Effect of lipoic acid on paraoxonase-1 and paraoxonase-3 protein levels, mRNA expression and arylesterase activity in liver hepatoma cells

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Running Title: Effects of lipoic acid on PON1 and PON3 in liver

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Abstract:

Paraoxonase-1 and paraoxonase-3 are anti-atherosclerotic enzymes, synthesized primarily in liver and bound to HDL in circulation. The aim of the present study was to investigate the effects of therapeutic doses of lipoic acid on paraoxonase-1 and paraoxonase-3 protein levels, mRNA expression and arylesterase activity in liver. We treated HepG2 cells with 0 (control), 10, 40 and 200 μM lipoic acid for 72 h. Cell viability was evaluated by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Paraoxonase-1 and paraoxonase-3 protein levels were measured by western blotting, their mRNA expression were measured by quantitative PCR and arylesterase activity was measured spectrophotometrically. 200 μM lipoic acid caused a significant increase on paraoxonase-1 and paraoxonase-3 protein levels and arylesterase activity as compared with control, 10 μM and 40 μM lipoic acid-treated cells. 200 μM lipoic acid also caused a significant decrease on paraoxonase-1 mRNA expression whereas on a significant increase paraoxonase-3 mRNA expression as compared with control, 10 μM and 40 μM lipoic acid-treated cells. Our study showed that although lipoic acid up-regulates paraoxonase-3 but down-regulates paraoxonase-1 mRNA expression, it increases both paraoxonase-1 and paraoxonase-3 protein levels and arylesterase activity in HepG2 cells. We can report that lipoic acid may be useful for preventing atherosclerosis at therapeutic doses.

Keywords: Lipoic acid; Paraoxonase-1; Paraoxonase-3; Arylesterase; Liver.

Introduction

Paraoxonase-1 (PON1) and paraoxonase-3 (PON3) are the members of paraoxonase enzyme family which consists of three genes: PON1, PON2 and PON3 and are mainly synthesized by the liver (Précourt et al. 2011). All three proteins have peroxidase, lactonase and arylesterase activity and are capable of hydrolyzing oxidized phospholipids. In circulation, both PON1 and PON3 enzymes are bound to HDL and shows antioxidant properties (Précourt et al. 2011; Draganov et al. 2000; Rosenblat et al. 2003). PON1 prevents the development of atherosclerosis by protecting LDL against to oxidation and reducing macrophage foam cell formation (Aviram and Rosenblat 2004). On the other hand, it is known that the formation of mildly oxidized LDL and therefore the induction of monocyte chemotactic activity is prevented by PON3 (Reddy et al. 2001). PON1 activities were shown to be regulated both genetically and by diet (Précourt et al. 2011).

Lipoic acid is synthesized from octanoic acid and cysteine in mitochondria (Golbidi et al. 2011) and is primarily metabolized in the liver (Goraca et al.2011). Lipoic acid shows direct and indirect antioxidant properties (Rochette et al. 2013) and has been proposed as a potent antioxidant especially for diabetes mellitus, cardiovascular and autoimmune diseases and cancer treatment (Goraca et al.2011).

In our previous study, we reported that lipoic acid prevents an increase in lipid peroxidation of the liver; therefore it has protective effects against to hepatic ischemia/reperfusion injury in rats (Ozgun et al. 2014). In another study, we also reported that lipoic acid treatment causes an increase in serum PON1 and PON3 protein levels and enzyme activities in diabetic rats (Ozgun et al. 2016). Despite several previous reports on the effects of lipoic acid on paraoxonase enzymes protein and activity, no study has ever been performed to specifically examine the effect of lipoic acid on PON1 and PON3 mRNA expression.

The aim of the present study was to investigate the effects of therapeutic doses of lipoic acid on PON1 and PON3 protein levels, mRNA expression and arylesterase activity in liver, the major source of these enzymes. For this purpose, we used the HepG2 cells which are commercial human liver hepatoma cells. HepG2 cells are frequently used as in vitro models for human hepatocytes in previous studies (Kockar et al. 2010; Jaichander et al. 2008; Cheng et al. 2013).

Following oral administration of 600 mg lipoic acid, its mean peak plasma level has been found as approximately 10 μM. On the other hand, following intravenous administration of 200 and 600 mg lipoic acid, its mean peak plasma level has been found as approximately 40 and 200 μM, respectively (Moini et al. 2002). Taking into consideration the above findings, we investigated the effect of 10, 40 and 200 μM lipoic acid on PON1 and PON3 protein, gene expression and arylesterase activity in HepG2 cells.

The present study is also the first report investigating the effect of therapeutic plasma concentrations of lipoic acid on PON1 and PON3 protein, gene expression and arylesterase activity in liver.

