Biochemical Characterization of H$_2$O$_2$-Induced Oxidative Stress in E.coli

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List of 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; PM: Plasma Membrane; ROS: Reactive Oxygen Species; PUFA: Polynsaturated Fatty Acid; 2D-TLC: Two Dimensional Thin Layer Chromatography; O$_2^-$: Superoxide Ion; OH$: Hydroxyl Ion; SOD: Superoxide Dismutase.

Introduction

Aerobic bacteria such as E. coli are subjected to a variety of extrinsic and intrinsic oxidative stress such as exposure to toxic chemicals, ionizing radiation, hyperbaric oxygen and incomplete reduction of O$_2$ during metabolism. H$_2$O$_2$ is also generated in cells as a by-product of water radiolysis after exposure to ionizing radiation. The consequent univalent reduction of molecular oxygen to water produces three active intermediates: superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$), collectively termed as reactive oxygen species (ROS) [1]. Reaction of H$_2$O$_2$ with transition metal ions like Fe$^{2+}$ and Cu$^{2+}$ accelerates oxidative damage of cellular constituents by producing reactive hydroxyl (OH$^-$) ions through Fenton reaction and Haber-Weiss reaction [2-4].

These ROS react with cellular components such as lipid, protein and nucleic acid that trigger a series of reactions culminating in cellular oxidative damage [5,6]. In bacteria, these detrimental consequences of oxidative damage can be lethal or mutagenic [7-11]. Increasing evidences suggest that in human, the cumulative damage caused by ROS contributes to numerous degenerative diseases associated with aging, such as atherosclerosis, rheumatoid arthritis and cancer [12].

The detailed mechanism of H$_2$O$_2$-induced cytotoxicity is not yet completely explored. In E. coli (DH5α), two pathways of H$_2$O$_2$-mediated cytotoxicity are proposed that are distinguishable by metabolic, kinetic and genetic criteria [13]. Mode one is characterized by a greater rate of killing exhibited by low (1-3 mM) concentration of H$_2$O$_2$. However, mode two is characterized by a broad shoulder of H$_2$O$_2$ that is exhibited by intermediate concentration (3-10 mM) of H$_2$O$_2$. While mode one killing appears to result from DNA damage, the detailed pathway of lethal cell damage has not been identified for mode two killing [13].

Additional possible targets of H$_2$O$_2$ remain to be investigated [10].

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Lipids are among the most vulnerable group of biomolecules that are prone to oxidative damage by ROS. PLs, the predominant class of lipids in E. coli constitutes ~89% of the cell envelope in Gram negative bacteria like E. coli. Phosphatidyl ethanolamine (PE) is the predominant PL that constitutes 69% of total PLs, 19% being phosphatidyl glycerol (PG) and 6.5% is cardiolipin (CL) [14]. Rest of the PL (including unidentified PLs) constitute ~6% of the total PL. Phosphatidyl choline (PC) and phosphatic acid (PA) constitute minor PLs in E. coli that are normally not detected on TLC [14]. However, variation in cellular PL composition is observed in response to extreme conditions such as high osmotic stress, heavy metal toxicity and growth phase of E. coli [15-18]. CL content of E. coli is known to be altered in multitudes of growth inhibitory conditions [19]. CL synthesis is upregulated in stationary phase, extreme pH and ionic strength etc. However, the effect of oxidative stress on CL content of bacteria has remained unexplored.

Recent investigation shows the importance of lipid-mediated regulatory pathways that control multiple cellular responses to extreme environmental conditions [20]. Alteration in CL composition affects lipid organization and lipid-protein interaction in plasma membrane of bacteria and inner mitochondrial membranes of eukaryotes [21,22]. Hence, cells might respond to oxidative stress by regulating CL composition in these membranes. However, the lipid-mediated cellular response to H$_2$O$_2$-induced oxidative stress remains to be understood. In the present work, we used E. coli (DH5α) as a model system to investigate the effect of H$_2$O$_2$-induced cytotoxicity on cellular lipid composition and lipid-mediated cellular responses to H$_2$O$_2$-induced toxicity.

