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Cobalt-Induced Cytotoxicity in *E. coli* (DH5α) is Mediated by Modulation of Cellular Phospholipid Composition

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Abstract: The mechanism of Co^{2+}-induced cytotoxicity in bacteria was evaluated using *E. coli* (DH5α) as a model system. In *E. coli* (DH5α), Co^{2+}-induced cytotoxicity was characterized by a dose-dependent (i) reduction of growth rate, (ii) decrease in catalase activity and (iii) decrease in cytosolic free iron content, showing that Co^{2+}-induced cytotoxicity enhances oxidative stress in *E. coli*. Co^{2+} induced lipotoxicity in *E. coli* (DH5α) as indicated by a dose-dependent enhancement of total cellular phospholipid content and increased lipid peroxidation. Lipid peroxidation was mediated through formation of conjugated diene and lipid hydoperoxides folds. A quantitative analysis of total phospholipids using 2D-TLC showed that Co^{2+}-induced toxicity led to modulation of cellular phospholipid (PL) content in *E. coli* (DH5α). Sum total of phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) (PE+PG) content was increased by 15 % that was accompanied by ~2 fold increase in cardiolipin (CL) content. As CL is known to be augmented in multiple growth inhibitory conditions, we hypothesize that enhanced CL content is a regulatory mechanism of *E. coli* to survive Co^{2+}-induced cytotoxicity.

Key words: Phospholipid, heavy metal, lipotoxicity, cardiolipin, ROS, catalase, peroxidation, lipid hydroperoxide, conjugated diene, membrane fluidity.

Abbreviations

BSA, bovine serum albumin; CL, cardiolipin, IDR; iron-detection reagent, IRR, iron releasing reagent; PC, Phosphatidyl choline; PE, Phosphatidyl ethanolamine; PG, phosphatidyl glycerol; CL, cardiolipin; PL, phospholipid; PM, plasma membrane; ROS, reactive oxygen species, PUFA, polyunsaturated fatty acid; 2D-TLC, two dimensional thin layer chromatography; $O_2^{\bullet-}$, superoxide ion; $OH^{\bullet-}$, hydroxyl ion; SOD, superoxide dismutase.

Introduction

Cobalt (Co), a redox-active transition metal is a cofactor in multiple enzymes (e.g. vitamin B_{12} requiring enzymes) and proteins (e.g. RcnA and RcnB) contains Co as essential cofactor [1]. Co and its derivatives are toxic to all life forms due to its non-biodegradability, bio-accumulation and ability to interfere with bio-molecules [2-3]. Co-induced toxicity in human is known to affect thyroid, lungs, skin and immunosystem that results in multiple disorders such as goiter, asthma (hard-metal disease), dermatitis, lethal cardiomyopathy and lung cancer [4]. Co enters into human body primarily through contaminated water, consumption of vegetables and fishes grown in Co-enriched environment, co-containing body implants (e.g. hip-implants) and Co-containing nanoparticles used as medicine [2]. Hence, removal of Co^{2+} from

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water, air and soil is a challenging environmental problem.

Removal of Co from water using chemical methods (e.g. chelators) is expensive and produces toxic byproducts [5-6]. Hence, it is essential to develop effective and eco-friendly bio-remediation technologies for removal of Co from contaminated water. Genetically modified *E. coli* with enhanced Co^{2+} tolerance is now used to remove Co from the environment [7]. However, the detailed mechanism of Co toxicity, tolerance and detoxification in bacteria remains unclear.