Materials and Methods

Chemicals

Human HepG2 cells were purchased from ATCC (Middlesex, UK). Minimum Essential Medium with Glutamin, fetal bovine serum, antibiotic-antimycotic, sodium pyruvate, trypsin-EDTA, RNA isolation kit, High-Capacity cDNA reverse transcription kit, TaqMan probes for PON1, PON3 and GAPDH and HRP chemiluminescent substrate were purchased from Thermo Fisher (Waltham, MA USA). PON1, PON3, alpha tubulin primary antibodies and goat anti-mouse IgG H&L horseradish peroxidase secondary antibody were purchased from Abcam (Cambridge, UK). RIPA lysis buffer system was purchased from

Santa Cruz (Heidelberg, Germany). Polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad (Hercules, CA, USA). Other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All reagents were of analytical grade.

Cell culture and experimental design

HepG2 cells were cultured in minimum essential medium with glutamin containing 10% fetal bovine serum, 1% sodium pyruvate and 1% antibiotic-antimycotic (100 units/mL penicillin and 100 µg/mL streptomycin and 25 µg/mL of Gibco Amphotericin B) in a humidified environment at 37 °C and 5% CO_2 atmosphere.

HepG2 cells were divided into four groups: control cells (cultured in medium without lipoic acid for 72 h), 10 µM lipoic acid-treated cells (cultured with 10 µM of lipoic acid for 72 h), 40 µM lipoic acid-treated cells (cultured with 40 µM of lipoic acid for 72 h) and 200 µM lipoic acid-treated cells (cultured with 200 µM of lipoic acid for 72 h). Lipoic acid was dissolved in DMSO and DMSO concentration was 0.2% in all experimental mediums of groups. Culture mediums were refreshed every 24 hours in all 72-hours experiments. All the experiments were repeated at least three times.

Cell viability assays

Effect of lipoic acid on cell viability was evaluated by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann 1983). 10^4 cells were seeded into the 96 well plates. Cells were treated with lipoic acid (10, 40 and 200 µM) for 72 hours. At the end of treatment, mediums were removed and 10 µl of MTT (5 mg/mL) solved in PBS and 100 µl of medium without phenol red were added to each well. Cells were then incubated for 4 hours in a humidified environment at 37 °C and 5% CO_2 atmosphere. MTT-containing medium was then removed and formazan crystals were then dissolved by adding 200 µL DMSO and 25 µL Sorensen buffer (0.1 M glycine, 0.1 M sodium chloride equilibrated to pH

10.5 with 0.1 M NaOH). Optical density of plates were measured using a microplate reader at 570/630 nm (Ahmadian et al. 2009). Optical density of each sample was then compared with the mean optical density value of control group optical density.

Western blot analysis of PON1 and PON3 proteins

Cells were seeded into 75 cm^2 flask. After the cells reached 70-80% confluence, they were treated with lipoic acid (10, 40 and 200 μM) for 72 hours. Following the treatments, cells were scrapped with RIPA lysis buffer system. Samples were homogenized and then centrifuged at 4 °C for 10 minutes at 15,000 × g (Beltowski et al. 2005). Supernatants were used for protein determination and western blotting. Protein concentrations were measured according to Lowry et al. (Lowry et al. 1951) by using bovine serum albumin as standard.

30 μg of total protein was separated by 4-8% sodium dodecyl sulfate-polyacyrlamide gel electrophoresis and transferred to PVDF membrane by using semi-dry blotting system. Membranes were blocked with 5% skim milk powder for 1 hour at room temperature. After blocking, membranes were incubated with monoclonal primary antibodies (PON1:1/1000 dilution and PON3:1/1000 dilution) overnight at 4 °C and then with secondary antibody (HRP goat anti-mouse: 1/10000 dilution) at room temperature for one hour. PON1 and PON3 protein bands were visualised by using ECL detection system with HRP chemiluminescent substrate and were quantified by Image J (Schneider et al. 2012). Results were calculated relative to alpha-tubulin as loading control and expressed as fold change relative to control for each blot.

PON1 and PON3 mRNA expression

Cells were seeded into 25 cm^2 flask. After the cells reached 70-80% confluence, they were treated with lipoic acid (10, 40 and 200 μM) for 72 hours. RNA isolations and PCR reactions were performed at the laboratories of the Technology Research and Development Centre of Trakya University (TUTAGEM). RNA was isolated from cells by using commercial

RNA isolation kit and cDNA was generated from 1 μg of total RNA by using commercial high-capacity cDNA reverse transcription kit. These reaction products were subject to quantitative PCR by using TaqMan gene expression assays for PON1 and PON3. GAPDH was used as housekeeping gene.