**Materials and Methods**

**Materials**

E. coli (DH5α) was a gift from Dr. R.N. Munda from 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). H$_2$O$_2$, Silica gel GF 254, Folin’s reagent and iodine balls were purchased from Merck (India). Butylated Hydroxy Toluene (BHT) was obtained from Sisco Research Laboratory (SRL), India. All organic solvents (Chloroform, Methanol, Acetic acid, and Ammonia solution (25%), Acetone) were purchased from Merck (India). Inorganic acids: hydrochloric acid and Perchloric acid were purchased from Merck (India).

**Growth of E. coli (DH5α) and induction of H$_2$O$_2$-mediated toxicity**

E. coli (DH5α) was grown in LB or LB containing different concentration of H$_2$O$_2$ by inoculating 100 ml broth in 250 ml Erlenmeyer flask with 1 ml seed culture grown for 12h at 25°C and 200 rpm. The cells were grown for 16h at 25°C and 200 rpm.

**Collection and re-suspension of cells**

The 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 ~10 mg/ml total protein (cells from 10 ml saturated culture broth was re-suspended to 1 ml) in re-suspension buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM BHT) and used immediately for further experiments.

**Estimation of protein**

Total protein from E. coli (DH5α) was quantitated by Lowry’s method with modification [23]. Briefly, 16 µl of re-suspended cells was incubated with 10 µg lysozyme at 25°C for 30 min with intermittent mixing to lyse bacterial cell wall. Cell membrane was lysed by incubating with 1% Triton-X-100 at 25°C for 30 min with intermittent mixing. Whole cell lysate was mixed sequentially with Lowry’s reagent I [2% Na$_2$CO$_3$ in 0.2 N NaOH (48 parts), 1% sodium-potassium tartarate (1 part) and 0.5% copper sulfate (1 part) by volume followed by Lowry’s reagent II [Folin Ciocalteu reagent (1 part) + distilled water (1 part) by volume] in a final assay volume of 2.6 ml and incubated for 1h at 25°C. The assay mix was centrifuged at 5000 × g to settle down the white precipitate resulting from triton-X-100. Absorbance of the supernatant was measured in 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 earlier [24]. Briefly, 0.5 mg cell in 0.5 ml of 20 mM Tris-HCl, pH 8.0 was mixed with 1.9 ml CHCl$_3$-CH$_2$OH (1:2 v/v) followed by 0.625 ml CHCl$_3$. Aqueous and organic phases were separated by adding 0.625 ml H$_2$O. Cells were lysed and separated as aqueous and organic phases by rigorous mixing for 1 min and centrifuging at 3000 × g at 25°C using a table top Sorval (REMI, India). Lower phase was collected and upper phase including the protein ring was re-extracted with 0.625 ml CHCl$_3$. CHCl$_3$ was evaporated in the rotary evaporator overnight. The dried lipid samples were dissolved at approximately 1 µmol/ml PL in CHCl$_3$ and stored at -20°C for further analysis.

**Quantitation of phospholipid**

Phospholipid (PL) content in total lipid extract was quantitated by phosphate assay [25]. Briefly, 100 µl of total lipid extract in CHCl$_3$ was dried at 50°C, added with 325 µl of perchloric acid (16M) and incubated at 150°C for 2 h to hydrolyze the phosphate group. Phosphate thus released was added sequentially with 2.5% ammonium molybdate (0.25 ml) and 10% ascorbic acid (0.25 ml) that upon incubation at 100°C, yielded a blue colored ammonium-phosphomolybdate complex that absorbed at 797nm. Absorbance of the samples was measured using a Systronics double beam spectrophotometer (Model 2202, Japan) and compared with the standard curve obtained from KH$_2$PO$_4$ standard solution (1 nmol/µl) to calculate total PL content in the lipid extract.
Quantitation of conjugated-diene

Diene conjugation in total lipid extract was quantitated following the procedure of Howlett and Avery with modification [26]. Briefly, total lipid containing 1 µmol PL was completely dried and dissolved in 3ml cyclohexane. Absorbance of the samples was scanned from 200 nm to 400 nm. Two absorbance peaks were observed at 230 nm (peak1) \(A_{230\text{nm}}\) and 274 nm \(A_{274\text{nm}}\) (peak 2) respectively. The ratio \(A_{230\text{nm}}/A_{274\text{nm}}\) gives the relative amount of conjugated-dienes formed in the lipid.