Genotoxic and carcinogenic effects of Co and its compounds are reported by many investigators [2]. However, the mechanism of lipid-mediated cytotoxicity remains unclear. Lipids are prone to oxidative damage by reactive oxygen species (ROS) [8]. Phospholipids (PLs), the predominant class of lipids in *E. coli*, constitute ~89 % of total lipids. Phosphatidyl ethanolamine (PE), the predominant PL in *E. coli*, constitutes 69 % of total PL, 19 % being phosphatidyl glycerol (PG) and 6.5 % is cardiolipin (CL) [9]. Rest of the PL (including unidentified PLs) constitutes ~6 % of the total PL. However, variation in cellular PL composition is observed in response to extreme conditions such as osmotic stress, metal ion toxicity and growth phase of *E. coli* [10-11]. CL, the most variable PL in *E. coli* is known to be altered in multiple growth inhibitory conditions such as the stationary phase, extreme pH and ionic strength etc [11].

In the present work, we used *E. coli* (DH5α) as a model system to investigate the effect of Co^{2+} on cellular lipid composition and lipid-mediated cellular responses in bacteria.

Materials and methods

Materials and reagents

E. coli (DH5α) was a gift from Mr. R.N. Munda of department of Biotechnology, North Orissa University. PL standards: PC, PE, PG and CL were obtained from Sigma (India). Lysozyme, bovine serum albumin (BSA), Triton-X-100, $FeCl_3$, Ferrozine, Neucoproine, ammonium acetate, ascorbic acid, ammonium molybdate, potassium permanganate, sodium hydroxide, sodium chloride, Sodium carbonate, sodium potassium tartarate, copper sulphate, Tris Buffer and components of LB media (Yeast extract, Tryptone and Agar) were obtained from Himedia (India). $CoCl_2$, Silica gel GF 254, Follin's reagent and Iodine balls were purchased from Merck (India). Butylated Hydroxy Toluene (BHT) was obtained from Sisco Research Laboratory (SRL). All organic solvents (Chloroform, Methanol, Acetic acid, and Ammonia solution (25 %), Acetone), Inorganic acids (Hydrochloric acid and Perchloric acid) were purchased from Merck (India).

Growth of *E. coli* (DH5α) and stimulation of Co^{2+}-induced cytotoxicity

E. coli (DH5α) was grown in LB medium or LB medium containing different concentration of Co^{2+} by inoculating 100 ml broth in 250 ml Erlenmeyer flask with 1 ml seed culture grown for 12 h at 25°C and 200 rpm. The cells were grown for 16h at 25°C and 200 rpm.

Collection and re-suspension of cells

Cells were collected at 16 h of growth (early saturation phase) by centrifugation at 5000 × g for 7 min at 25°C and re-suspended at 4 10 mg/ml total protein (cells from 10 ml saturated culture broth was re-suspended to 1 ml) in re-suspension buffer (50 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 5 m*M* BHT) and used immediately for further experiments.

Estimation of protein

Briefly, the cells were lysed with lysozyme and triton X-100 in re-suspension buffer. The whole cell lysate was used to estimate protein by Lowry's method [12]. The absorbance of the samples was measured using a Systronics double beam spectrophotometer (Model 2202, Japan) at 750 nm. Protein concentration was calculated from the standard curve using known concentration of BSA.

Extraction of total lipid from *E. coli* (DH5α)

Total lipid from *E. coli* (DH5α) was extracted using aqueous two phase method described by Bligh and Dyer [13]. Briefly, total lipid from 0.5 mg cells in 0.5 ml 20 mM Tris-HCl, pH 8.0 was extracted in aqueous two phase method made from methanol, chloroform and water. The bottom layer was collected, dried in rotary evaporator. The

dried samples were dissolved at approximately 1 µmol/ml PL in $CHCl_3$ and stored at -20°C for further analysis.

Quantification of phospholipid in total lipid extract

PL content in total lipid extract was quantified by phosphate assay described by Fiske and Subarrow [14]. Briefly, 100 µl of total lipid extract in chloroform was completely dried and the lipids were hydrolyzed by heating in 70 % perchloric acid at 160°C. PO_4 thus released was quantified with method of PO_4 estimation using ascorbic acid and ammonium molybdate.

Quantification of conjugated-diene content from total lipid extract

Diene conjugation of total lipid extract was quantified following the procedures of Howlett and Avery with modification [15]. Briefly, the A_{230}/A_{274} was calculated for total lipid extract containing 1 µM mol of PLs in cyclohexane.

