dysfunction in human prostate cancer cells

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Lithocholic acid (LCA) is a secondary bile acid that is selectively toxic to human neuroblastoma, breast and prostate cancer cells, whilst sparing normal cells. We previously reported that LCA inhibited cell viability and proliferation and induced apoptosis and necrosis of androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cells. In the present study, we investigated the roles of endoplasmic reticulum (ER) stress, autophagy and mitochondrial dysfunction in the toxicity of LCA in PC-3 and autophagy deficient, androgen-independent DU-145 cells. LCA induced ER stress-related proteins, such as CCAAT-enhancer-binding protein homologous protein (CHOP), and the phosphorylation of eukaryotic initiation factor 2-alpha (p-eIF2α) and c-Jun N-terminal kinases (p-JNK) in both cancer cell-types. The p53 upregulated modulator of apoptosis (PUMA) and B cell lymphoma-like protein 11 (BIM) levels were decreased at overtly toxic LCA concentrations, although PUMA levels increased at lower LCA concentrations in both cell lines. LCA induced autophagy-related conversion of microtubule-associated proteins 1A/1B light chain 3B (LC3BI to LC3BII), and autophagy-related protein ATG5 in PC-3 cells, but not in autophagy-deficient DU-145 cells. LCA (>10 μM) increased levels of reactive oxygen species (ROS) concentration-dependently in PC-3 cells, whereas ROS levels were not affected in DU-145 cells. Salubrinal, an inhibitor of eIF2α dephosphorylation and ER stress, reduced LCA-induced CHOP levels slightly in PC-3, but not DU-145 cells. Salubrinal pre-treatment increased the cytotoxicity of LCA in PC-3 and DU-145 cells and resulted in a statistically significant loss of cell viability at normally non-toxic concentrations of LCA. The late-stage autophagy inhibitor bafilomycin A1 exacerbated LCA toxicity at subtoxic LCA concentrations in PC-3 cells. The antioxidant α-tocotrienol strongly inhibited the toxicity of

LCA in PC-3 cells, but not in DU-145 cells. Collectively, although LCA induces autophagy and ER stress in PC-3 cells, these processes appear to be initially of protective nature and subsequently consequential to, but not critical for the ROS-mediated mitochondrial dysfunction and cytotoxicity of LCA. The full mechanism of LCA-induced mitochondrial dysfunction and cytotoxicity in the similarly sensitive DU-145 cells remains to be elucidated.

1   **Lithocholic acid induces endoplasmic reticulum stress, autophagy and**

2   **mitochondrial dysfunction in human prostate cancer cells**

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18 **Abstract**

19 Lithocholic acid (LCA) is a secondary bile acid that is selectively toxic to human neuroblastoma,

20 breast and prostate cancer cells, whilst sparing normal cells. We previously reported that LCA

21 inhibited cell viability and proliferation and induced apoptosis and necrosis of androgen-

22 dependent LNCaP and androgen-independent PC-3 human prostate cancer cells. In the present

23 study, we investigated the roles of endoplasmic reticulum (ER) stress, autophagy and

24 mitochondrial dysfunction in the toxicity of LCA in PC-3 and autophagy deficient, androgen-

25 independent DU-145 cells. LCA induced ER stress-related proteins, such as CCAAT-enhancer-

26 binding protein homologous protein (CHOP), and the phosphorylation of eukaryotic initiation

27 factor 2-alpha (p-eIF2α) and c-Jun N-terminal kinases (p-JNK) in both cancer cell-types. The

28 p53 upregulated modulator of apoptosis (PUMA) and B cell lymphoma-like protein 11 (BIM)

29 levels were decreased at overtly toxic LCA concentrations, although PUMA levels increased at

30 lower LCA concentrations in both cell lines. LCA induced autophagy-related conversion of

31 microtubule-associated proteins 1A/1B light chain 3B (LC3BI to LC3BII), and autophagy-

32 related protein ATG5 in PC-3 cells, but not in autophagy-deficient DU-145 cells. LCA (>10 μM)

33 increased levels of reactive oxygen species (ROS) concentration-dependently in PC-3 cells,

34 whereas ROS levels were not affected in DU-145 cells. Salubrinal, an inhibitor of eIF2α

35 dephosphorylation and ER stress, reduced LCA-induced CHOP levels slightly in PC-3, but not

36 DU-145 cells. Salubrinal pre-treatment increased the cytotoxicity of LCA in PC-3 and DU-145

37 cells and resulted in a statistically significant loss of cell viability at normally non-toxic

38 concentrations of LCA. The late-stage autophagy inhibitor bafilomycin A1 exacerbated LCA

39 toxicity at subtoxic LCA concentrations in PC-3 cells. The antioxidant α-tocotrienol strongly

40 inhibited the toxicity of LCA in PC-3 cells, but not in DU-145 cells. Collectively, although LCA

41 induces autophagy and ER stress in PC-3 cells, these processes appear to be initially of

42 protective nature and subsequently consequential to, but not critical for the ROS-mediated

43 mitochondrial dysfunction and cytotoxicity of LCA. The full mechanism of LCA-induced

44 mitochondrial dysfunction and cytotoxicity in the similarly sensitive DU-145 cells remains to be

45 elucidated.

46

48 **INTRODUCTION**

49 Prostate cancer is the second most common cancer worldwide in males and the fourth most

50 common cancer overall, with more than 1,112,000 new cases diagnosed in 2012, representing

51 15% of male cancer cases and 8% of all cancers (Ferlay *et al.*, 2015). In Western men, prostate

52 cancer diagnosis ranks first among male cancers and second as cause of cancer-related death

53 (Malvezzi *et al.*, 2015; Society, 2015a; Society, 2015b). Standard treatment of prostate cancer

54 consists of surgery (prostatectomy), antihormonal therapy and radiotherapy. Although these

55 treatments are successful for early-stage prostate cancer, they each have potentially serious side-

56 effects (Martin and D'Amico, 2014; Nguyen *et al.*, 2015), among which some that last a life-time

57 (Sanda *et al.*, 2008). Androgen-deprivation therapy uses drugs that blocking the action of male

58 sex hormones either through androgen receptor antagonism (bicalutamide, hydroxyflutamide) or

59 inhibition of androgen biosynthesis (finasteride, abiraterone). These treatments are initially

60 effective in controlling androgen-dependent prostate tumor growth, although side-effects include

61 increased insulin-resistance, bone density loss, hypogonadism, gynecomastia, muscle mass loss

62 and fatigue (Conde and Aronson, 2003; Nguyen *et al.*, 2015). In addition, a certain percentage of

63 tumors that have undergone androgen-deprivation therapy progresses to an androgen-

64 independent state, which is difficult to treat resulting in increased mortality. The limitations of

65 current standard treatments of prostate cancer has encouraged the search for safer and more

66 effective molecules based on naturally occurring compounds.

67 Lithocholic acid (LCA) is a secondary bile acid produced by microflora in the gut, which we

68 found to exhibit selective toxicity to human neuroblastoma cells and prostate cancer cells at

69 relatively low concentrations that did not affect normal cells (Goldberg *et al.*, 2011; Goldberg *et*

70 *al.*, 2013). LCA triggered both intrinsic and extrinsic pathways of apoptotic cell death that were,

71 at least in part, caspase-dependent. In addition, LCA selectively decreased the viability of human

72 breast cancer and rat glioma cells (Goldberg *et al.*, 2011). Various bile acids have been reported

73 to have anti-neoplastic and anti-carcinogenic properties in a number of cancer cell models:

74 chenodeoxycholic acid (CDCA) reduced growth of tamoxifen-resistant breast cancer cells by

75 downregulation of human epidermal growth factor receptor 2 (HER2) promoter activity

76 (Giordano *et al.*, 2011), LCA and several of its synthetic enantiomers reduced colon cancer cell

77 proliferation and viability (Katona *et al.*, 2009). Deoxycholic acid (DCA), ursodeoxycholic acid

78 (UDCA) and their taurine-derivatives delayed cell cycle progression in Jurkat human T leukemia

79   cells and DCA induced apoptosis (Fimognari *et al.*, 2009). These findings indicate that the bile

80   acid structure may form the basis for the development of potent and selective drugs for the

81   treatment of various cancers including those of the prostate.

82   The mechanisms underlying the cytotoxicity of LCA are not well understood and remain a

83   continuing topic of investigation. Studies have found that certain bile acids can induce apoptosis

84   via a variety of mechanisms including chronic endoplasmic reticulum (ER) stress (Perez and

85   Briz, 2009), autophagy (Gao *et al.*, 2014) or disruption of mitochondrial function (Goldberg *et*

86   *al.*, 2013). The endoplasmic reticulum is cell organelle responsible for the synthesis, folding and

87   maturation of proteins, the storage and release of intracellular calcium (Ca2+) and a large

88   number of biotransformation reactions. A variety of factors (radiation, pathogens, hypoxia,

89   disease states and chemical agents) can disrupt healthy ER function, resulting in a so-called

90   unfolded protein response (UPR), due to the accumulation of unfolded or misfolded proteins in

91   the lumen of the ER. As an adaptive response to these stress factors, the UPR aims to restore

92   normal cell function by halting protein translation, degrading misfolded proteins and increasing

93   the production of molecular chaperones involved in protein folding. However, chronic activation

94   of the UPR fails to promote cell survival and the cell is broken down by a proapoptotic ER

95   stress-mediated response pathway. CCAAT-enhancer-binding protein homologous protein

96   (CHOP) is a transcriptional regulator induced by ER stress, which is a modulator of ER stress-

97   mediated apoptosis (Marciniak *et al.*, 2004) and autophagy (Shimodaira *et al.*, 2014). CHOP

98   levels may be increased through activation of various ER stress sensor-pathways, including those

99   initiated by activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 alpha (IRE1α)

100   and protein kinase R-like endoplasmic reticulum kinase (PERK), the latter which phosphorylates

101   eukaryotic initiation factor 2-alpha (eIF2α), and the downstream transcription factor ATF4 which

102   in turn induces the transcription of CHOP.