Two dimensional thin layer chromatography (2D-TLC)

2D-TLC of total lipid extract was performed using methods described previously [27]. Lipid extract containing 500 nmol PL in 50µl CHCl\(_3\) was applied on a 20 cm × 20 cm × 0.002 cm silica gel GF 254 TLC plate. Samples were first developed in first dimension using solvent I (CHCl\(_3\):C\(_6\)OH:CH\(_3\)OH:H\(_2\)O in 50:20:10:10 by volume), air dried and developed in second dimension using solvent II (CHCl\(_3\):C\(_6\)OH:CH\(_3\)COOH:H\(_2\)O in 50:20:10:5 by volume). Plates were air dried and spots were detected using iodine vapor. PL was detected by the presence of phosphate in each spot from phosphate estimation and identified using PL standards developed in the same condition. Silica from each spot was scrapped into assay tubes for PL quantitation.

Quantitation of phospholipids from spots on TLC plates

PL content in spots obtained from TLC was quantitated by phosphate assay. Briefly, silica from the spots on plates was scrapped into 12 × 125 mm assay tubes and weighed. PL adsorbed to silica powder was hydrolyzed to release their phosphate by heating with 325 µl perchloric acid at 150°C for 2 h. Phosphate thus released was quantitated by method of Fiske and Subarrow [25]. PL in each spot on TLC plate was calculated by subtracting the error originated from silica using known weight of silica collected from places on TLC plates that were not stained.

Quantitation of total iron content

Cellular iron content was quantitated using method described previously [28]. Briefly, 1mg cells in 0.2 ml resuspension buffer was lysed in 0.8 ml of 10 mM HCl and neutralized with 1 ml of 50 mM NaOH. Bound iron was released by adding 1 ml iron releasing reagent (IRR) (2.25% KMnO\(_4\) in 0.7 M HCl) followed by incubation at 62°C for 2 h. The released iron was detected by 0.3 ml iron-detection reagent (IDR) (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5M ammonium acetate, and 1M ascorbic acid) followed by incubation at 25°C for 30 min to develop a purple colored complex with absorption maxima at 550 nm. Absorbance of the samples were measured using a Systronics double beam spectrophotometer (Model 2202, Japan) 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 method of Beers and Sizer [29]. Briefly, catalase activity was quantitated by measuring the time dependent depletion of H\(_2\)O\(_2\) as indicated by decrease of A\(_{240}\) in 3 ml assay mix (6.66 mM H\(_2\)O\(_2\), 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.3 ml cell lystate containing 2 mg total protein). Data obtained were analyzed by fitting them to Michaelis-Menten equation using Graph-Pad prism. Absorbance due to protein in the assay mix was corrected by subtracting A\(_{240}\) of the assay mix that didn’t contain H\(_2\)O\(_2\) for all samples. Percentage depletion of A\(_{240}\) per min was plotted against time and normalized against total protein content.

Results

H\(_2\)O\(_2\) in growth medium results in depletion of growth rate, reduction in catalase activity and regulation of cytosolic iron content in *E. coli* (DH5α).

