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

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Citation: Sahu SK, Behuria HG (2018) Biochemical Characterization of H$_2$O$_2$-Induced Oxidative Stress in E.coli. J Appl Microbiol Biochem. Vol.2 No.3:10

List of Abbreviations

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 consecutive 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\(^{3+}\) and Cu\(^{2+}\) accelerates oxidative damage of cellular constituents 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].
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 phosphatidic 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 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 H₂O₂-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 H₂O₂-induced cytotoxicity on cellular lipid composition and lipid-mediated cellular responses to H₂O₂-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, FeCl₃, 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). H₂O₂, Silica gel GF 254, Folin’s reagent and iodine 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 H₂O₂-mediated toxicity

E. coli (DH5α) was grown in LB or LB containing different concentration of H₂O₂ 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% Na₂CO₃ 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 [Follin 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 CHCl₃-CH₂OH (1:2 v/v) followed by 0.625 ml CHCl₃. Aqueous and organic phases were separated by adding 0.625ml H₂O. 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 CHCl₃. CHCl₃ was evaporated in the rotary evaporator overnight. The dried lipid samples were dissolved at approximately 1 μmol/ml PL in CHCl₃ 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 CHCl₃ 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 KH₂PO₄ 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 nm}) and 274 nm (A_{274 nm}) (peak 2) respectively. The ratio A_{230 nm}/A_{274 nm} gives the relative amount of conjugated-diienes 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 CHCl3 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 (CHCl3:CH3OH:25% ammonia solution 65:35:5 by volume), air dried and developed in second dimension using solvent II (CHCl3:C6H5OH:25% NaOH:CH3COOH:H2O in 50:20:10:10:5 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% KMnO4 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 FeCl3 (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 H2O2 as indicated by decrease of A_{240} in 3 ml assay mix (6.66 mM H2O2, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.3 ml cell lysate 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_{450 nm} of the assay mix that didn’t contain H2O2 for all samples. Percentage depletion of A_{240 nm} per min was plotted against time and normalized against total protein content.

Results

H2O2 in growth medium results in depletion of growth rate, reduction in catalase activity and regulation of cytosolic iron content in E. coli (DH5α).

H2O2-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 H2O2 in growth medium. However, the cultures were saturated at ~16 h of growth as indicated by equal OD_{600} for all doses of H2O2 and equal total protein content (Figure 1B). These results show that H2O2 is not bacteriocidal rather bacteriostatic at moderate (1 to 10 mM) concentration. A time-dependent adaptation to H2O2-induced toxicity was observed in E. coli (DH5α) that was proportional to concentration of H2O2 in the growth medium. An adaptive response to H2O2-induced cytotoxicity was characterized by depletion of catalase activity and regulation of cellular iron content. Catalase, the central enzyme that regulates intracellular level of H2O2, depleted by ~75% and remained almost invariable at all concentrations of H2O2 tested (1 mM to 10 mM). (Figure 1C). These results show that

Figure 1a

H2O2-induced toxicity in E. coli (DH5α) (A) Growth curve of the bacterium grown in LB containing different concentration of H2O2 as indicated to the right of the figure. The figure is the representative of three independent sets of experiments.
**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**
Discussion

H$_2$O$_2$ 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 H$_2$O$_2$ in imaging of pathological cells [36]. Apart from the normal physiological processes, a high level of H$_2$O$_2$ 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 H$_2$O$_2$ using E. coli (DH5α) as a model system. H$_2$O$_2$-induced formation of conjugated diene was saturable at 7.5 mM H$_2$O$_2$. These results indicate the existence of oxidative-stress regulatory mechanism in E. coli that buffers the conjugated diene level at 30% above the normal cellular level as a strategy to survive H$_2$O$_2$-induced toxicity.

**E. coli** (DH5α) regulates cellular PL composition as an adaptive mechanism to survive oxidative stress.

PLs that constitute ~89% of total lipids in E. coli are known to be altered in a multitude of stress conditions like high temperature, salinity, growth phase and toxic compounds [17-19,31-33]. CL is proposed to be the most unsaturated PL that possesses the most variable composition of fatty-acyl tails that is upregulated in multiple stress conditions [21]. Hence, we performed a quantitative analysis of PL composition of E. coli (DH5α) subjected to increasing concentration of H$_2$O$_2$ (from 1 to 10 mM). 2D-TLC shows three major PLs that constituted up to 95% of total PL loaded on the plate (Figure 3A). Three major PLs were identified as PE, PG and CL using PL standards (not shown). In control cells, PE and PG together constituted ~90% of the total PL, CL being ~7%. Increasing doses of H$_2$O$_2$ up to 10 mM led to augmentation of CL up to 15% (two fold) accompanied by corresponding depletion in PG+PE content to 82% (Figure 3B and 3C).