Quantification of LHP content from total Lipid extract

Lipid hydroperoxide (LHP) formed in *E. coli* (DH5α) due to Co^{2+}-cytotoxicity was quantified by Fukuzawa et al with modification [16]. Briefly, 3.0 µmols PL from total extract was used to form vesicles. Oxidized lipids in the vesicles that were used for conversion of Fe^{2+} to Fe^{3+} that is detected by xylenol orange (XO).

Two dimensional thin layer chromatography (2D-TLC)

2D-TLC of total lipid extract from *E. coli* (DH5α) was performed using methods described previously [17]. Lipid extract containing 500 nmol PL in 50 µl $CHCl_3$ was applied on a 20 cm × 20 cm × 0.0002 cm silica gel GF 254 TLC plate. The sample was first developed in solvent I and II sequentially and the spots were detected using iodine vapour. The spots were identified using PL standards developed in the same condition.

Quantification of phospholipids from spots on TLC plates

Known weight of silica from the spots identified on TLC plates was scrapped into 12 × 125 mm assay tubes. PL adsorbed to silica powder was hydrolyzed to release phosphate by heating with perchloric acid at 160°C for 2 h. Phosphate thus released was quantified by the method of Fiske and Subarrow [14].

Quantification of cytosolic iron content

Cellular iron content was quantified using methods of Reimer *et al*. [18]. Briefly, the total iron content from 1mg resuspended cells was released by iron releasing reagent (IRR) and detected by iron detection reagent (IDR). Absorbance of the purple color developed was measured at 550 nm and total iron was calculated from standard curve of $FeCl_3$ (3 nmol/µl).

Catalase assay

Catalase assay was performed on freshly collected cells using the methods of Beers and Sizer [19]. Briefly, catalase activity from lysate of 1 mg cells was quantified by measuring the time dependent depletion of H_2O_2 concentration A_{240} in 3 ml assay mix (6.66 m*M* H_2O_2, 50 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, and 0.3 ml cell lysate). Data obtained were analyzed by fitting them to Michelis-Menten equation using Graph-pad prism.

Statistical analysis

The statistical significance between the treatments was evaluated by Student's "t" test for the biochemical studies and one-way ANOVA test was used to compare the results whenever more than two experimental groups were compared. All the data are expressed as Mean ± SEM (standard error of the mean).

Results

Co^{2+}-induced toxicity reduces growth rate, decreases catalase activity and depletes cytosolic iron content in E. coli (DH5α).

Co^{2+}-induced cytotoxicity in *E. coli* (DH5α) reduced growth rate, enhanced oxidative stress and depleted cytosolic iron content (Figure 1 A-D). No growth reduction was observed up to 1 mM $CoCl_2$ and a sharp reduction in growth rate was observed at 2.5 mM. However, growth was abol-