Arylesterase activity

Cells were seeded and treated, cell lysates were prepared and protein concentrations were measured in western blotting analysis as described above, except cells were scrapped with 50 mM Tris-HCl buffer (pH:8) containing 1mM $CaCl_2$ and 1% protease inhibitor cocktail, instead of RIPA lysis buffer system to avoid the inhibition of enzyme activity. Arylesterase activity was measured according to the method of Gan et al. (Gan et al. 1991) by modifying substrate concentration. Arylesterase activity was determined by the measuring initial rate of substrate hydrolysis at 270 nm in the assay mixture containing 1 mM $CaCl_2$, 10 mM phenylacetate in 50 mM Tris-HCl buffer (pH 8.0). Measurements were performed at 25 °C and the blank sample containing incubation mixture without cell lysate was run simultaneously for correction of spontaneous substrate breakdown. Results were calculated relative to total protein levels and expressed as fold change relative to control.

Statistical analysis

Results were given as means ± standard deviation (SD). The One-Way ANOVA test was used for comparison of biochemical parameters among the groups, and then, Tukey or Tamhane post-hoc tests were used for multiple comparisons when the significant difference obtained. SPSS 20.0 (IBM SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. P value < 0.05 was considered as statistical significant.

Results

The mean percentage of cell viabilities of lipoic acid-treated groups were 139% for 10 μM lipoic acid-treated cells, 130% for 40 μM lipoic acid-treated cells and 124% for 200 μM lipoic acid-treated cells. Lipoic acid (10, 40, 200 μM) treatment caused a significant increase on cell viabilities as compared with control ($P<0.001$ for all). Cell viabilities caused by 200 μM lipoic acid was significantly lower than those of 10 μM lipoic acid ($P<0.001$ for all) (Fig. 1).

PON1 protein levels of lipoic acid-treated groups were 1.06-fold, 1.13-fold and 1.28-fold for 10, 40 and 200 μM lipoic acid, respectively. PON1 protein levels in 200 μM lipoic acid-treated cells were significantly increased as compared to those in control, 10 μM and 40 μM lipoic acid-treated cells ($P<0.001$, $P<0.05$ and $P<0.05$, respectively) (Fig. 2A).

PON3 protein levels of lipoic acid-treated groups were 0.94-fold, 1.35-fold and 3.09-fold for 10, 40 and 200 μM lipoic acid, respectively. 200 μM lipoic acid caused a significant increase on PON3 protein levels as compared with control, 10 μM and 40 μM lipoic acid ($P<0.05$ for all) (Fig. 2A).

PON1 mRNA expression of lipoic acid-treated groups were 1.03-fold, 1.10-fold and 0.74-fold for 10, 40 and 200 μM lipoic acid, respectively. PON1 mRNA expression in 200 μM lipoic acid treated cells were significantly decreased as compared to those in control, 10 μM and 40 μM lipoic acid-treated cells ($P<0.05$ for all) (Fig. 2B).

PON3 mRNA expression of lipoic acid-treated groups were 0.97-fold, 0.92-fold and 1.44-fold for 10, 40 and 200 μM lipoic acid, respectively. 200 μM lipoic acid caused a significant increase on PON3 mRNA expression as compared with control, 10 μM and 40 μM lipoic acid ($P<0.001$, $P<0.05$ and $P<0.05$, respectively) (Fig. 2B).

Arylesterase activities of lipoic acid-treated groups were 1.00-fold, 1.06-fold and 1.36-fold for 10, 40 and 200 μM lipoic acid, respectively. 200 μM lipoic acid caused a significant

increase on arylesterase activity as compared with control, 10 μM and 40 μM lipoic acid ($P<0.001$ for all) (Fig. 3).

Discussion

Paraoxonases are antioxidant enzymes which are able to catalyze hydrolysis of oxidized phospholipids (Ng et al. 2005). Although most known activities of paraoxonase enzyme family are paraoxonase and arylesterase activities, these enzymes are primary lactonases and unlike PON1, PON3 have no paraoxonase and limited arylesterase activity (Précourt et al. 2011). PON1 and PON3 both have antioxidant properties and by protecting LDL against to oxidation, they prevent the development of atherosclerosis (Aviram and Rosenblat 2004; Reddy et al. 2001). Oxidized lipids in oxidized LDL are known to inactivate PON1. Dietary antioxidant can effect paraoxonase levels and activities by reducing oxidative stress (Aviram 2003). Antioxidants significantly reduce the content of lipoprotein-related lipid peroxides and protect PON1 activities (Aviram et al. 1999). Dietary antioxidant can also effect the gene expression of paraoxonases (Aviram 2003).

