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DIFFERENTIAL EFFECT OF PARAQUAT AND HYDROGEN PEROXIDE ON THE OXIDATIVE STRESS RESPONSE IN *VIBRIO CHOLERAE* NON O1 26/06

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ABSTRACT

Vibrio cholerae non O1 26/06, a non-pathogenic strain, was subjected to treatment by different concentration of paraquat (PQ) and H_2O_2. Exposure to PQ for 1 h caused induction of reactive oxygen species (ROS) such as superoxide anion radical ($^{\bullet}O_2^-$) and H_2O_2. At the same time, second stress factor significantly inhibited $^{\bullet}O_2^-$ production and enhanced the intracellular H_2O_2 content. The enhanced ROS generation resulted in a significant increase in the levels of oxidatively damaged proteins in comparison to the control variant. Thus, the exposure of V. cholerae cells to PQ and H_2O_2 promoted oxidative stress. Cell response against this stress includes activation of antioxidant enzyme defence. The treatment with PQ concentrations in the range of 0 - 5 mM resulted mainly in activation of SOD, but not noticeably changed CAT activity in V. cholerae non-O1 26/06. In contrast, effect of H_2O_2 treatment on antioxidant enzyme synthesis in our Vibrio strain was still much more pronounced for CAT than for SOD. Therefore, oxidative stress responses induced by $^{\bullet}O_2^-$ (generated intracellularly by PQ) and H_2O_2 demonstrated differential adaptation of Vibrio cells to different toxic agents.

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Introduction

The genus *Vibrio* consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum (40). Microorganisms that biochemically resemble *V. cholerae* but lack the O1 antigen (*V. cholerae* non-O1) are believed to be nonpathogenic (28). They are widely distributed in marine environments, especially bays, estuaries and other brackish waters, and in shellfish. It is now known, however, that some strains of the non-O1 group of *V. cholerae* are pathogens. These species have been associated with cholera-like diseases and other extraintestinal infections, not only in humans but also in higher aquatic organisms (15).

Efficient killing of *Vibrios* by host macrophages depends on a number of mechanisms including production of reactive oxygen species (ROS) by the phagosomal NADPH oxidase. ROS like superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) are toxic compounds produced by the incomplete reduction of oxygen during oxidative metabolism (21). These compounds are cytotoxic oxidants that can initiate lipid peroxidation, cause DNA strand breaks and indiscriminately oxidize organic molecules, leading to cell damage or death. ROS are also thought to be important in the aging process and in the pathogenesis of various diseases.

Cells have acquired the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. The protective mechanisms include both non-enzymatic (ascorbic acid, β-carotene, glutathione, and α-tocopherol) and enzymatic (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)) antioxidant systems. Bacteria employ mainly enzyme mechanisms to eliminate the damaging effects of oxidative stress, such as superoxide dismutase (23, 27, 29, 31), NADH oxidase (10, 19), CAT (18, 22, 30), GPx (8), glutathione reductase (32), thiol peroxidase (12) and alkyl hydroperoxidase (33). The presence of one or several of these enzymes has been shown in many pathogenic bacteria, and they have been linked to microbial virulence (12, 38).

The question of the mechanisms by which certain *Vibrio* species survive oxidative stress has been under intense investigation (35). Our previous results described the relationship between temperature stress and antioxidant enzyme defense in *V. cholerae* non-O1 strains (1). In the present study, we studied the effect of oxidative stress induced by paraquat and H_2O_2 on the production of ROS and antioxidant enzyme activities in *V. cholerae* non-O1 26/06. Paraquat (1,1-dimethyl-4,4-bipyridinium), a widely used nonselective herbicide, has been found to induce oxidative stress by production of superoxide anion and cause toxicity to aerobic cells.

Material and Methods

Bacterial strains, media, cultivation and cell-free extracts preparation

The model strain *Vibrio cholerae* non-O1 26/06 was grown statically in 100 ml Erlenmeyer flasks containing 30 ml Tryptic soy broth (TSB), pH 8.0, at 30 ºC. The stress-factors paraquat and hydrogen peroxide were added to the cultures in different concentrations at the 40th hour of cultivation. Two control cultures with no stress-factors were provided. After another hour of cultivation at the same conditions all cultures

were centrifuged at 14 500 rpm, 4 ºC. The cell pellets were resuspended in 0.05 M phosphate buffer with pH 7.8 and centrifuged again. The collected cells were stored at -20 °C until use. Frozen cells suspended in phosphate buffer at pH 7.2 were homogenized with a mortar and pestle using quartz sand. The supernatant was collected after centrifuging at 5 000 × *g* for 5 min and was used as a cell-free extract for enzyme and protein assays.