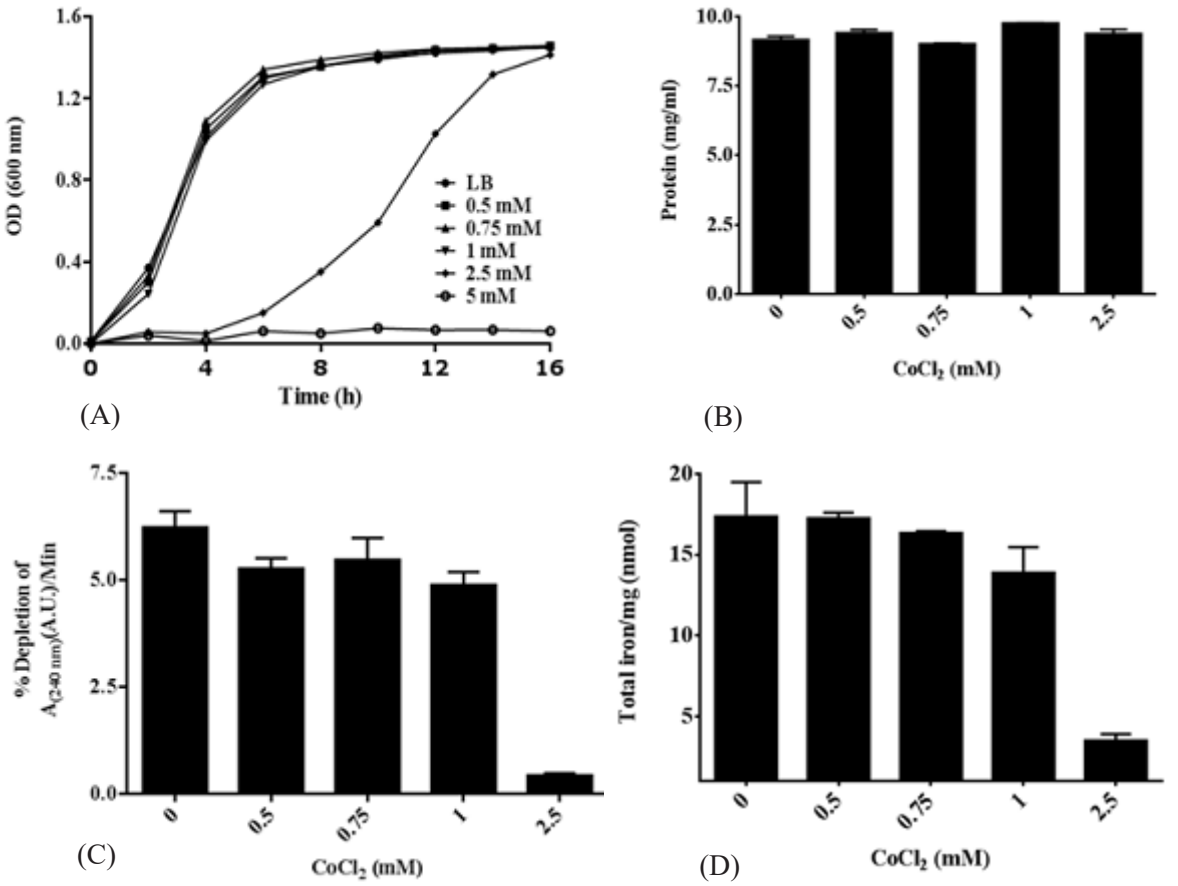

Figure 1. Co^{2+}-induced toxicity in *E. coli* (DH5α). (A) Growth curve of the bacterium grown in LB containing different concentration of Co^{2+} as indicated on the right of the figure. The figure is the representative of three independent sets of experiments. (B) Total protein of cells collected at early saturation phase (16th h) of growth and quantified as described in methods. (C) Effect of Co^{2+} on catalase activity. Rate of depletion of A_{240} was analyzed using Graph-pad prism after fitting the curves in to Michelis-Menten equation. (D) Cytosolic iron content of cells grown in LB containing different concentration of Co^{2+}. The graphs presented here show the mean ± SEM (n=3)

ished at 5 mM $CoCl_2$. Co^{2+}-induced growth inhibition was due to prolonged lag phase and log phases at 2.5 mM $CoCl_2$ compared to non-treated control (Figure 1A). Cells grown in LB containing up to 2.5 mM Co^{2+} were saturated at ~16 h compared to 8 h saturation time of control cells. However, $CoCl_2$ didn't affect the total protein content of cells at saturation (Figure 1B).

As oxidative stress induction is a primary mechanism of metal-induced cytotoxicity and catalase is the central oxidative stress regulatory enzyme, we quantified the catalase activity in the cells. We observed a slow, dose dependent depletion of catalase activity up to 1 mM $CoCl_2$. However, 2.5 mM $CoCl_2$ almost nullified the catalase activity (Figure 1C). Depletion of catalase activity in response to increasing concentration of $CoCl_2$ shows enhanced formation of cytosolic ROS content in *E. coli* (DH5α).