Lipoic acid is found in very small amounts in mammalian tissues, not in free form but bound to particular dehydrogenase as a cofactor (Golbidi et al. 2011). Because it is able to quench reactive oxygen species, chelate metal ions and reduce the oxidized forms of other antioxidants, it is known as an important antioxidant (Packer et al. 2001). There is an increasing interest in using lipoic acid as a potent antioxidant for the treatment of diabetes and cardiovascular diseases (Goraca et al. 2011).

Only a few studies have focused on the effect of lipoic acid on serum PON1 enzyme activities (Yi and Maeda 2006; Gavrovskaia et al. 2008). We previously showed for the first time that lipoic acid causes an increase in serum PON1 and PON3 protein levels and activities in diabetic rats (Ozgun et al. 2016). Despite several previous reports on the beneficial effects

of lipoic acid on cardiovascular diseases, (Goraca et al. 2011; Rochette et al. 2013; Koufaki 2014) no study has ever been performed to specifically examine the effects of lipoic acid on PON1 and PON3 gene and protein expression and activities in liver. The present study was designed to investigate the effects of therapeutic doses of lipoic acid on liver PON1 and PON3 gene and protein expression and arylesterase activity in vitro.

Lipoic acid was non-toxic and caused a significant increase on cell viability in HepG2 cells at the dose of 10, 40 and 200 μM. The present study showed that 200 μM lipoic acid has an increasing effect on PON1 protein levels and arylesterase activity while it has a decreasing effect on PON1 mRNA expression in HepG2 cells. The fold increases in PON1 protein levels and arylesterase activities were similar. PON1 is regulated by oxidative stress and has buffer action (Précourt et al. 2011). While PON1 protects LDL from oxidation (Aviram and Rosenblat 2004), it becomes inactivated by oxidized lipids (Reddy et al. 2001). Trudel et al. (Trudel et al. 2005) reported that lipid peroxidation induced by iron-ascorbic acid causes a decrease on the protein mass of PON1 in liver. In our previous study, we showed that lipoic acid prevents an increase in liver lipid peroxidation in hepatic ischemia/reperfusion injury (Ozgun et al. 2014).

Lipoic acid may prevent PON1 degradation and therefore repress PON1 mRNA expression by reducing oxidative stress. Our findings indicating that there is an increased in PON1 protein levels and arylesterase activity support to above idea.

PON3 is not a well-known protein such as PON1. It was reported that PON3 is not regulated by oxidized lipids (Reddy et al. 2001). In our study 200 μM lipoic acid caused a significant increase on both PON3 protein level and mRNA expression. This finding indicates that lipoic acid induces PON3 gene expression and supports our previous study which showed that lipoic acid increases serum PON3 protein levels and the increase percent of serum PON3

protein is higher than that of serum PON1 protein in lipoic acid-treated diabetic rats (Ozgun et al. 2016).

Our study showed that, although lipoic acid up-regulates PON3 but down-regulates PON1 mRNA expression, it increases both PON1 and PON3 protein levels and arylesterase activity in HepG2 cells. We can report that lipoic acid may be useful for preventing atherosclerosis at therapeutic doses.

Lipoic acid cause an increase in protein levels of PON3 by the induction of PON3 mRNA expression. On the other hand, an increase in the protein levels of PON1 caused by lipoic acid may be resulted from a decrease in the degradation of PON1 because of a reduction in oxidative stress.

Our findings indicating that lipoic acid induces PON3 mRNA expression but represses PON1 mRNA expression, point out that the regulation of PON1 gene expression may be differ from that of PON3 gene expression. Further research should address the precise regulation mechanisms of PON1 and PON3 gene expression in liver.

The limitation of this paper is not to able to show lactonase which is the main activity of PON3 enzyme. Although we attempted to measure paraoxonase activity using paraoxon according to Gan et al. (Gan et al. 1991) and lactonase activity using dihydrocoumarin and decanolactone according to Draganov et al. (Draganov et al. 2000), we were not able to find any results because the activities were below or very close to detection limits of our assays.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1

Effect of lipoic acid treatment for 72 hours on the viability in HepG2 cells.

Results are expressed as mean ± SD for experimental groups (n=12 for all groups).

Effects of lipoic acid was analyzed by one-way analysis of variance.

Tukey test was performed for multiple comparisons between experimental groups.

a: P<0.001, compared with control.

b: P <0.001, compared with 10 µM lipoic acid-treated cells.

Figure 2

Effect of lipoic acid treatment for 72 hours on PON1 and PON3 **A)** protein and **B)** mRNA levels in HepG2 cells.

Results are expressed as mean ± standard deviation for experimental groups (n=4 for protein and n=6 for mRNA levels for all groups).