### Figure 3b

Quantitative analysis of PG+PE content on the TLC plates.

### Figure 3c

Quantitative analysis of CL content of the samples grown in LB containing different concentration of H$_2$O$_2$. All analyses were performed using Graph-pad prism.
investigation shows that catalase activity is reduced by 75% in response to exogenous H$_2$O$_2$ at all concentration tested (Figure 1C). Catalase is the central oxidative stress regulatory enzyme in _E. coli_ that is involved in maintenance of cytosolic H$_2$O$_2$-homeostasis. Reduced activity of catalase is observed in multiple cell types with increasing cytosolic H$_2$O$_2$, including developing rat oligodendrocytes [48] and aging sarcopenia [46]. H$_2$O$_2$-induced depletion in catalase activity was also observed as early as 1931 [49]. Depletion of catalase activity at elevated cytosolic H$_2$O$_2$ is a regulatory mechanism to maintain the oxidative stress at minimum, as H$_2$O$_2$ is known to be the less reactive compared to superoxide anion (O$_2^-$) and hydroxyl radical (OH$^-$).

A depletion of cellular iron content was observed in cells cultured in presence of low (1 to 2.5 mM) concentration of H$_2$O$_2$ in growth medium. At low concentration of H$_2$O$_2$, cells are known to reduce cytosolic free iron content by converting them into tight, protein-bound form as observed in many different cell types including bacteria, plants and animals [50-52]. Reports suggest that oxidative stress induced by O$_2^-$ and H$_2$O$_2$ lead to downregulation of iron regulatory protein (IRP) causing transient decrease of cytosolic free iron that otherwise would convert them into more potent oxidants such as hydroxyl radicals or equally aggressive iron-peroxo complexes [53]. H$_2$O$_2$-induced oxidative stress probably by over expression of high-affinity iron-binding proteins like Dps, Dpr, ferritins, IRR and OxyR that are known to scavenge free cytosolic iron leading to reduced detection of cytosolic iron [50, 53-58].

However, higher (> 2.5 mM) doses of H$_2$O$_2$ is known to destroy the iron-sulfur (FeS) centers of many proteins containing iron-sulfur clusters, releasing the protein-bound iron that leads to augmentation of cytosolic pool of free iron content (Figure 1D) [30,59,60]. Fe-S enzymes (e.g. aconitate, succinate dehydrogenase, and ubiquinol-cytochrome c oxidoreductase), as well as cytosolic Fe-S enzymes (sulfite reductase and isopropylmalate isomerase) are known to release iron in response to elevated level of oxidative stress [30]. Our results indicate a biphasic effect of H$_2$O$_2$ on cytosolic iron of _E. coli_ corresponding to low and high concentration of the oxidant as proposed in earlier studies.

Conjugated diene is one of the initial products of lipid peroxidation that is formed due to abstraction of hydrogen from double bonds of unsaturated fatty acyl chains of lipids [26,61]. Our results show that lipid peroxidation is negligible up to 2.5 mM H$_2$O$_2$ and is initiated beyond this concentration. However, higher concentration (≥ 5 mM) of H$_2$O$_2$ increased conjugated diene content of lipids. No alteration in total lipid content was observed (Figure 2A and 2B), implying that phospholipid biosynthesis remains unaltered under all the concentration of H$_2$O$_2$ tested. A 30% enhancement in conjugated diene content was observed at 7.5 mM H$_2$O$_2$, beyond which the cells resisted further enhancement in conjugated diene content. This phenomenon is explained by assuming either strict regulation of diene conjugation or by conversion of conjugated dienes into terminal products (e.g. lipid hydroperoxides and lipid peroxy radicals) of lipid peroxidations.

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 signaling processes under multiple physiological conditions that enhance cytosolic \( \text{H}_2\text{O}_2 \). To cite some examples, eukaryotic immune-defense mechanism uses augmentation of cytosolic \( \text{H}_2\text{O}_2 \) against the invading microbes and plant cells upregulate \( \text{H}_2\text{O}_2 \) under the effect of transfecting *Agrobacterium*. Further investigation is required to reveal the effect of \( \text{H}_2\text{O}_2 \)-induced toxicity on membrane-based mechanisms such as membrane biogenesis and membrane asymmetry and their role in oxidative stress signaling in different cells.

**References**


**Acknowledgements**

We thank Department of Science and Technology, Govt of Odisha, India for funding. H.G. Behuria thanks Department of Science and Technology, Govt of India for fellowship.

**Conflict of Interest Statement**

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