As oxidative stress leads to alteration of cytosolic iron homeostasis, we analyzed the cytosolic iron content of *E. coli* (DH5α) grown in LB containing increasing doses of Co^{2+}. Our results show that *E. coli* (DH5α) grown in LB possess 15-20 nmol of iron/mg of protein (Figure 1D). Intracellular iron is slowly depleted (by 15 %) up to 1 mM of Co^{2+} followed by a sharp depletion (by 80%) at 2.5 mM Co^{2+}. These results show that $CoCl_2$-induced toxicity leads to loss of cytosolic iron content.

Lipotoxic effects of Co^{2+} on *E. coli* (DH5α)

As PL constitutes ~90 % of the total lipid in *E. coli* that is altered in multiple growth inhibitory conditions such as heavy metal toxicity, we analyzed the effect of Co-toxicity in *E. coli* by quantifying total PL content and lipid peroxidation (Figure 2). Our results show that *E. coli* possess ~130 nmol/mg protein (Figure 2A). $CoCl_2$ led to a dose-dependent depletion of total cellular PL content, leading to 25 % depletion of total cellular PL at 2.5 mM. These results show that Co^{2+}-induced toxicity affects PL biosynthesis in *E. coli* (DH5α).

Lipid peroxidation in *E. coli* was quantified by measuring the level of diene conjugation and LHP content of total lipid extract. Co^{2+} up to 2.5 mM showed no significant alteration in conjugated diene content (Figure 2B). However, we observed a dose-dependent increase in LHP content (Figure 2C). A 3.5 fold augmentation in LHP content was observed in cells grown at 2.5 mM $CoCl_2$. These results indicate that Co^{2+} induced toxicity leads to a faster oxidation of cellular lipids, quickly converting conjugated dienes to terminal products of lipid peroxidation (LHP), hence, reducing the probability of observing conjugated diene in the saturation phase (e.g. 16th h).

Co^{2+}-induced cytotoxicity alters PL composition of *E. coli* (DH5α)

As alteration of cellular PL composition is one of the bacterial regulatory mechanism to survive heavy metal-induced toxicity, we investigated the Co^{2+}-induced change in cellular PL composition