Effects of lipoic acid was analyzed by one-way analysis of variance.

Tukey test was performed for multiple comparisons between experimental groups for PON1 protein levels.

Tamhane test was performed for multiple comparisons between experimental groups for PON1 mRNA levels and PON3 protein and mRNA levels.

a: P <0.001, compared with control

b: P <0.05, compared with control

c: P <0.05, compared with 10 µM lipoic acid-treated cells

d: P <0.05, compared with 40 µM lipoic acid-treated cells

Figure 3

Effect of lipoic acid treatment for 72 hours on arylesterase activity in HepG2 cells.

Results are expressed as mean ± standard deviation for experimental groups (n=9 for all groups).

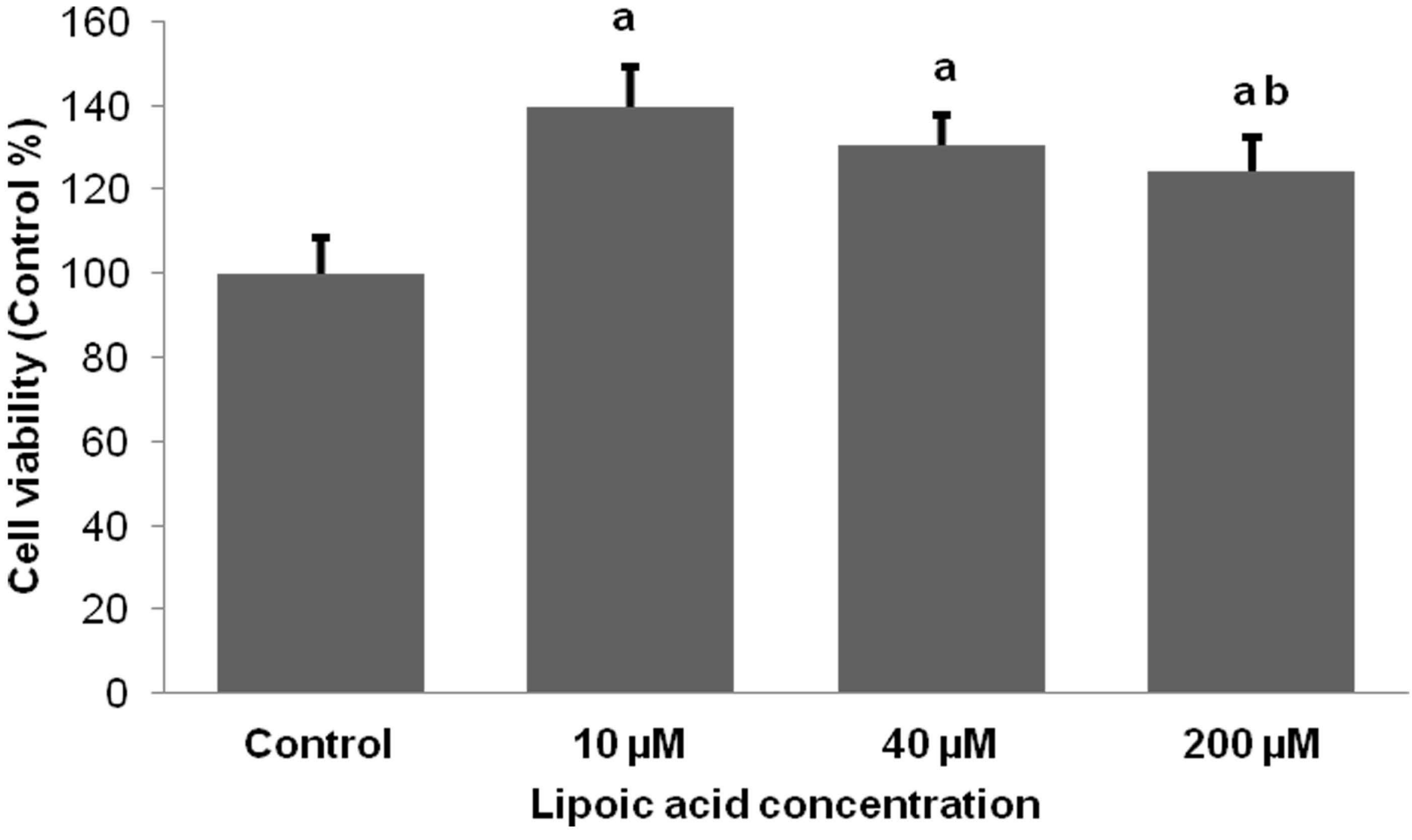
Effects of lipoic acid was analyzed by one-way analysis of variance.