103   Autophagy is a catabolic process for the autophagosomic/lysosomal degradation of bulk

104   cytoplasmic contents (Reggiori and Klionsky, 2002; Codogno and Meijer, 2005). Autophagy is

105   generally activated by nutrient deprivation but is also important in physiological processes such

106   as fetal development and cell differentiation, as well as diseases such as neurodegeneration,

107   infection and cancer (Levine and Yuan, 2005). The molecular machinery of autophagy was

108   largely uncovered in yeast by the discovery of autophagy-related genes (Atg). Formation of the

109   autophagosome involves a ubiquitin-like conjugation system in which Atg12 is covalently bound

110 to Atg5 and targeted to autophagosomal vesicles (Mizushima *et al.*, 1998a; Mizushima *et al.*,

111 1998b). Upon induction of autophagy, a fraction of microtubule-associated proteins 1A/1B light

112 chain 3 (LC3-I) is conjugated to phosphotidylethanolamine (PE) to produce LC3-II proteins,

113 which are required for autophagosome membrane expansion and fusion. (Tanida I, Ueno T,

114 Kominami E (2004). LC3-I-to-II conversion is reliable marker of autophagosome formation.

115 (Mizushima N. et al (2001)

116 Bile acids have also been reported to induce apoptosis via disruption of mitochondrial function,

117 ligand-independent activation of death receptor pathways and modulation of certain members of

118 the Bcl2 protein family. We have previously shown that LCA induces intrinsic and extrinsic

119 apoptosis in LNCaP and PC-3 prostate cancer cells that involved a decrease in the mitochondrial

120 protein Bcl-2 and cleavage of Bax, concomitant with an increase of mitochondrial outer

121 membrane permeability. It has been suggested that the well-known solubilising properties of bile

122 acids could explain disruption of (mitochondrial) membranes and induction of mitochondrial

123 dysfunction leading to cell death. However, the lack of or far poorer toxicity of several

124 enantiomers of toxic bile acids suggests physico-chemical properties alone cannot explain cell

125 toxicity (Katona *et al.*, 2009) and that a specific three-dimensional structure is required to

126 explain the selectivity of LCA-mediated toxicity in cancer cells.

127 Our present study aims to investigate to which extent the involvement of ER stress, autophagy or

128 disruption of mitochondrial function is critical to LCA-induced prostate cancer cell death.

129

130 **MATERIALS AND METHODS**

131 **Cell lines and reagents**

132 PC3 and DU-145 cells were obtained from the American Type Culture Collection (Manassas,

133 VA). PC-3 cells were grown in 1:1 (v/v) Dulbecco's modified Eagle medium/Ham's F-12

134 nutrient mix (DMEM/F12, Life Technologies, Grand Island, NY) supplemented with 10% fetal

135 bovine serum (FBS; Mediatech, Corning, Manassas, VA) and 1% penicillin/streptomycin (Life

136 Technologies). DU-145 and RWPE-1 cells were cultured in RPMI-1640 medium (Life

137 Technologies) supplemented with 10% FBS, 1% HEPES , 1% sodium pyruvate (Sigma-Aldrich,

138 St. Louis, MO) and penicillin/streptomycin. All cells were incubated in a humidified atmosphere

139   of 95% air and 5% CO_2 at 37°C. LCA was purchased from Sigma-Aldrich and dissolved in

140   DMSO as 100 mM a stock solution and 1000-fold concentrated serial dilutions were prepared in

141   DMSO for treatment of the cells. Bafilomycin A1, salubrinal and D-α-tocotrienol (Sigma-

142   Aldrich) were dissolved in DMSO at 1000-fold stock solutions of 2 μM, 20 mM and 20 mM,

143   respectively

144   **Cell viability**

145   Each cell type was added to 96-well plates at a density of 1×10^4 cells/well in 200 μl of

146   complete medium. After 24 h, medium was replaced with fresh medium containing 2% dextran-

147   coated charcoal-treated (stripped) FBS and various concentrations of LCA (0, 5, 10, 25, 50 and

148   75 μM) in a final DMSO concentration in culture medium of 0.1%. Cell viability was assessed

149   using a WST-1 Cell Proliferation Reagent kit (Roche, Laval, QC) according to the

150   manufacturer's instructions. Absorbance was measured at 440 nm using a SpectraMax M5

151   multifunctional spectrophotometer (Molecular Devices, Sunnydale, CA).

152   **Fluorescence microscopy**

153   PC-3 and DU-145 cells were added to 24-well plates at a density of 1×10^5 cells/well in 1 ml of

154   complete medium. After 24 h, cells were treated with several concentrations of LCA (0, 1, 3, 10

155   and 30 μM) in fresh medium containing 2% stripped FBS and another 24 h later, Hoechst 33342

156   (Sigma-Aldrich) and propidium iodide (Invitrogen, Carlsbad, CA) were each added at a

157   concentration of 1 μg/ml per well. After a 15 min incubation at 37°C, cells were observed and

158   counted under a Nikon Eclipse (TE-2000U) inverted fluorescent microscope at 20 X

159   magnification. Hoechst- and propidium iodide-positive cells were made visible using filter cubes

160   with excitation wavelengths of 330–380 nm and 532–587 nm, respectively. To measure

161   autophagy, PC-3 cells were exposed to LCA (0, 3, 10, 30 and 50 μM) for 24 h and then stained

162   with Hoechst 33342 and 2 μL of Cyto-ID® Green Detection Reagent (ENZ-51031-K200, Enzo

163   Life Science, Farmingdale, NY). After a 15 min incubation at 37°C, cells were observed and

164   counted under a Nikon Eclipse (TE-2000U) inverted fluorescent microscope at 20 X

165   magnification.

166   **SDS–PAGE and immunoblot analysis**

167   Cells were added to 6-well Cell-Bind plates (Fisher Scientific, Ottawa, ON) at a density of 7.5 x

168   10^5 cells/well in 2 ml of complete culture medium and allowed to adhere for 24 h. Cells were

169   then exposed to LCA (0, 3, 10, 30 and 50 μM) in fresh medium with 2% stripped FBS for 1, 8 or

170   24 h, dependent on the experiment. Adherent cells were collected using a cell scraper, then

171   rinsed three times in cold phosphate-buffered saline (PBS) followed by centrifugation at 700 × g

172   for 5 min. After removing the PBS, the cell pellets were lysed in RIPA buffer containing 1 ×

173   protease and phosphatase inhibitor cocktail. Then, cell lysates were centrifuged at 15,000 rpm for

174   15 min at 4°C and protein concentrations in the supernatant were determined using a BCA

175   protein assay kit (Pierce Biotechnologies, Rockford, IL). Proteins (40 μg) were diluted with

176   loading buffer and boiled for 5 min, then loaded onto 10% sodium dodecyl sulfate-

177   polyacrylamide gels. After electrophoresis, gels were transferred to polyvinylidene diflouride

178   (PVDF) membranes using a Trans-Blot Turbo System (Bio-Rad, Mississauga, ON). Membranes

179   were then blocked using Tris-buffered saline (TBS) containing 5% milk powder (blocking

180   buffer) for 1 h at room temperature, after which the membranes were incubated overnight in

181   blocking buffer with the appropriate primary antibodies (anti CHOP, eIF2α, p- eIF2α, JNK, p-

182   JNK, PUMA, BIM, cleaved caspase 3, LC3BI/II, ATG5 and β-actin at 1:1000 dilution; Cell

183   Signaling, Beverly, MA) at 4°C. The next day, membranes were washed three times with Tris-

184   buffered saline containing 0.1% Tween (TBS-T) followed by a 1-h incubation with the

185   appropriate secondary antibody at room temperature. Membranes were washed another three

186   times with TBS-T and then incubated with Immobilon Western Chemiluminescent Horseradish

187   Peroxidase Substrate (EMD Millipore, Billerica, MD) for 5 min to make the bands visible;

188   membranes were sealed in plastic wrap and photographed using a ChemiDoc gel documentation

189   system (Bio-Rad). *B*-actin was used as reference protein and loading control.