The experiments with each substance and concentration were carried out in duplicate.

Determination of ROS

For measurement of $^{\bullet}O_2^-$ production rate, the method of superoxide dismutase-inhibitable reduction of cytochrome *c* was used (17) with some modifications, as described by Abrashev et al. (1). For measurement of hydrogen peroxide production, the method of Pick and Mizel (34) was used.

Measurement of protein carbonyl content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using DNPH binding assay (24), slightly modified by Adachi and Ishii (2).

Growth and protein content determination

Growth was estimated turbidimetrically (and expressed as optical density [OD]) at 650 nm with a spectrophotometer *Specol 11* (Carl Zeiss Jena, Germany). Protein was estimated by the Lowry procedure (26) using bovine serum albumin as standard.

Enzyme assay

SOD activity was measured by the nitro blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (5). One unit of SOD activity was defined as the amount of SOD required for inhibition of NBT reduction by 50% (A_{560}) and was expressed as units per mg protein [U (mg protein)$^{-1}$]. CAT was assayed by the method of Beers and Sizer (7). The decomposition of H_2O_2 was followed directly by measuring the decrease in absorbance at 240 nm. One unit of CAT is defined as the amount that will decompose 1 µmol of H_2O_2 in 1 min at pH 7.0 and 25 C. Specific activity is given as U (mg protein)$^{-1}$.

PAGE electrophoresis

The SOD isoenzyme profile was performed on polyacrylamide gels. Forty µg total protein was applied to 10% nondenaturing PAGE and was stained for superoxide dismutase activity, as described by Beauchamp and Fridovich (5).

Results and Discussion

Our previous investigations demonstrated the ability of the bacterial strain *V. cholerae* non O1 26/06 to produce antioxidant enzymes SOD and CAT under normal physiological conditions and temperature stress (1).

PQ and H_2O_2 affect ROS production

We used ROS as a marker of oxidative stress, testing changes in the level of $^{\bullet}O_2^-$ and H_2O_2 in *V. cholerae* non-O1 26/06 treated by different concentrations of PQ and H_2O_2. Exposure to PQ resulted in enhanced ROS levels in bacterial cells in a dose-dependent manner (**Table 1**). Superoxide anion radical level (µM per mg d.w. per 1 h) increased steadily in PQ concentration range of 0.1 - 3 mM. Treatment with herbicide, even in low concentration (0.1 – 0.5 mM), induced 1.2 - 1.8-fold higher generation of $^{\bullet}O_2^-$ compared with the control. In the presence of 3 mM PQ, bacterial cells accumulated 3.5-fold more $^{\bullet}O_2^-$ than the control cells. At the same time, PQ induced about 2.4-fold increase in H_2O_2 content (mM per mg d.w. per 1 h) compared with the control.

The results of exposure to H_2O_2 (**Table 1**) demonstrated an opposite trend of $^{\bullet}O_2^-$ changes to that seen in the experiments with PQ. After 60 min in the presence of H_2O_2 in the concentration range of 0.1 – 3.0 mM, a 20 to 50% decrease in $^{\bullet}O_2^-$ production was observed. On the other hand, 2-fold increased H_2O_2 content was detected in treated *Vibrio* cells compared with the control.

Above mentioned results indicate that exposure of *V. cholerae* cells to PQ and H_2O_2 promoted oxidative stress. As is known, even the unstressed bacterial cells seemed to produce

TABLE 1

Increase in ROS generation ($^{\bullet}O_2^-$ and H_2O_2) in the cells of *V. cholerae* non 01 26/06 treated by enhanced concentration of PQ and H_2O_2

Variant	PQ [mM]		H_2O_2 [mM]	
	$^{\bullet}O_2^-$ [µg/mg cells/h]	H_2O_2 [µg/mg cells/h]	$^{\bullet}O_2^-$ [µg/mg cells/h]	H_2O_2 [µg/mg cells/h]
Control	6.2 ± 0.52	5.3 ± 0.34	8.8 ± 0.1	5.3 ± 0.34
0.1	7.5 ± 2.23	8.5 ± 0.71	4.5 ± 0.23	8.5 ± 0.71
0.3	9.8 ± 1.71	10.8 ± 1.02	5.8 ± 1.71	8.8 ± 1.02
0.5	11.2 ± 5.42	12.1 ± 0.78	6.2 ± 0.42	9.1 ± 0.78
1.0	14.6 ± 3.23	12.7 ± 0.98	5.6 ± 0.23	9.7 ± 0.98
2.0	18.4 ± 2.76	11.5 ± 0.82	4.4 ± 0.76	9.5 ± 0.82
3.0	21.6 ± 3.82	12.4 ± 1.01	3.6 ± 0.82	10.4 ± 1.01