Tukey test was performed for multiple comparisons between experimental groups.

a: P <0.001, compared with control

b: P <0.001, compared with 10 µM lipoic acid-treated cells

c: P <0.001, compared with 40 µM lipoic acid-treated cells


160
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Cell viability (Control %)
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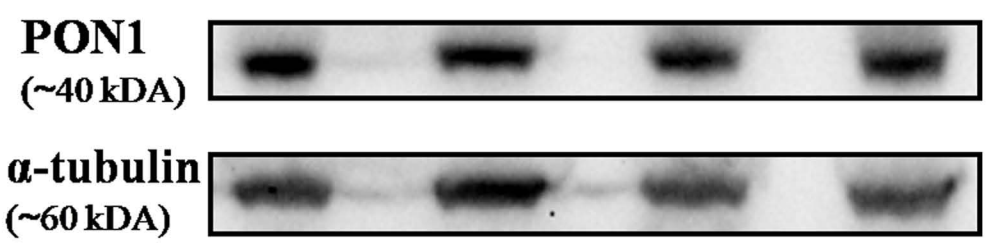
10 µM
40 µM
200 µM
Lipoic acid concentration

A)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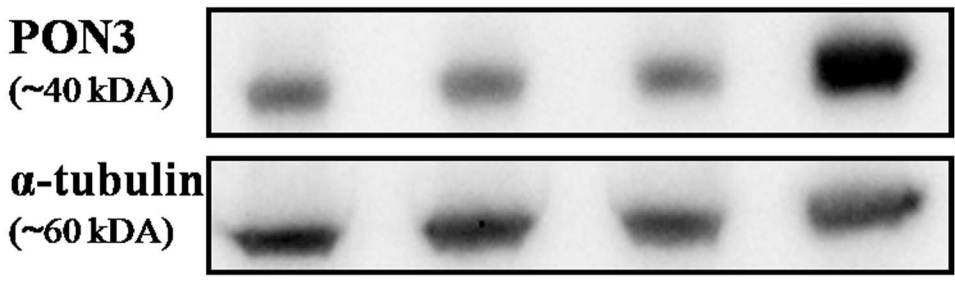
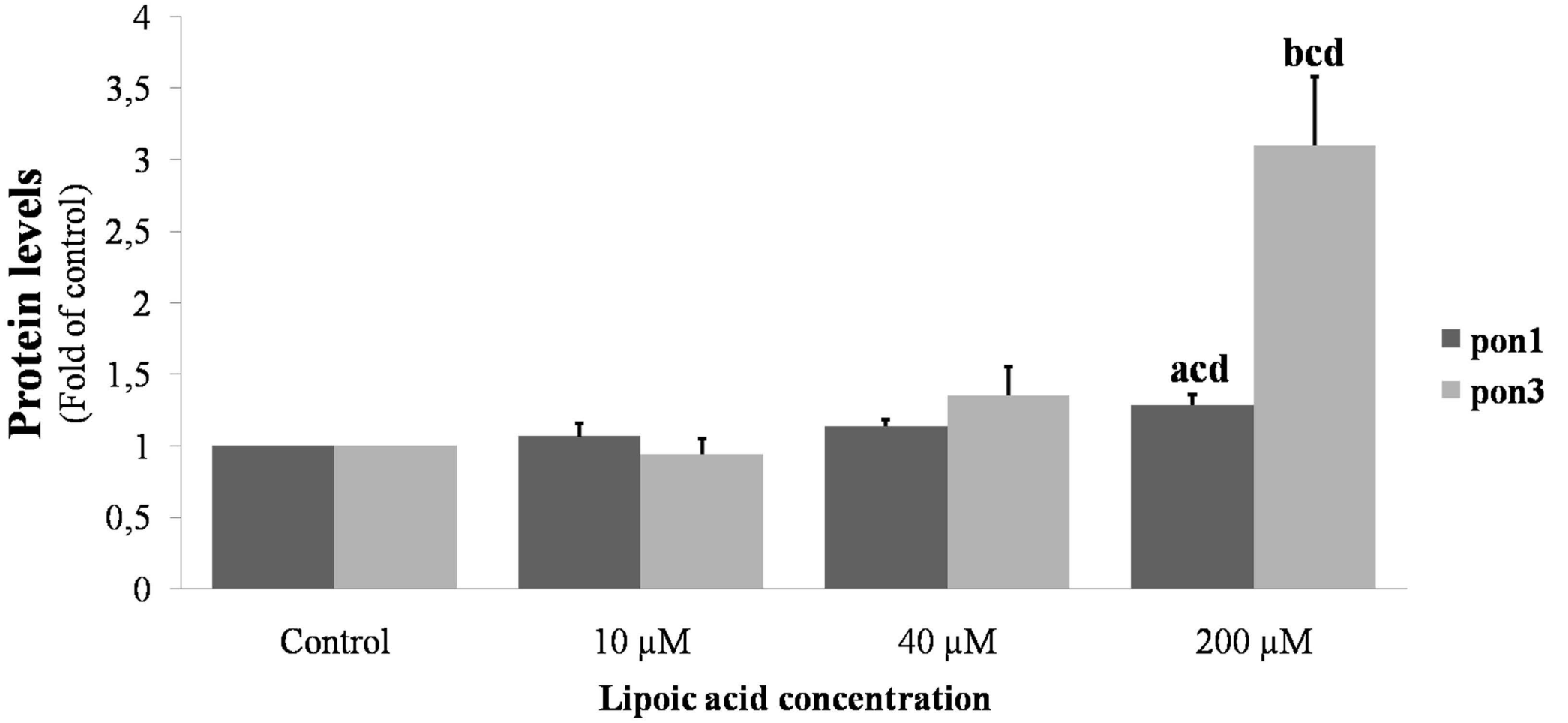
α-tubulin
(~60 kDA)