190   **Gene-silencing using small interfering RNA (siRNA)**

191   CHOP expression was silenced by transfecting PC-3 and DU-145 cells with SMARTpool ON-

192   TARGETplus siRNA oligonucleotides selective for CHOP (Dharmacon, Lafayette, CO) using

193   lipofectamine RNAiMAX (Life Technologies, Burlington, ON) in serum free Opti-MEM

194   according to manufacturer's protocols. ON-TARGETplus Non-targeting Control siRNA was

195   used as negative control. After a 24-h transfection period, cells were exposed to various

196 concentrations of LCA (0, 10 and 30 µM) for 24 h. CHOP protein levels were evaluated by

197 immunoblotting as described above.

198 **Measurement of reactive oxygen species (ROS)**

199 PC3 and DU-145 cells were added to 96-well plates at a concentration of 1×10^4 cells/well in

200 200 µl of their respective culture medium containing 2% stripped FBS. After 24 h, medium was

201 removed and the cells were incubated in prewarmed PBS at 37°C containing 10 mM fluorescent

202 ROS probe (CM-H2DCFDA; Life Technologies). After 30 minutes, the PBS mixture was

203 removed and cells were exposed to various concentrations of LCA or 1 µM H_2O_2 for 60 minutes

204 at 37°C temperature. In experiments with α-tocotrienol and N-acetylcysteine. cells were

205 preincubated with the antioxidants for 4 h prior to exposure to LCA. ROS production was

206 quantified using a SpectraMax M5 multifunctional spectrophotometer (Molecular Devices,

207 Sunnydale, CA) with an excitation wavelength of 490 and emission wavelength of 545 nm.

208

209   **Statistical analysis**

210   Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San

211   Diego, CA). Results are presented as means ± standard deviations of at least three experiments.

212   IC_{50} values were determined from concentration-response curves by non-linear curve-fitting.

213   Statistically significant differences of LCA treatments compared to vehicle control were

214   determined by one-way analysis of variance (ANOVA) and a Dunnett post-hoc test or by two-

215   way ANOVA and a Bonferroni post-hoc test when assessing differences between concentration-

216   response curves. A p-value less than 0.05 was considered statistically significant.

217

RESULTS

LCA decreases the viability and induces apoptosis and necrosis of PC-3 and DU-145 human prostate cancer cells

A 24-h exposure to LCA reduced the viability of PC-3 and DU-145 cells concentration-dependently, with IC_{50} values of 32.0 µM and 30.4 µM, respectively (Fig 1). The viability of RWPE-1 immortalized normal prostate epithelial cells was not affected by concentrations of LCA between 5 and 75 µM (Fig 1). Hoechst 33342 and propidium iodide-staining of PC-3 and DU-145 cells exposed for 24 h to LCA showed a significant concentration-dependent increase in staining, with both necrotic (and late-apoptotic) and early-apoptotic cells starting to appear at a concentration at or above 3 µM (Fig 2).

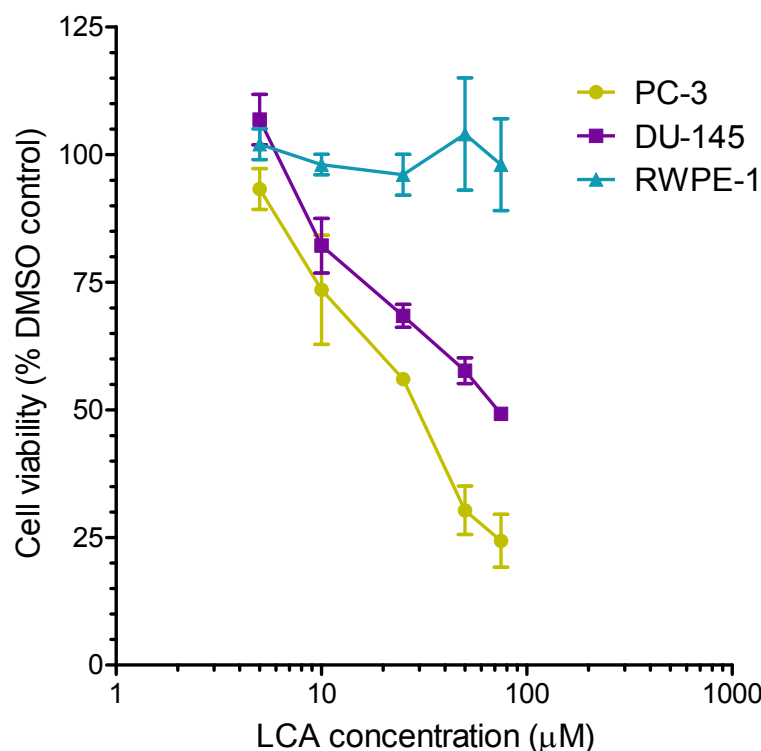



Figure 1. Lithocholic acid (LCA) decreases the viability of PC-3 and DU-145 human prostate cancer cells, but not RWPE-1 immortalized normal prostate epithelial cells. *Cells were exposed to increasing concentrations of LCA (5-75 µM) for 24 h. IC_{50} values for LCA-induced cytotoxicity in PC-3 and DU-145 cells were 32.0 µM and 30.4 µM, respectively. Experiments were performed three times; per experiment, each concentration was tested in triplicate.*

234   **See high-definition Figure 2**

235   ***Figure 2. Lithocholic acid (LCA) induces apoptotic and necrotic death of PC-3 and DU-145 prostate cancer cells.*** *Apoptotic*

236   *nuclear morphology (chromatin condensation) was observed with Hoechst 33342 staining using fluorescence microscopy. Propidium*

237   *iodide staining was used to distinguish apoptotic from necrotic (and late-apoptotic) cell death. The concentration-response*

238   *experiment was performed three times using different cell passages. Per experiment, concentrations were tested in triplicate.*

239   **LCA induces ER stress in PC-3 and DU-145 cells**

240   To determine whether the ER stress pathway was involved in LCA-induced prostate cancer cell

241   death, we determined the concentration- and time-dependent effects of LCA on p-JNK, JNK, p-

242   eIF2α, eIF2α and CHOP protein levels, as well as on levels of BIM and PUMA in PC-3 and DU-

243   145 cells exposed for 24 h to sub-cytotoxic (3 and 10 μM) and overtly cytotoxic (30 and 50 μM)

244   concentrations of LCA. Levels of BIM and PUMA were decreased concentration-dependently by

245   LCA in PC-3 and DU-145 cells, although in DU-145 cells PUMA levels increased at 3 and 10

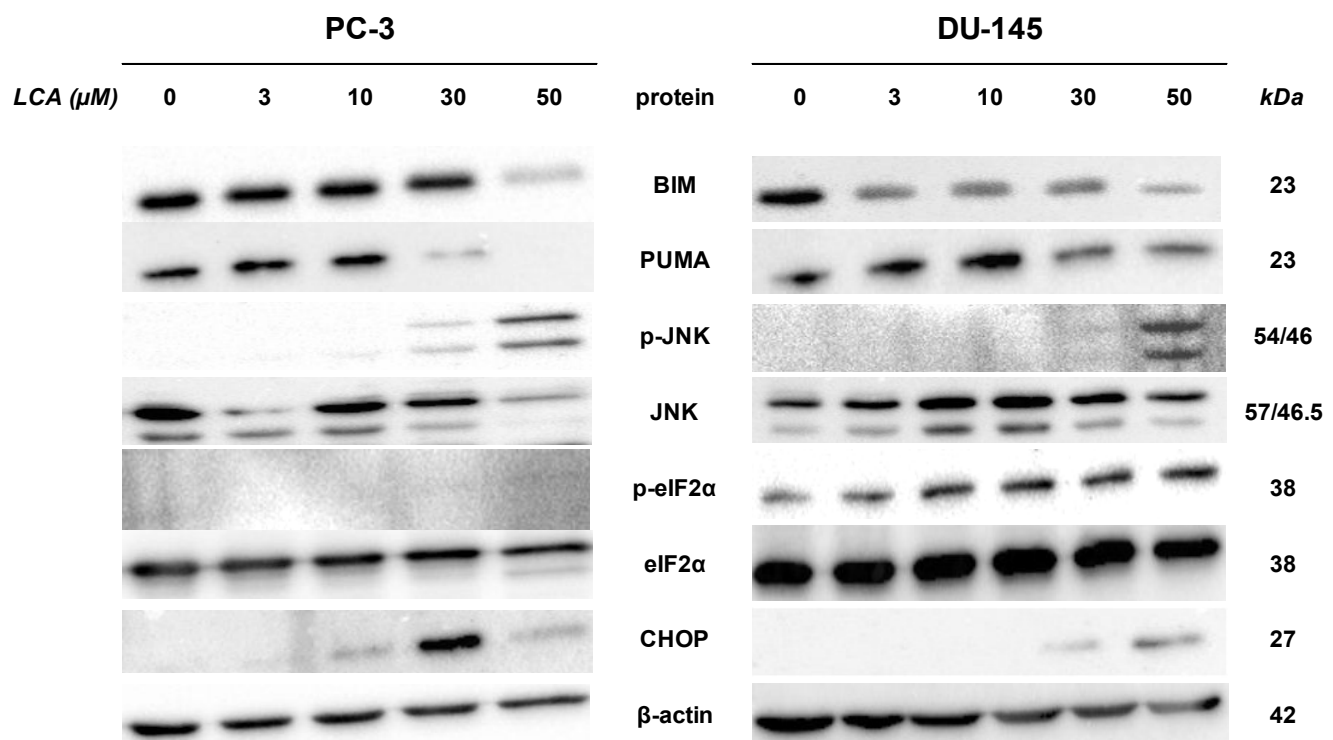
246   μM before decreasing strongly at overtly cytotoxic concentrations (Fig 3). LCA concentration-

247   dependently increased levels of p-JNK (46 and 54 KDa) and CHOP (27 kDa) in PC-3 and DU-

248   145 cells (Fig 3). Phosphorylation of eIF2α was increased in a concentration-dependent manner

249   in DU-145 cells, but was poorly detectable in PC-3 cells after a 24 h exposure to any of the LCA

250   concentrations (Fig 3).