H\(_2\)O\(_2\)-induced toxicity was characterized by a dose-dependent depletion of growth rate (Figure 1A). Reduction in growth rate of *E. coli* (DH5α) was due to proportionately prolonged lag phase induced by increasing doses of H\(_2\)O\(_2\) in growth medium. However, the cultures were saturated at ~16 h of growth as indicated by OD\(_{600}\) for all doses of H\(_2\)O\(_2\) and equal total protein content (Figure 1B). These results show that H\(_2\)O\(_2\) is not bacteriocidal rather bacteriostatic at moderate (1 to 10 mM) concentration. A time-dependent adaptation to H\(_2\)O\(_2\)-induced toxicity was observed in *E. coli* (DH5α) that was proportional to concentration of H\(_2\)O\(_2\) in the growth medium. An adaptive response to H\(_2\)O\(_2\)-induced cytotoxicity was characterized by depletion of catalase activity and regulation of cellular iron content. Catalase, the central enzyme that regulates intracellular level of H\(_2\)O\(_2\), depleted by ~75% and remained almost invariable at all concentrations of H\(_2\)O\(_2\) tested (1 mM to 10 mM) (Figure 1C). These results show that...
*E. coli* (DH5α) regulates the toxic level of cytosolic ROS content by reducing degradation of H$_2$O$_2$, that is the less toxic compared to O$_2$$^*$ and OH$^*$ [1]. Recent investigation shows that oxidative stress has a profound effect on cellular iron concentration [30]. Hence, we analyzed the intracellular iron content of *E. coli* (DH5α) grown in LB containing different concentration of H$_2$O$_2$. Our results show that *E. coli* (DH5α) grown in LB possess 30-40 nmol of iron/mg protein (Figure 1D). Intracellular iron is depleted at lower doses (1-2.5 mM) of H$_2$O$_2$ and increased at higher doses (5-10 mM) of H$_2$O$_2$. These results show that *E. coli* (DH5α) regulates intracellular iron content as an adaptive mechanism to survive the H$_2$O$_2$-induced toxicity.

**H$_2$O$_2$ increases lipid peroxidation through formation of conjugated dienes**

Diene conjugation is an initial step in the mechanism of lipid peroxidation. Extraction and quantitation of total lipid from *E. coli*

![Figure 1b](image)

**Figure 1b** Total cellular protein of cells collected at 16th h of growth and quantitated as described in methods.

![Figure 1c](image)

**Figure 1c** Effect of H$_2$O$_2$ on catalase activity. The rate of depletion of H$_2$O$_2$ as indicated by A$_{240}$ was analyzed using GraphPad prism after fitting the curves in to Michalis-Menten equation. Concentration of H$_2$O$_2$ in the growth medium for each curve is shown to the lower-right corner of the figure. The inset (bar diagram) shows the relative amount of catalase activity of *E. coli* (DH5α) at different doses of H$_2$O$_2$.

![Figure 1d](image)

**Figure 1d** Iron content of cells grown in LB containing different concentration of H$_2$O$_2$.

![Figure 2a](image)

**Figure 2a** Analysis of the effect of H$_2$O$_2$ on phospholipids (PL) of *E. coli*(DH5α). Total PL content of samples grown in LB containing different concentration of H$_2$O$_2$.

![Figure 2b](image)

**Figure 2b** Analysis of the relative amount of conjugated diene content (A$_{228}$ nm/A$_{274}$ nm) of total lipids extracted from the cells grown in LB containing different concentration of H$_2$O$_2$. All the samples contained 1 µmol PLs.
H2O2 is consistently generated in almost all cell types by several enzymes (including superoxide dismutase, glucose oxidase, and monoamine oxidase) that is detrimental to the cells and must be degraded to prevent oxidative damage [34,35]. Recent investigation reveals the use of exogenously administered H2O2 in imaging of pathological cells [36]. Apart from the normal physiological processes, a high level of H2O2 has been implicated in many pathological conditions including diabetes, cardiovascular diseases, neurodegenerative disorders and cancer [37-41]. Oxidative stress is recognized as a major contributor to aging and age-associated disease [42-46] and evidence suggests its involvement in the development of sarcopenia [47].