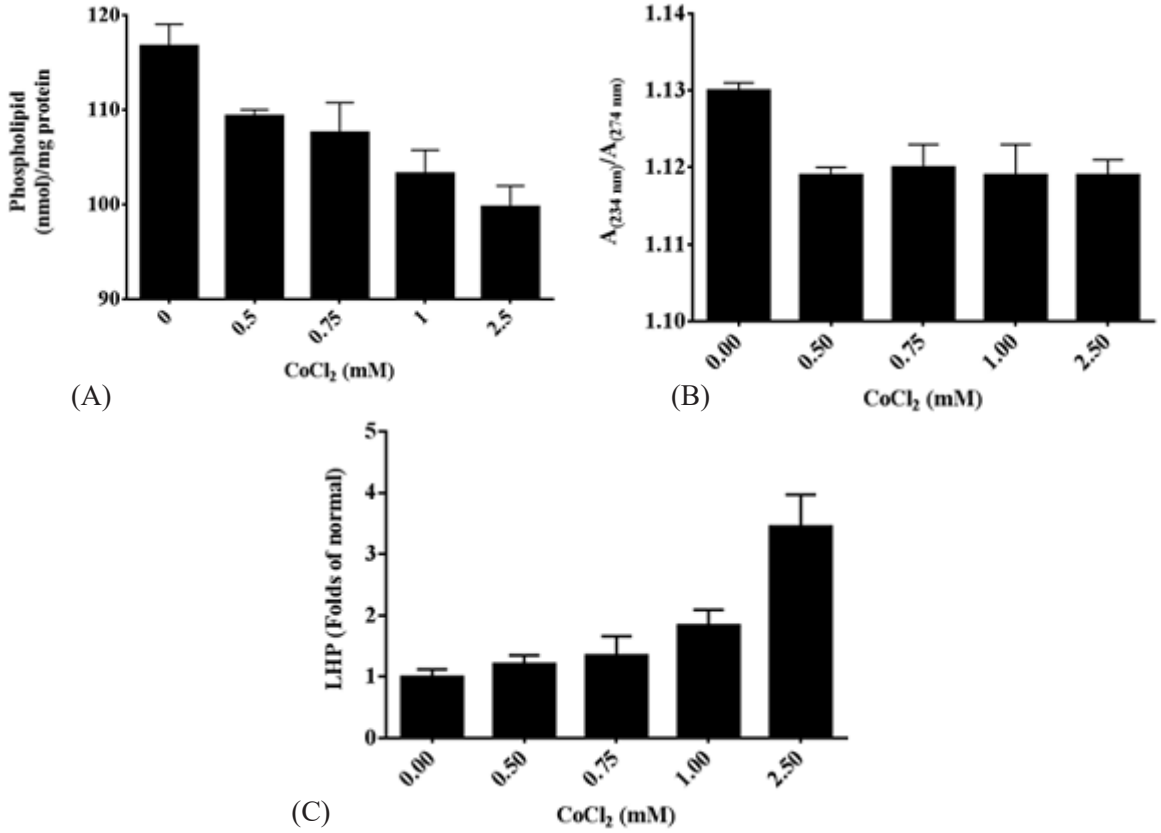

Figure 2. Analysis of the effect of Co^{2+} on lipids of *E. coli* (DH5α) (A) Total phospholipid (PL) content of the samples grown in LB containing increasing concentration of Co^{2+} (B) Analysis of the relative amount of conjugated diene content (A_{234nm}/A_{274nm}) of total lipids extracted from the cells grown in LB containing different concentration of Co^{2+}. (C) Relative amounts of lipid hydroperoxides (LPH) formed in *E. coli* (DH5α) at different concentration of Co^{2+}. All the samples contained 1 µmol PLs. The graphs presented here show the mean ± SEM (n=3)

(Figure 3). 2D-TLC of total lipid showed three major PLs that constituted up to 98% of the total PL (Figure 3A). In control cells, PE and PG together constituted ~92 % and CL constituted ~7 % of total PL (Figure 3B). Increasing doses of Co^{2+} up to 2.5 mM led to augmentation of CL content up to ~14 % (2 fold). Increase in CL content was found to be saturable, implying an acute regulation of CL content in *E. coli* (DH5α). PE+PG content was depleted by 10-15 %, show-