$^{\bullet}O_2^-$ and H_2O_2, presumably due to a single electron reduction of 2% of the consumed oxygen as was previously suggested (14). ROS can be generated via a variety of physiological and pathological conditions, including PQ and H_2O_2 exposure (10, 16, 36, 39). Our direct assay of ROS showed that all variants of PQ treatment of bacterial cells for 1 h do clearly cause oxidative stress, which induced both $^{\bullet}O_2^-$ and H_2O_2 generation. In contrast, presence of second stress agent significantly inhibited $^{\bullet}O_2^-$ production and enhanced intracellular H_2O_2 content. Excessive ROS level has been linked to lipid peroxidation of the cell membrane, resulting in a loss of membrane fluidity, structure and function (14). On the other hand, the increased ROS level present in our experiment could be a direct consequence of the exogenous H_2O_2, but could also result from the generation of ROS by damaged cells and mitochondria (10). It is possible that the exogenous H_2O_2 causes cells to become damaged in turn causing the mitochondria to leak high levels of free electrons. Similar direct analysis of ROS content in bacterial cells is not often published. Becerra and Albesa (6) reported that oxidative stress agents increased $^{\bullet}O_2^-$ and H_2O_2 levels in *Staphylococcus aureus*. There is more information in the field of temperature-induced ROS on the model of higher eukaryotes (25). Our results extend previous information about pathogenic bacteria such as *Vibrio cholerae*.

PQ and H_2O_2 induce protein oxidation

Reaction of proteins with oxygen radicals leads to the appearance of carbonyl groups in polypeptide chains (13). Measuring the content of these groups in intracellular proteins is one of the accepted assays for oxidative damage in microbial cells. When *Vibrio* strain was exposed to enhanced concentration of PQ and H_2O_2, the amount of carbonyl groups in cell proteins was changed in a dose-dependent manner (**Fig. 1**). PQ caused a gradual increase in carbonyl content by up to 1 mM concentration (**Fig. 1A**). Oxidatively damaged proteins in treated cells were almost 4-fold higher than in control cells. A similar trend was demonstrated by the H_2O_2-treated cultures (**Fig. 1B**).

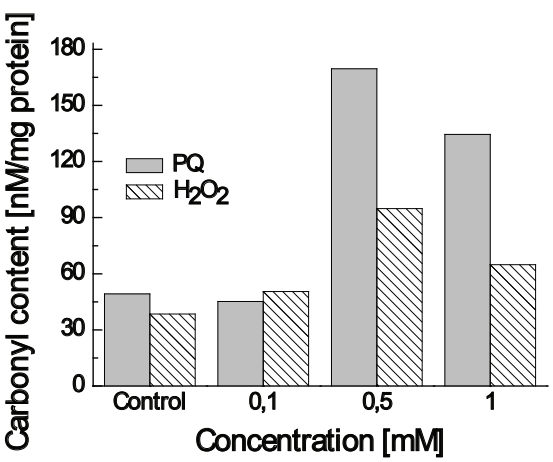

Fig. 1. Oxidative damage of proteins in *V. cholerae* non 01 26/06 upon PQ (full bars) and H_2O_2 (hatched bars) exposure.

The enhanced carbonylation damage to intracellular proteins is a marker for accumulation of oxidatively modified proteins. Environmental agents such as ionising, near-UV radiation, or numerous compounds that generate intracellular $^{\bullet}O_2^-$ (redox-cycling agents such as menadione and paraquat) can cause oxidative stress, which accelerates oxidation of proteins in pro- and eukaryotic cells (10). According to Tamarit et al. (37), an increase in oxidative stress may contribute to the development of oxidative protein damage in *E. coli*, grown in presence of menadione, PQ and H_2O_2.