PON3
(~40 kDA)
α-tubulin
(~60 kDA)


Protein levels
(Fold of control)
4
3,5
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2
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Control
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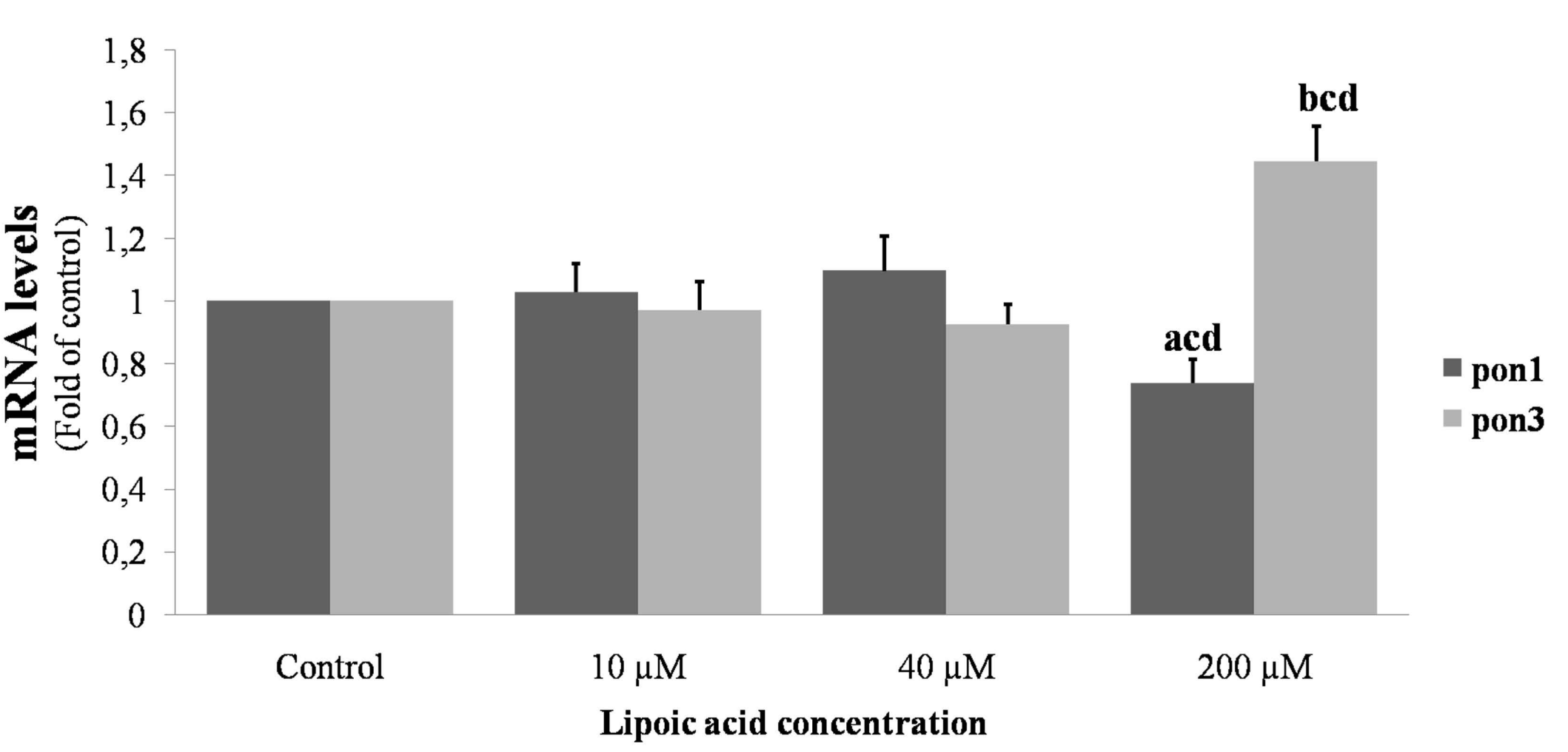
200 μM
Lipoic acid concentration
acd
bcd
pon1
pon3