251

252   ***Figure 3. Lithocholic acid (LCA) induces ER stress in PC-3 and DU-145 prostate cancer cells.***

253   *Cells were exposed to 3, 10, 30 or 50 μM of LCA for 24 h. BIM, PUMA, p-JNK, JNK, eIF2α, p-*

254   *eIF2α, CHOP and β-actin were detected by immunoblotting; one representative gel of three is*

255   *shown.*

256

257   To determine the effects of LCA on the ER stress response at earlier time-points, PC-3 and DU-

258   145 cells were exposed to cytotoxic concentrations (30 and 50 μM) of LCA for 1 and 8 h (Fig 4).

259   BIM and PUMA levels were decreased concentration-dependently by LCA in both cell lines. In

260   PC-3 cells BIM levels were somewhat higher at 8 h than 1 h (Fig 4), which appeared to be an

261   effect of the vehicle control, although they were, nevertheless, decreased by LCA, as was

262   observed after 24 h exposure (Fig 3). In DU-145 cells BIM levels were detectable at 1 h but not

263   at 8 h. PUMA levels were decreased concentration-dependently by LCA in both cell lines,

264   although basal levels in each cell line increased between 1 and 8 h of culture (Fig. 4). Levels of

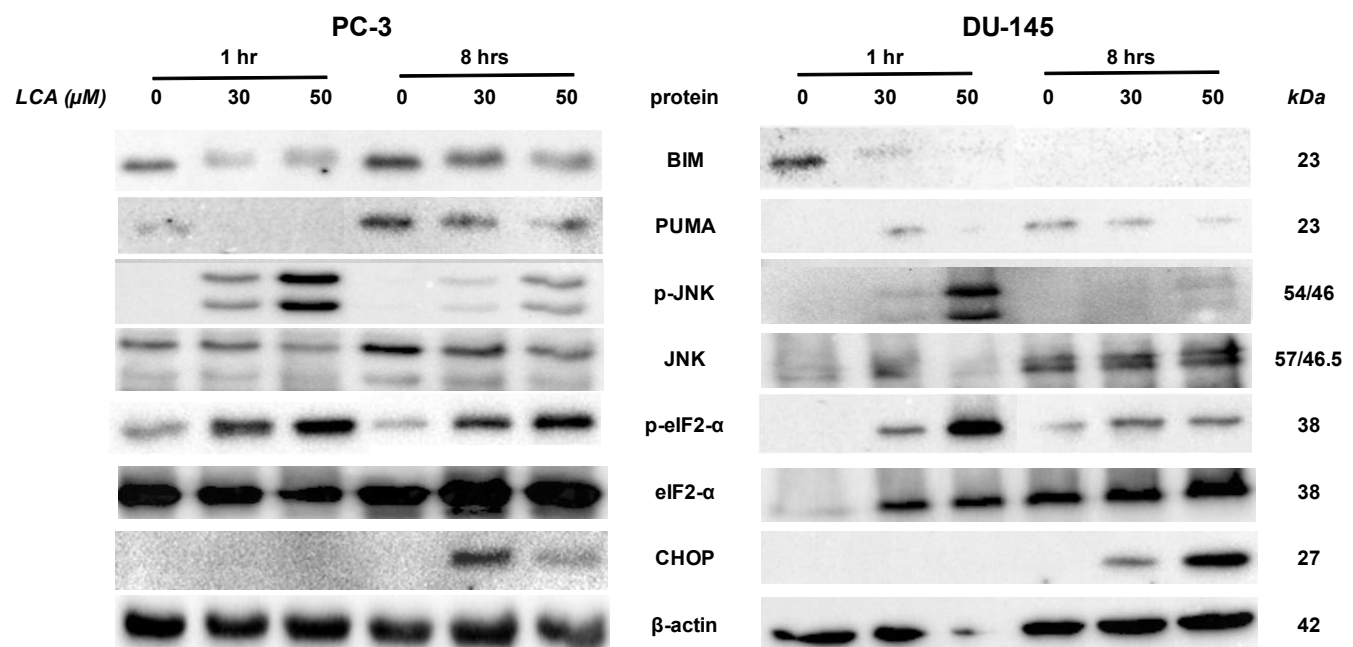
265   p-JNK underwent a biphasic response in both cell lines with expression levels appearing lower

266   after 8 h than 1h of exposure to LCA, whereas levels were increased again after 24 h of

267   exposure, in particular to 50 μM LCA. Levels of p-eIF2α increased concentration-dependently

268   after a 1 h and 8 h exposure of PC-3 and DU-145 cells to LCA (Fig. 4), but decreased time-

269   dependently in both cell lines and, after 24 h of exposure, to non-detectable levels in PC-3 cells

270   (Fig. 3). LCA (30 and 50 μM) visibly increased CHOP levels after 8 h in both cell lines.

271

272   *Figure 4. Time-dependent induction of ER stress by overtly cytotoxic concentrations of*

273   *lithocholic acid (LCA) in PC-3 and DU-145 prostate cancer cells. Cells were exposed to 30 or*

274 *50 μM LCA for 1 and 8 h. BIM, PUMA, p-JNK, JNK, p-eIF2α, eIF2α, CHOP and β-actin were*

275 *detected by immunoblotting; one representative gel of three is shown.*

276

277 **ER stress-inhibitor salubrinal and CHOP gene-silencing do not abrogate LCA-induced**

278 **cytotoxicity or apoptosis**

279 To determine the role of ER stress in causing the cytotoxicity of LCA to PC-3 and DU-145 cells,

280 each cell type was pretreated for 4 h with salubrinal, a selective inhibitor of eIF2α

281 dephosphorylation, before exposure to toxic concentration of 30 or 50 μM LCA. After an 8-h

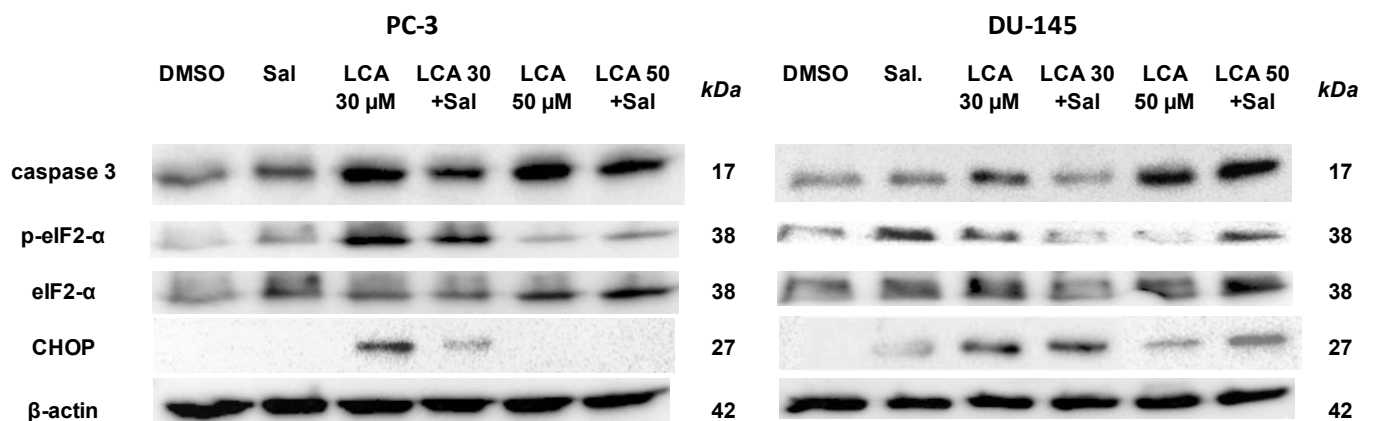
282 exposure, LCA increased levels of cleaved caspase 3, p-eIF2α and CHOP in both cell lines (Fig

283 5). Salubrinal pretreatment reduced each of these LCA-mediated increases in PC-3 cells,

284 although in DU-145 cells salubrinal pretreatment increased CHOP levels induced by 50 μM

285 LCA (Fig 5). In addition, salubrinal pretreatment did not alleviate LCA-induced death of PC-3

286 and DU-145 cells, but exacerbated the toxicity of LCA statistically significantly at most test

287 concentrations (Fig 6).