Antioxidant enzyme response to PQ and H_2O_2

To assess the roles of the antioxidant enzymes in *Vibrio* tolerance to oxidative stress agents, the time- and dose-dependent effect of PQ and H_2O_2 was evaluated. Time-dependent changes in SOD and CAT activities are shown in **Fig. 2**. PQ induced significant increase in the activity of both enzymes up to 60 min of incubation, followed by a constant *steady-state* level of *SOD activity* and a sharp decrease in CAT activity (**Fig. 2A**). In contrast, peroxide stress caused 2.5 to 4-fold increase in catalase activity compared to the control, while SOD sustained only modest increase with H_2O_2 up to 30 min of incubation, followed by about 2-fold reduction in the activity compared to the control variant (**Fig. 2B**).

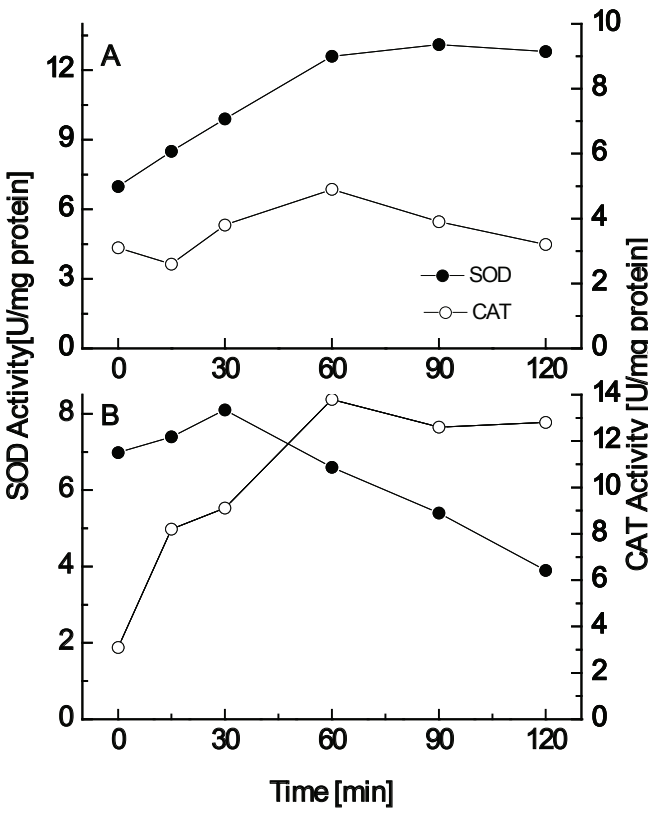

Fig. 2. Effect of time exposure of PQ (**A**) and H_2O_2 (**B**) on SOD (●) or CAT (○) activity of *V. cholerae* non 01 26/06.

The experiments also demonstrated that the antioxidant enzyme level is influenced by PQ and H_2O_2 in a dose-dependent manner (**Fig. 3**). As seen from **Fig. 3A**, 1 h exposure to PQ concentration between 0.1 and 1 mM resulted in increased superoxide scavenging activity (about 2-times in comparison with control variant). In the presence of higher concentration, 2 and 5 mM, 1.9- and 1.4-fold elevated activity was determined, respectively. At the same time, the level of CAT did not change noticeably in treated cells. As displayed in **Fig. 3B**, CAT activity was significantly increased in *Vibrio* cells that were treated by H_2O_2. The maximal rise was about 3-fold in the variants exposed to 2 mM peroxide. In contrast, no significant differences in SOD activity were observed under peroxide stress condition.

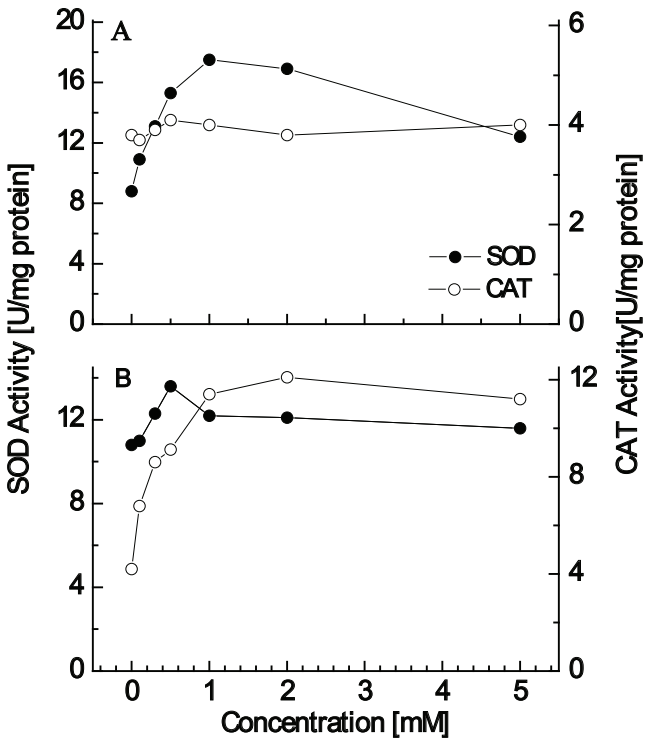

Fig. 3. Effect of PQ (**A**) and H_2O_2 (**B**) concentration on SOD (●) or CAT (○) activity of *V. cholerae* non 01 26/06.