Our investigation shows a lipid-mediated cellular regulatory mechanism in response to exogenously added H2O2, using E. coli (DH5α) as a model system. H2O2 induces cytotoxicity in E. coli by reducing growth rate, however, without altering saturation density of cells or protein content indicating a bacteriostatic effect of H2O2 on the bacterium (Figure 1A and 1B). Our investigation shows a lipid-mediated cellular regulatory mechanism in response to exogenously added H2O2, using E. coli (DH5α) as a model system. H2O2 induc
A previous report suggested an important role of lipid in resistance of apoptotic cells to H$_2$O$_2$-induced [62]. Alteration of PL composition in biological membranes is a regulatory mechanism for maintenance of optimal packing and fluidity essentially required for function of many membrane proteins [63]. Cells respond to extreme environmental conditions by altering PL composition or by altering fatty-acyl composition of membrane lipids [17,19,31-33]. Biological membranes are known to reorganize their lipids in response to perturbations that modifies their polar head groups [64]. Our results show that higher concentration of H$_2$O$_2$ (5 mM to 10 mM) leads to oxidation of lipids in E. coli (DH5α) indicating a detrimental effect on plasma membrane (Figure 2B). We hypothesize that augmentation of CL content accompanied by depletion of PG+PE is a regulatory mechanism to adapt to oxidative membrane damage induced by high concentration of H$_2$O$_2$.

CL is essential for the function of multiple membrane-bound proteins and organization of electron transport chain [21,65]. Further, oxidative stress induced-disruption of iron homeostasis is partially due to loss of cardiolipin from inner bacterial and mitochondrial membranes resulting in damage to Fe-S centers of their proteins [22]. CL is required for biogenesis of proteins containing Fe-S cluster and maintenance of mitochondrial and bacterial iron homeostasis [22]. Hence, a twofold enhancement of CL content in response to higher concentration (5 mM to 10 mM) of H$_2$O$_2$ might be an adaptive mechanism to compensate for oxidative modification of membrane lipid and proteins.

In summary, our results support the previous findings by Imlay et al. that H$_2$O$_2$ shows a biphasic toxic effect on E. coli [13]. Our present investigation suggests that at low concentration of H$_2$O$_2$, (i) no lipid peroxidation was initiated, (ii) no protein-bound iron was released and (iii) no significant alteration in PL composition was observed. These results imply that low conc. of H$_2$O$_2$ doesn’t exhibit lipotoxicity in E. coli (DH5α). Hence, the observed growth reduction at 1-2.5 mM H$_2$O$_2$ was probably due to the genotoxic effects of H$_2$O$_2$ [2,66]. However, higher concentration (>2.5 mM) of H$_2$O$_2$ exerts its cytotoxicity in part by lipid oxidation. Our previous work shows that treatment with toxic heavy metals such as Hg and Co that induce lipid peroxidation in bacteria, also alters their PL composition [67,68]. However, more studies are required to confirm if similar changes in PL composition is a general oxidative stress response mechanism in Gram negative bacteria.

**Conclusion**

In conclusion, our results show that in E. coli, H$_2$O$_2$-induced toxicity leads to lipid peroxidation and alters cellular lipid composition. Lipid peroxidation is mediated through formation of conjugated dienes, depletion of catalase activity and oxidative attack on Fe-S clusters that releases the protein-bound iron. At 10 mM H$_2$O$_2$, CL content increases by twofold, whereas PG+PE is depleted by 20%. Recent evidences show that ROS affect organization of rafts in mammalian cells under oxidative stress [20,65]. Our findings provide the scope of understanding the membrane-based oxidative stress response mechanism in Gram negative bacteria.
oxidative stress signaling processes under multiple physiological conditions that enhance cytosolic H$_2$O$_2$. To cite some examples, eukaryotic immune-defense mechanism uses augmentation of cytosolic H$_2$O$_2$ against the invading microbes and plant cells upregulate H$_2$O$_2$ under the effect of transfecting Agrobacterium. Further investigation is required to reveal the effect of H$_2$O$_2$-induced toxicity on membrane-based mechanisms such as membrane biogenesis and membrane asymmetry and their role in oxidative stress signaling in different cells.

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Conflict of Interest Statement

The authors of the present work declare no conflict of interest.

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