288

289 *Figure 5. The effects of salubrinal-pretreatment on lithocholic acid-(LCA)-induced cleaved*

290 *caspase 3, p-eIF2α and CHOP levels in PC-3 and DU-145 prostate cancer cells. PC-3 and DU-*

291 *145 were exposed to LCA (30 and 50 μM) for 8 h in the presence or absence of 20 μM*

292 *salubrinal. The expression of caspase-3, p-eIF2α and CHOP was determined by*

293 *immunoblotting; one representative gel of three is shown.*

294

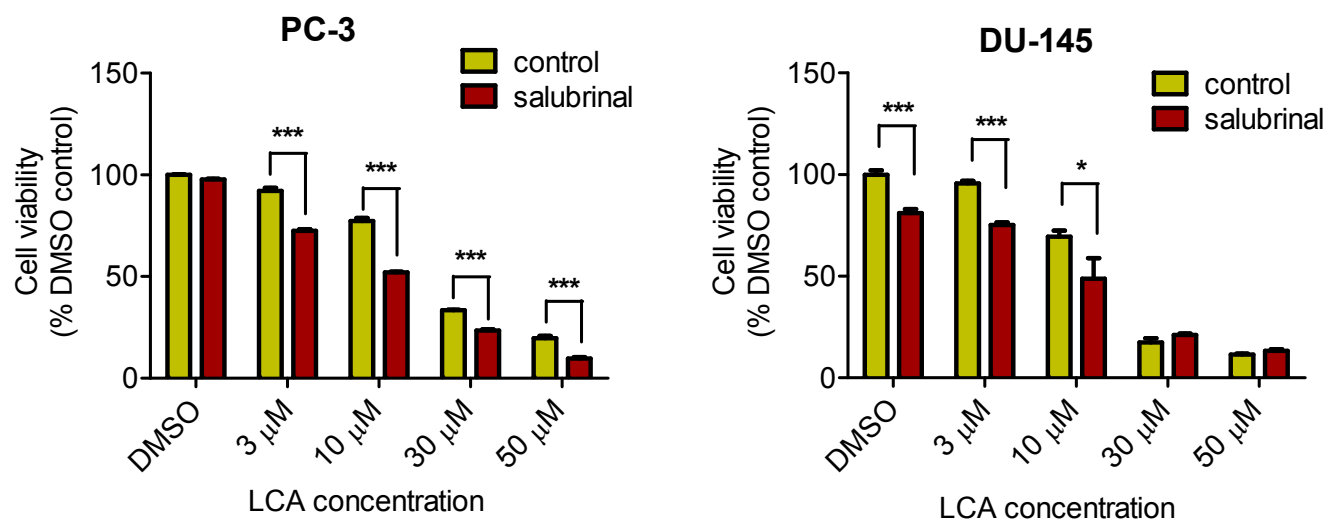



Figure 6. Salubrinal-pretreatment exacerbates the cytotoxicity of lithocholic acid (LCA) in

PC-3 and DU-145 prostate cancer cells (24 h exposure). Statistically significant differences in

cell viability between salubrinal-treated and vehicle control-treated cells were observed by two-

way ANOVA and Bonferroni post-hoc test (* p<0.05; *** p<0.001). Experiments were

performed in triplicate using different cell passages; per experiment each concentration was

tested in triplicate.

301

Given that salubrinal-pretreatment further increased levels of LCA-induced CHOP in DU-145

cells, we assessed the effect of blocking CHOP gene expression using CHOP-selective siRNA.

Gene silencing reduced LCA-induced levels of CHOP protein to undetectable levels in DU-145

cells (Fig 7A). However, no effect of CHOP silencing on LCA-induced cytotoxicity in DU-145

cells was observed (Fig 7B). This was confirmed using Hoechst staining to evaluate the effect of

CHOP silencing on LCA-induced apoptosis in both DU-145 and PC-3 cells (Fig 8).

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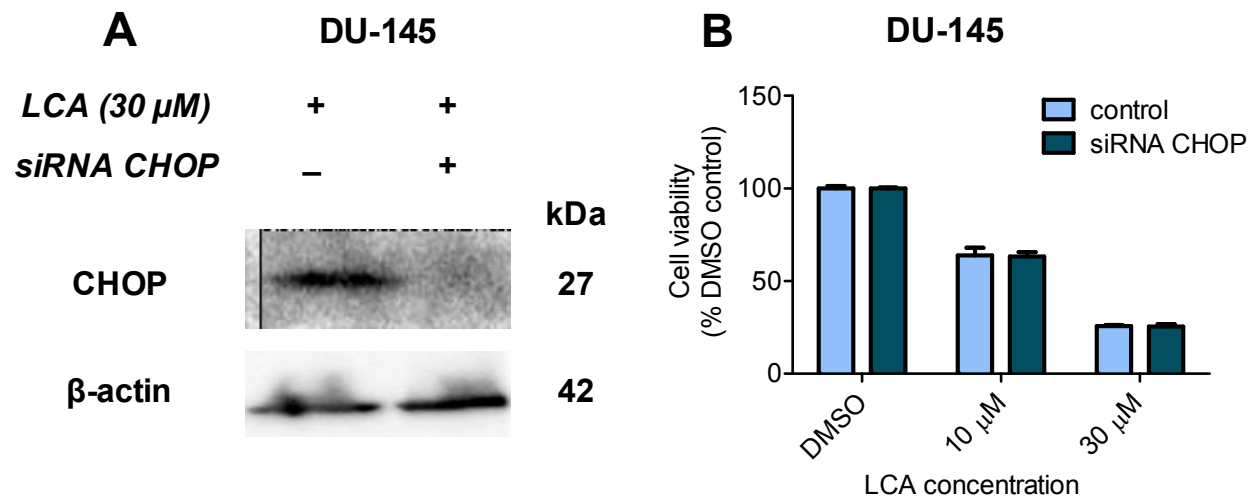


Figure 7. CHOP gene silencing does not affect lithocholic acid-(LCA)-induced cytotoxicity in

DU-145 prostate cancer cells. *No statistically significant effects were observed of siRNA*

treatment on control or LCA-decreased DU-145 cell viability by two-way ANOVA (p = 0.9;

n=3).

314

315

316

See high-definition Figure 8

318

Figure 8. CHOP gene silencing does not affect lithocholic acid-(LCA)-induced apoptosis in PC-3 and DU-145 prostate cancer cells. *Apoptotic nuclear morphology (chromatin condensed nuclei) was observed by Hoechst 33342 staining using fluorescence microscopy. The concentration-response experiment was performed three times using different cell passages; per experiment, concentrations were tested in triplicate.*

LCA induces autophagy in PC-3 cells

PC-3 cells exposed to increasing concentrations of LCA for 24 h were stained with Cyto ID
Green to detect the formation of autophagic vacuoles. A significant concentration-dependent
increase of green fluorescence signal was observed starting at an LCA concentration as low as 1
µM) (Fig 9). Further confirming the autophagic response, a concentration-dependent increase of
the conversion of LC3B I to LC3B II was observed in PC-3 cells (Fig 10). A time-course
experiment indicated that noticeable conversion of LC3B was seen as early as 1 h after exposure
to 30 or 50 µM LCA (Fig 10). When PC-3 cells were pretreated with the autophagy inhibitor
bafilomycin A1, the toxicity of relatively non-toxic concentrations of LCA (3 and 10 µM) was
increased to a statistically significant degree, whereas no effects on the toxicity of LCA were
observed at overtly toxic concentrations of 30 and 50 µM (Fig 11A). Similarly, silencing LC3B
gene expression also increased the toxicity of LCA at lower concentrations (Fig 11B). To
establish if there was a link between induction of CHOP by LCA and that of autophagy, PC-3
cells were treated with siRNA to silence CHOP and then exposed to 30 or 50 µM LCA (Fig 12).
CHOP silencing did not alter the increased conversion of LC3BI to II or alter the levels of ATG5
protein that were increased by LCA.

See high-definition Figure 9

Figure 9. Lithocholic acid (LCA) induces autophagy in PC-3 prostate cancer cells. *Cells were
exposed to increasing concentrations of LCA for 24 h and then stained with Cyto-ID® Green dye
for 10 minutes to detect autophagic vacuoles. LCA concentration-dependently increased the
accumulation of autophagic vacuoles (bright green fluorescence) as detected by Cyto-ID®
Green dye staining using fluorescence microscopy. The concentration-response experiment was
performed three times using different cell passages; per experiment, concentrations were tested
in triplicate.*