Fig. 4 shows the results of native PAGE, stained for SOD activity of cell-free extracts of *V. cholerae* 26/06 grown under both control and stress conditions. Only one band of fast migrating SOD was apparent. Our preliminary (data not shown) and present results (lane 4 in **Fig. 5**) indicate that the enzyme is Fe-containing SOD. As seen also in **Fig. 5**, the enzymatic activity of SOD was significantly increased in PQ treated cells in dose-dependent manner. At the same time, SOD activity did not change significantly in the response to the H_2O_2 treatment.

Our results demonstrated that the treatment with PQ concentrations in the range of 0 - 5 mM results mainly in the activation of SOD. Data on SOD induction by PQ has been reported also in bacteria (20), filamentous fungi (4), and plants (25). At the same time, PQ did not noticeably change CAT activity in *V. cholerae* non-O1 26/06. Our data confirm the general suggestion concerning cell response to $^{\bullet}O_2^-$-generators. Exposing bacteria to PQ (well known generator of superoxide anion) enhances the synthesis of SOD but not of catalase (3). SOD null mutants in *Escherichia coli* exhibit increased sensitivity to PQ suggesting that this enzyme is responsible for the principal superoxide dismutating activity during oxidative stress (11). As an exception to the rule, PQ has been shown to strongly induce a particular CAT activity in *Pseudomonas* sp. strain O1 (20).

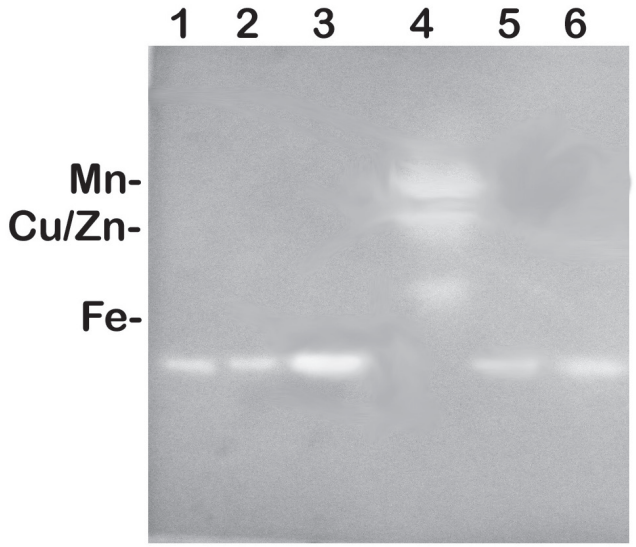

Fig. 4. Polyacrylamide gel electrophoresis (10% gel) of cell-free extracts from *Vibrio* cells cultivated under oxidative stress conditions, stained for SOD activity. Lane 1 – control, lane 2 - 0.3 mM PQ, lane 3 – 1.0 mM PQ, lane 4 standard – Mn-, Cu/Zn- and Fe-containing SOD, lane 5 – 0.3 mM H_2O_2, and lane 6 – 1.0 mM H_2O_2.

In contrast, the effect of H_2O_2 treatment on antioxidant enzyme synthesis in our *Vibrio* strain was still much more pronounced for CAT than for SOD. Similar data have been published for the archaeon *Methanosarcina barkeri* (9). According to Davies (13), *E. coli* has independent multiple responses to the two kinds of oxidative stress: excess H_2O_2 triggers the oxyR regulon, and excess $^{\bullet}O_2^-$ triggers the soxRS regulon.

The observations by PAGE suggest that *V. cholerae* SOD is involved in the bacterial line of defence under conditions of superoxide-induced stress.

Conclusions

Overall, the present findings underlined the sharp contrast between two apparently similar oxidative stress responses induced by $^{\bullet}O_2^-$ (generated intracellularly by PQ) and H_2O_2 that conferred differential adaptation of *Vibrio* cells to different toxic agents.

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