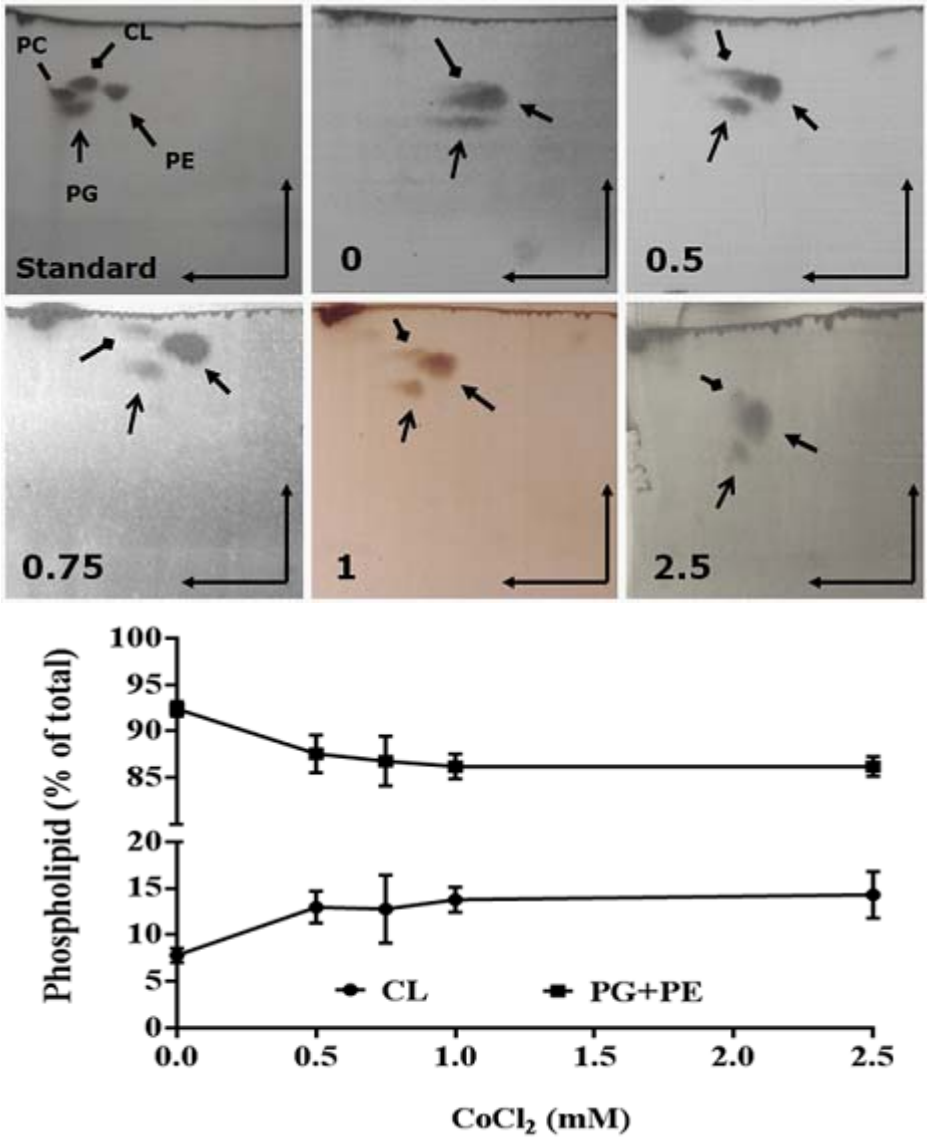

Figure 3. Analysis of PL composition of *E. coli* (DH5α) by two-dimensional TLC. The plates contain three major PLs, PE (triangular head), CL (diamond head) and PG (normal arrow head). The spots were identified using known PL standards (Sigma) developed in the same condition as those of unknown lipid mixture. Other minor spots visible on the plates are not identified. The horizontal and vertical arrows at the bottom-right corner of each plate show the first and second dimension of development of TLC (in solvent I and II) respectively with the origin showing the point of application of samples. The numbers at the bottom-left corner of the plates show the concentration of Co^{2+} in the growth medium. The figure shows the representative of four independent sets of experiments (B) Quantitative analysis Co^{2+}-induced alteration in PL content. The quantification of PLs was performed by phosphate estimation. The graphs presented here show the mean ± SEM (n=4).

ing that augmentation of CL content is compensated by depletion of PE+PG content. Our results show that Co^{2+}-induced oxidative stress leads to reorganization of PL in bacteria.

Discussion

In the present investigation, we used *E. coli* (DH5α) as a prokaryotic model system to investigate the Co^{2+}-induced cytotoxicity in bacteria. Co at 400 μM is optimal for growth and over expression of proteins in *E. coli* [20]. MIC of Co for *E. coli* (DH5α) was proposed to be >1 mM for each strain of *E. coli* [21]. Our investigation supports this finding as $CoCl_2$ at 1 mM didn't inhibit the growth *E. coli* (DH5α). $CoCl_2$ at 2.5 mM reduced the growth rate by ~50 % of the normal growth rate however, without affecting total cellular protein content at saturation (Figure 1A and B).