348

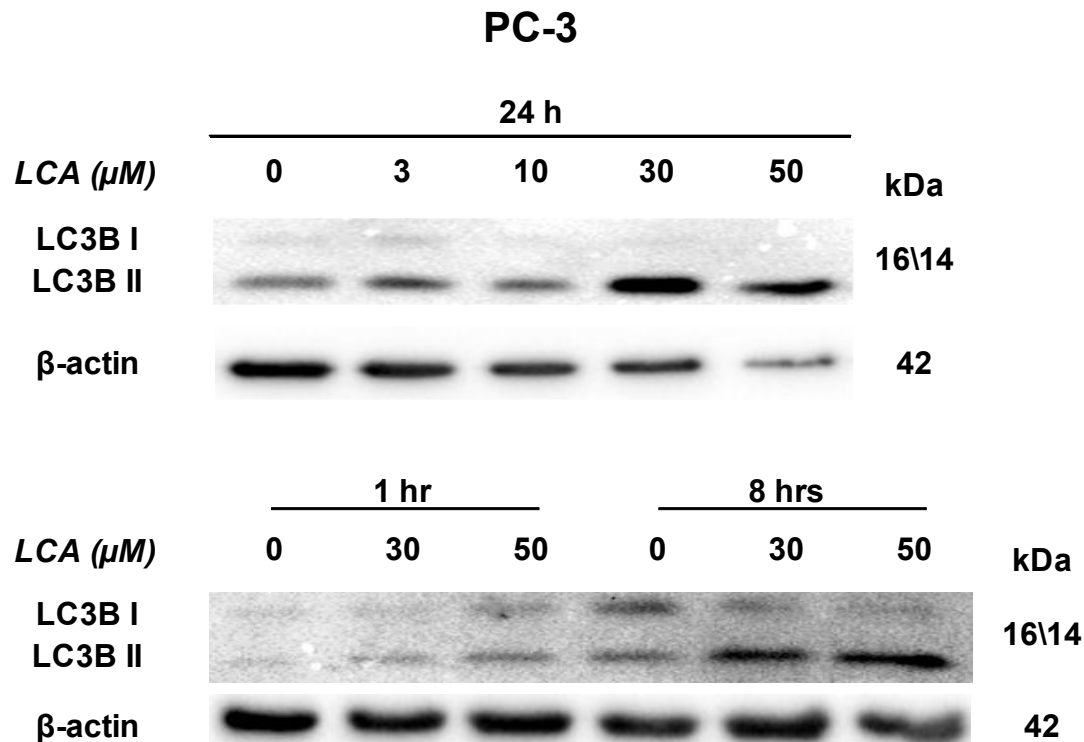
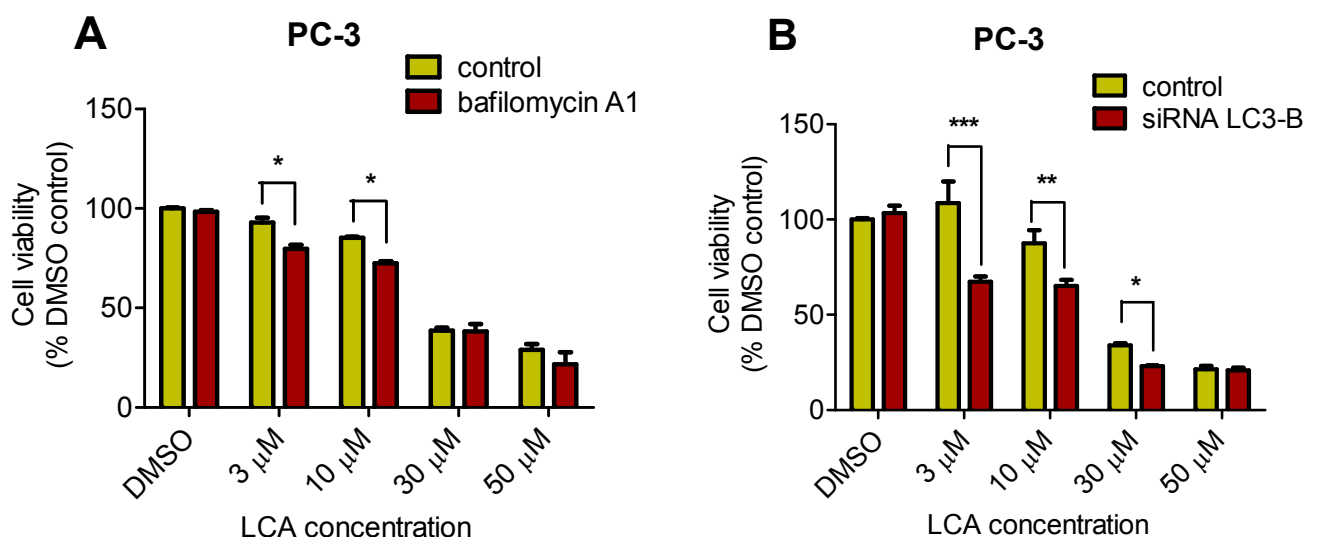



Figure 10. Lithocholic acid (LCA) induces LC3B conversion in PC-3 prostate cancer cells.

Cells were exposed to increasing concentrations of LCA for 1, 8 or 24 h. Proteins were detected

by immunoblotting; one representative gel of three is shown.

352

353
354 *Figure 11. Bafilomycin A1-pretreatment (A) or LC3B gene silencing (B) enhanced the*

355 ***cytotoxicity of lithocholic acid (LCA) in PC-3 prostate cancer cells.*** *Statistically significant*

356 *differences in cell viability between bafilomycin A1- or LC3B siRNA-treated PC-3 cells and*

357 *vehicle control-treated cells were determined by two-way ANOVA and Bonferroni post-hoc test*

358 *(* p<0.05; ** p<0.01; *** p<0.001). Experiments were performed in triplicate using different*

359 *cell passages; per experiment, each concentration was tested in triplicate.*

360

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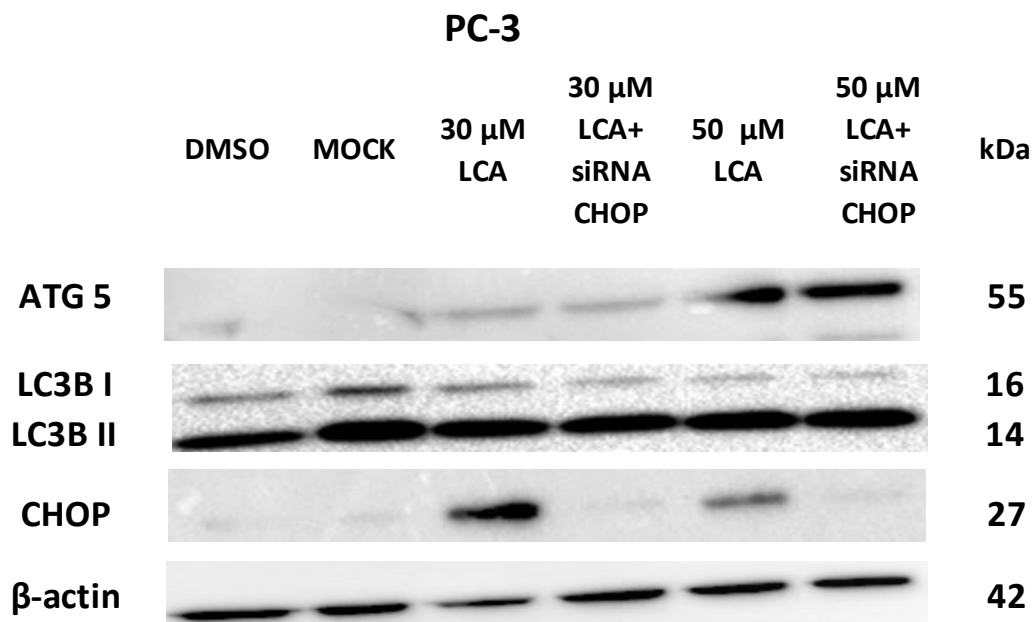

362 **Figure 12. *CHOP silencing had no affect on LCA-mediated induction of the autophagic***

363 ***markers LC3B conversion or ATG5 expression.*** *Proteins were detected by immunoblotting; one*

364 *representative gel of three is shown.*

365

366 **LCA induces mitochondrial dysfunction in PC-3 and DU-145 cells**

367 Lithocholic acid induced mitochondrial dysfunction in PC-3 and DU-145 as measured using

368 TMRE dye (Fig 13), which is sequestered by active mitochondria, but fails to accumulate in

369 mitochondria that have reduced or lost their outer membrane potential. PC-3 and DU-145 were

370 exposed to different concentration of LCA (0, 1, 3, 10 and 30 μM) for 8 h and observed a

371 concentration-dependent decrease in TMRE sequestration, which was most apparent at 30 μM

372 LCA (Fig 13). The loss of mitochondrial membrane potential coincided with an increase in

373 nuclear staining with Hoechst 33342 (Fig 13).