B)


mRNA levels
(Fold of control)
1,8
1,6
1,4
1,2
1
0,8
0,6
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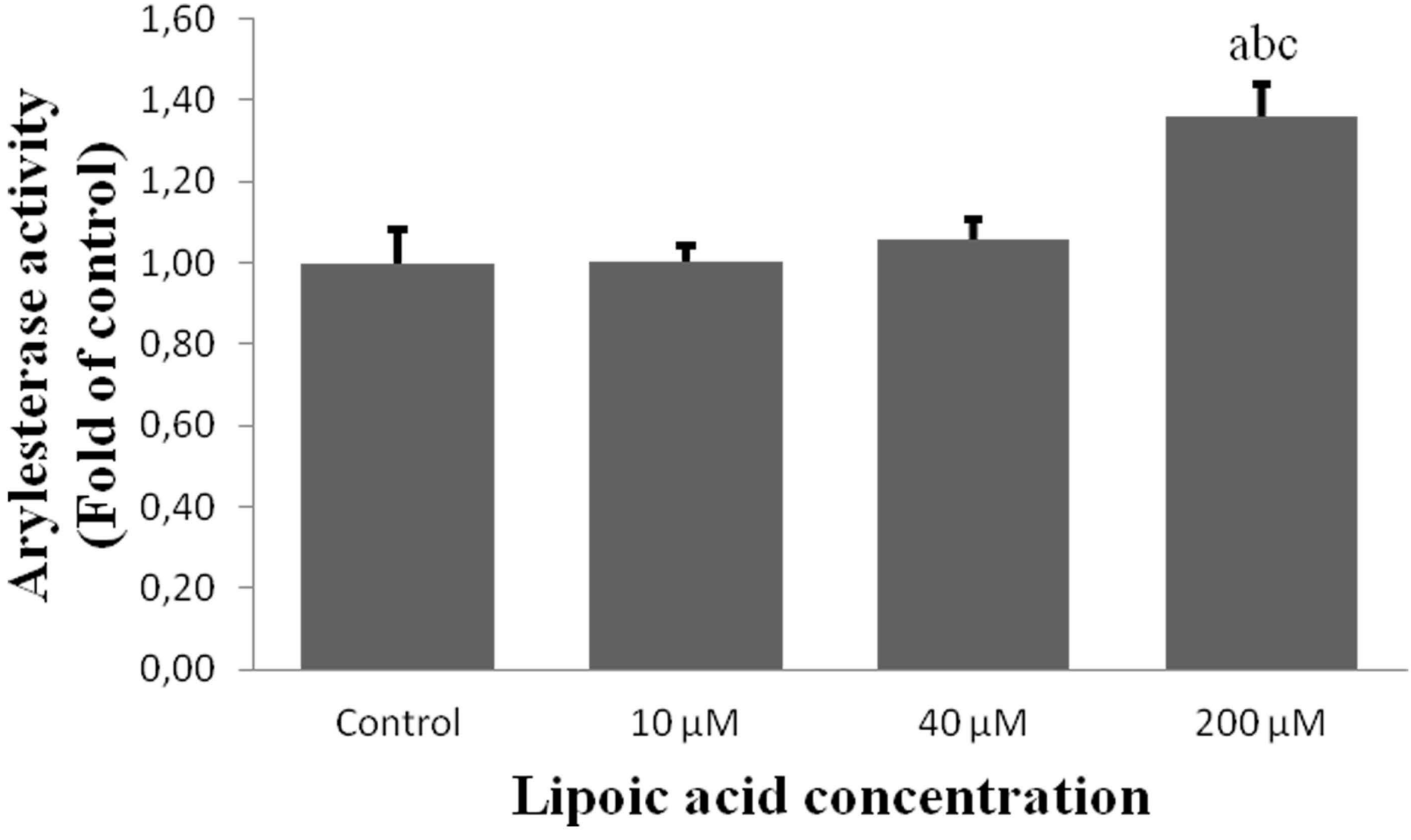
0,2
0
Control
10 μM
40 μM
200 μM
Lipoic acid concentration
acd
bcd
pon1
pon3


1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Arylesterase activity
(Fold of control)
abc
Control
10 µM
40 µM
200 µM
Lipoic acid concentration