We observed a slow, dose dependent depletion of catalase activity up to 1 mM $CoCl_2$. However, 2.5 mM $CoCl_2$ almost nullified the catalase activity (Figure 1C). Similar findings were observed rat liver and in livers of Lewis lung carcinoma-bearing mice in response to $CoCl_2$ [22-23]. Depleted catalase activity was also observed in toxicity induced by other heavy metals such as Cd, Pb, and Hg toxicity in different cell types [24-25]. Activity of catalase is known to be inhibited by H_2O_2 [26]. Hence, Co^{2+}-induced depletion of catalase activity in *E. coli* (DH5α) may be due to increased production of H_2O_2, non-specific binding of Co^{2+} to catalase, a cellular regulatory mechanism to minimize cytosolic ROS production or a concerted mechanism of all the three processes. Oxidative stress has a profound effect on free cytosolic iron [27]. Our results show that $CoCl_2$ up to 1 mM of Co^{2+} leads to depletion of cytosolic iron content by 15 %, followed by a sharp depletion (by 80 %) at 2.5 mM Co^{2+} (Figure 1D). Similar effect on cytosolic iron content was observed in yeast [28]. Co^{2+} is known to displace Fe from FeS clusters of multiple proteins 100 μM [3, 21]. Hence, depletion of cytosolic iron may due to the loss of protein-bound Fe stimulated by Co toxicity.

A dose-dependent depletion of total PL content was observed in presence of $CoCl_2$. 2.5 mM Co^{2+} led to 25 % depletion of total PL (Figure 2A). These results show that Co^{2+}-induced toxicity leads to reduction in PL biosynthesis in *E. coli* (DH5α) that supports the previous findings [29]. Studies on analysis of PL-biosynthesis in yeast suggested iron to be an essential component of PL biosynthetic enzymes (e.g. Sur2, Ole1, and Scs7) [30-32]. As Co^{2+} toxicity is known to disrupt FeS clusters of multiple proteins and enzymes, it is plausible that Co^{2+}-induced inhibition in activity of enzymes of PL biosynthetic pathways [3].

Co^{2+} induced peroxidation of cellular lipids mainly, through formation of LHP. We observed a slow increase in LHP content up to 1 mM $CoCl_2$ that rapidly increased at 2.5 mM. This rapid increase in cytosolic LHP that may be explained by assuming a synergistic action of Co^{2+}, Co^{2+}-induced ROS production and oxidized lipid intermediates on cellular lipids (Figure 2 B-C). Analysis of total cellular PL composition showed a two folds enhancement in CL content accompanied by 15 % depletion of other two PLs (PE+PG) (Figure 3B). Co^{2+}-induced lipid peroxidation leads to decreased fluidity and increased permeability PM [33]. For instance, Co^{2+}-induced cytotoxicity is known to enhance membrane fluidity by altering the fatty acyl composition and enhancing unsaturation of fatty acids in plasma membrane of *S. chartarum* [29]. Regulation of membrane lipid composition is proposed to be a compensatory mechanism for maintenance of optimal membrane packing and fluidity under conditions of oxidative stress [34]. Further, oxidative stress induced-disruption in iron homeostasis is partially due to loss of CL from inner bacterial and mitochondrial membrane. As CL is essential for biogenesis of proteins containing FeS cluster(s), maintenance of cellular iron homeostasis, increase in CL content might be a bacterial adaptive mechanism to compensate for Co^{2+}-induced damage to PM [35].

Conclusion

In conclusion, the present investigation provides new insight into the mechanism of Co^{2+}-induced cytotoxicity in *E. coli* by enhancing oxidative stress, inhibiting catalase activity, decreasing cytosolic iron content, decreasing total PL content, increasing lipid peroxidation and increasing CL content. As Co-induced cytotoxicity is implicated

in multiple heavy metal mediated disorders in human (e.g. hard metal disease), our findings will lead to further research on membrane damage and membrane-based signaling mechanisms associated with Co-toxicity.

Conflict of interest statement

The authors of the present work declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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