374

375   **See high-definition Figure 13**

376

377 *Figure 13: Lithocholic acid induces mitochondrial dysfunction in PC-3 and DU-145 cells.*

378 *Cells were exposed to different concentrations of LCA for 8 hrs. Apoptotic nuclear morphology*

379 *(chromatin condensed nuclei) was observed by Hoechst 33342 staining and mitochondrial*

380 *membrane permeability was measured using TMRE fluorescent dye by fluorescence microscopy.*

381 *The concentration-response experiment was performed three times using different cell passages;*

382 *per experiment, each concentrations was tested in triplicate.*

383

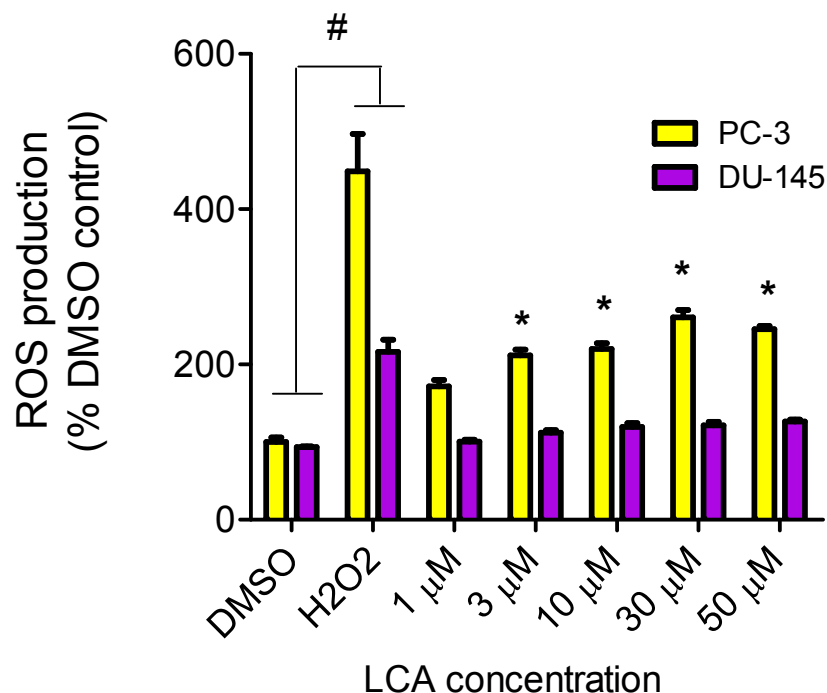
384 ***LCA induces reactive oxygen species (ROS)***

385 LCA increased the production of ROS concentration dependently in PC-3 but not DU-145 cells

386 at concentrations between 1 and 50 μM. (Fig 14). To determine if the antioxidant α-tocotrienol

387 (T-3; 20 μM) could reduce the cytotoxicity of LCA, PC-3 and DU-145 cells were incubated with

388 T-3 four hours prior to a 24-h exposure to LCA. T-3 protected significantly against LCA-induced

389 cytotoxicity in PC-3 cells whereas in DU-145 cells, T-3 had no effect (Fig 15).

390

391 **_Figure 14. LCA induces reactive oxygen species (ROS) concentration dependently in PC-3 but_**

392 **_not DU-145 cells._** *Cells were exposed to LCA for 60 minutes in culture medium containing 10*

393 *mM fluorescent probe dye (CM-H2DCFDA). H_2O_2 (20 μM) was used as a positive control for*

394 *ROS production, which was measured using a fluorescence spectrophotometer. #) A statistically*

395 *significant difference between DMSO- and H_2O_2-treated cells. *) A statistically significant*

396 *diffference between DMSO- and LCA-treated cells determined by one-way ANOVA followed by a*

397 *Dunnett test. One of three experiments is shown; each concentration was tested in triplicate.*

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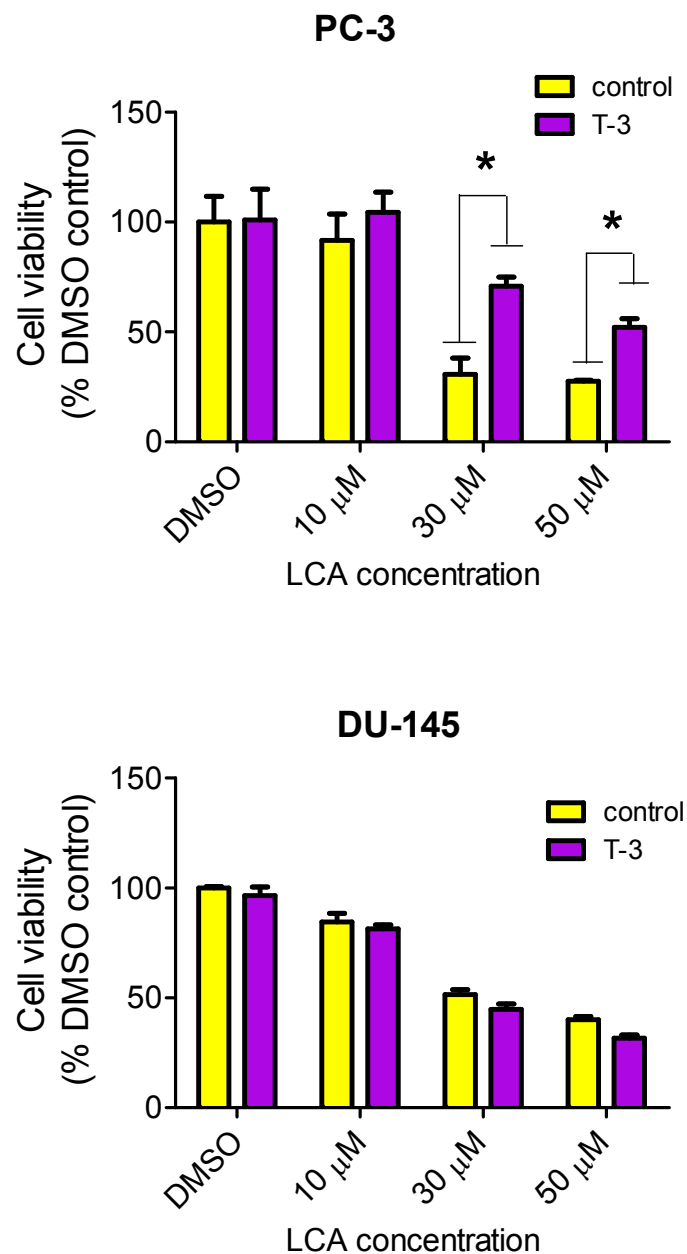
399

400 ***Figure 15.*** *Effects of a 4-hour pretreatment with the antioxidant* α-tocotrienol (T-3; 20 μM) *on the*

401 *cytotoxicity of LCA (24 h exposure) in (PC-3 cells or DU-145 cells. Statistically significant differences in*

402 *cell viability between antioxidant-treated and vehicle control-treated cells were observed by two-way*

403 *ANOVA and Bonferroni post-hoc test (* p<0.05). One of three experiments is shown; each concentration*

404 *was tested in triplicate.*

405

406   **DISCUSSION**

407   **LCA induces selective cancer cell death**

408   In our study, we found that LCA reduces the viability of androgen-independent DU-145 and PC-
409   3 human prostate cancer cells, but not RWPE-1 immortalized human prostate epithelial cells (Fig
410   1), confirming and expanding upon our previous observations in prostate cancer cells that
411   included androgen-dependent LNCaP cells (Goldberg *et al.*, 2013). LCA triggered
412   concentration-dependent death of PC3 and DU-145 cells via apoptotic and necrotic pathways
413   (Fig 2). The selectiveness of LCA in killing cancer cells has recently been demonstrated in
414   hepatocytes, where galactosylated poly(ethylene glycol)-conjugated LCA was toxic to HepG2
415   human hepatocarcinoma cells, but not to immortalized human LO2 liver cells (Gankhuyag *et al.*,
416   2015). Furthermore, we have previously shown that LCA killed neuroblastoma cells, whilst
417   sparing normal neuronal cells (Goldberg *et al.*, 2011).

418   **LCA induces ER stress in prostate cancer cells**

419   We show for the first time that LCA induces ER stress in human androgen-independent prostate
420   cancer cells in a time- and concentration-dependent manner (Fig. 3 and 4). Toxic concentrations
421   of LCA reduced BIM and PUMA, and increased CHOP levels and the phosphorylation of eIF2α
422   and JNK in both cancer cell types. Increased phosphorylation of eIF2α and JNK were early (1 h)
423   responses to toxic concentrations of LCA, whereas concentration-dependent decreases of BIM
424   and PUMA were sustained between 1 and 24 h of exposure (Fig. 3 and 4). The increased
425   cleavage of caspase 3 by LCA (Fig. 5) likely explains why BIM and PUMA levels decreased at
426   toxic concentrations of LCA, as it is known that active caspase 3 downregulates PUMA (Hadji *et*
427   *al.*, 2010) and BIM (Wakeyama *et al.*, 2007) expression in other cell types. At lower LCA
428   concentrations and at earlier exposure durations, on the other hand, PUMA is initially increased,
429   suggesting that PUMA is involved in triggering mitochondrial apoptosis (as discuss later) and
430   caspase 3 activation that ultimately results in its breakdown. The up-regulation of PUMA is
431   clearly p53-independent in PC-3 cells as these cells are p53-deficient (Rubin *et al.*, 1991).

432   LCA caused sustained induction of CHOP at 30 μM in PC-3 cells, although levels were sharply
433   lower at 50 μM, possible due to excessive cell death (Fig 3-5). In DU-145 cells CHOP levels
434   were increased by 30 and 50 μM LCA, but levels declined between 8 and 24 h of exposure (Fig.

435   3 and 4). Our observations suggest that LCA-induced ER stress involves activation of the eIF2α

436   phosphorylation pathway and subsequent induction of p-JNK (early response) and CHOP (later

437   response), resulting in caspase 3-dependent apoptosis. However, an attempt to block this

438   particular pathway with salubrinal reduced CHOP induction in PC-3 cells only, although it

439   decreased LCA-induced caspase 3 in both cell lines (Fig 5). Yet, salubrinal pretreatment resulted

440   in increased toxicity of LCA in both cell lines (Fig 6). We have previously shown that direct

441   inhibition of the catalytic activity of caspase 3 did result in partial protection against LCA-

442   induced cytotoxicity in LNCaP and PC-3 prostate cancer cells (Goldberg *et al.*, 2013), and in

443   neuroblastoma cells (Goldberg *et al.*, 2011). It is possible that the observed decreases in cleaved

444   caspase 3 protein levels do not reflect a significant change in its catalytic activity.

445   Furthermore, blocking CHOP expression using *CHOP*-selective siRNA had no effect on the

446   reduced viability (Fig 7) or apoptosis (as determined by measuring chromatin condensation and

447   fragmentation using the fluorescent dye Hoechst 33342) (Fig 8) of DU-145 and PC-3 cells after

448   exposure to increasing concentrations of LCA). Therefore, inhibition of ER stress signaling alone

449   does not appear to be essential for LCA-induced prostate cancer cell death.

450   Other studies have observed the induction of ER stress by bile acids. In HepG2 cells, the

451   secondary bile acids LCA and DCA were the most toxic, followed by CDCA, although they

452   induced cell death at concentrations of 100 μM and above (Adachi *et al.*, 2014), which are

453   significantly greater than the concentrations of LCA that we have found to be toxic to prostate

454   cancer cells. The same investigators detected increased expression of genes involved in ER

455   stress, such as *GRP78* and *CHOP* after 24 h exposures to 100 μM of LCA, DCA or CDCA.

456   Using CDCA as a prototype bile acid, it was found to increase caspase 3 activity at 200 μM, but

457   not 100 μM. Although cytotoxicity and CHOP induction, but not caspase 3 activation, appeared

458   to occur concurrently after exposure to certain bile acids (Adachi *et al.*, 2014), a direct link

459   between ER stress and HepG2 cell death was not established. Glycochenodeoxycholic acid

460   (GCDCA) has been shown to induce ER stress in freshly isolated rat hepatocytes and this study

461   interestingly showed that ER stress-mediated activation of caspase 12 occurred at a later stage

462   than mitochondrial apoptosis mediated by cytochrome c release and caspase 3 activation

463   (Tsuchiya *et al.*, 2006), suggesting induction of ER stress may not be critical to cell death. In a

464   follow-up study, the investigators determined that caspase 8 activation via the extrinsic Fas

465   pathway triggered ER stress in response to 300 µM GCDCA in HepG2 human hepatocarcinoma

466   cells (Iizaka *et al.*, 2007). It is unclear how critical caspase activation is for bile acid-induced cell

467   death. Glycodeoxycholate induced caspase 3-dependent apoptosis in rat hepatocytes after a 2 h

468   exposure and inhibition of caspase 3 activity resulted in less apoptosis, but whether this

469   translated into less cell death was not reported (Webster *et al.*, 2002). We point out that these

470   previous studies were performed with remarkably high concentrations of bile acids and whether

471   cells were dying due to excess necrosis was never reported. We have previously shown in

472   LNCaP and PC-3 prostate cancer cells that LCA (50 and 75 µM, respectively) activates caspases

473   8, 9 and 3, and that caspase 9 activation was likely secondary to caspase 8-induced truncation of

474   Bid (Goldberg *et al.*, 2013), a finding consistent with those of (Iizaka *et al.*, 2007). Inhibition of

475   caspases 8 or 3 resulted in partial protection against LCA induced cytotoxicity, suggesting that

476   the cytotoxicity of LCA is, at least in part, caspase-dependent (Goldberg *et al.*, 2013). However,

477   we are currently performing studies to show that necrotic signaling pathways may play a

478   significant role in LCA-induced death of prostate cancer cells.

479   **LCA induces autophagy in PC-3 cells.**

480   We found that LCA induces a general autophagic response in PC-3 cells based on a time- and

481   concentration-dependent increase of LC3B conversion observed in these cells (Fig. 9 and 10). To

482   delineate the protective or cytotoxic nature of the autophagic response to LCA, cells were

483   exposed to LCA after pre-incubation with the autophagy inhibitor bafilomycin A1. Cells were

484   also treated with siRNA specific for LC3B to silence the expresssion of this protein. Inhibiting

485   autophagy in PC-3 cells in either of these manners enhanced the toxicity of normally sub-

486   cytotoxic concentrations of LCA (Fig 11A and 11B). This observation indicates that the

487   autophagic response of PC-3 cells to LCA exposure is, at least initially, of a protective nature.

488   Similarly, autophagy was shown to provide protection against cell death of rat hepatocytes

489   induced by glycochenodeoxycholate, as its inhibition using the autophagy inhibitor chloroquine

490   exacerbated toxicity whereas induction of autophagy using rapamycin provided protection

491   against cell death (Gao *et al.*, 2014). Our laboratory has also recently shown that blocking

492   autophagy in LNCaP and LNCaP C4-2B prostate cancer cells, resulted in a strong sensitization

493   of these cells to the cytotoxicity of diindolylmethane and a series of ring-substituted

494 dihalogenated DIM derivatives again demonstrating the protective nature of the autophagic

495 process in these cells (Goldberg *et al.*, 2015).

496 To our knowledge, this is the first reported observation that LCA induces autophagy in human

497 (prostate) cancer cells, although a link between bile acids and autophagy has been recently

498 proposed via activation of the farnesoid X receptor (FXR) (Nie *et al.*, 2015). The FXR is a

499 cytoplasmic receptor and an important target for hydrophilic primary bile acids, but is unlikely to

500 play a large role in the biological effects of LCA, which is very hydrophobic and remains almost

501 entirely outside the cell (Goldberg *et al.*, 2013). More likely targets for LCA are cell membrane

502 surface receptors such as the death receptors or the G-protein-coupled bile acid receptor

503 (GPBAR1), the latter for which LCA has a particularly strong affinity. Although the role of the

504 GPBAR1 in LCA mediated signaling in healthy cells is currently under intense investigation

505 (Tiwari and Maiti, 2009; Stepanov *et al.*, 2013; Fiorucci and Distrutti, 2015; Li and Chiang,

506 2015; Perino and Schoonjans, 2015), nothing is known about its functions in prostate cancer

507 cells. Our preliminary results show strong expression of GPBAR1 protein in LNCaP, PC-3 and

508 DU-145 cells and we are currently investigating the role of this receptor in triggering various cell

509 death or survival pathways in these prostate cancer cells.

510 We did not establish a link between the induction of ER stress by LCA and its induction of

511 autophagy. CHOP silencing did not alter the autophagic response of PC-3 cells to LCA at the

512 tested concentration of 30 and 50 µM as we observed no changes in the induction of LCB3

513 conversion or ATG5 protein levels (Fig 12). A recent study showed that whether triggering ER

514 stress resulted in induction of either autophagy or apoptosis depended on the type of trigger.

515 They found that triggering ER stress with thapsigargin only resulted in induction of apoptosis,

516 whereas the ER stress inducer tunicamycin only caused autophagy (Matsumoto *et al.*, 2013).

517 However, it was not made clear whether the induction of either autophagy or apoptosis was

518 directly mediated by ER stress or could have been due to off-target effects of the typical ER

519 stress inducers. Our results indicate that the induction of ER stress by LCA was not directly

520 responsible for the induction of either cell death or autophagy, and that likely these effects are

521 secondary to the disruption of mitochondrial function by LCA.

522 **LCA induces mitochondrial dysfunction in PC-3 and DU-145 cells**

523   We have shown that LCA impairs mitochondrial function by increasing mitochondrial outer-
524   membrane permeability (Fig 13). These results confirm our earlier finding that LCA impairs
525   mitochondrial membrane potential in PC-3 and LNCaP cells as early as 1 h after exposure and
526   was sustained for at least 8 h (Goldberg *et al.*, 2013). In the present study, we found that
527   induction of ROS by LCA (Fig 14) appeared to be a key trigger of cell death in PC-3 cells as the
528   antioxidant T-3 was able to protect these cells against the cytotoxicity of LCA (Fig 15).
529   Interestingly LCA did not induce ROS in DU-145 cells (Fig 14) and consistent with this,
530   antioxidant pretreatment had no protective effect against LCA-mediated cytotoxicity in these
531   cells (Fig 15). These remarkable differences in (anti)oxidative responses between the two cell
532   lines warrant further investigation.

533

534   **CONCLUSIONS**

535   In summary, we have found that LCA induces an ER stress response in PC-3 and DU-145 human
536   prostate cancer cells via a p-eIF2α-dependent pathway and an autophagic response in autophagy-
537   capable PC-3 cells. These pathways appear to play a cytoprotective role against LCA-induced
538   cell death, and are rather a response to the underlying, yet to be precisely elucidated mechanisms
539   of LCA-induced prostate cancer cell death. These underlying mechanisms appear to involve
540   induction of ROS and subsequent mitochondrial dysfunction in PC-3 cells, whereas in DU-145
541   cells LCA-induced mitochondrial dysfunction and cell death occurred at similar LCA
542   concentrations, yet in the absence of ROS formation.

543

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