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Effects of Copper-phenanthroline on Pentachlorophenol-induced Adaptation and Cell Death of *Escherichia coli* [1]

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Objective To evaluate the effects of copper-phenanthroline (CuOP) on pentachlorophenol (PCP)-induced adaptation and cell death of *Escherichia coli.* **Methods** Bacterial growth and adaptation to PCP were monitored spectrophotometrically at 600 nm. Inactivation of bacterial cells was determined from colony count on agar dishes. Cellular ATP content and accumulation of PCP were assessed by chemiluminescence and HPLC analysis respectively. The formation of PCP-Cu-OP complex was shown by UV-visible spectra. **Results** *Escherichia coli* (*E. coli*) could adapt to PCP, a wood preservative and insecticide used in agriculture. The adaptation of *E. coli* to PCP prevented its death to the synergistic cytotoxicity of CuOP plus PCP and declined cellular accumulation and uncoupling of oxidative phosphorylation of PCP. Furthermore, CuOP and PCP neither produced reactive oxygen species (ROS) nor had a synergistic effect on uncoupling of oxidative phosphorylation in *E. coli*. The synergistic cytotoxicity of CuOP and PCP in *E. coli* might be due to the formation of lipophilic PCP-Cu-OP complex. **Conclusion** Our data suggested that adaptation of *E. coli* to PCP decreased the synergistic effects of CuOP and PCP on prokaryotic cell death due to the formation of lipophilic PCP-Cu-OP complex, but it had no effect on the uncoupling of oxidative phosphorylation and production of reactive oxygen species in *E. coli*.

Key words: Adaptation; Cell death; Cytotoxicity; *Escherichia coli*; Oxidative phosphorylation; Pentachlorophenol; 1,10-phenanthroline-copper

INTRODUCTION

Pentachlorophenol (PCP) is mainly used in wood industries as a wood preservative. It is also employed as an insecticide in agriculture[1-2]. Though its use is banned or restricted in many countries, PCP remains an important pesticide from a toxicological perspective[3]. PCP has led to substantial environmental contamination and accumulated in humans especially PCP-exposed workers[2]. Chronic occupational exposure to PCP may produce a syndrome similar to acute systemic poisoning, together with conjunctivitis and irritation of the upper respiratory and oral mucosae. Long-term exposure has also been reported to result in chronic fatigue or neuropsychiatric features in combination with skin infections, chronic respiratory symptoms, neuralgic pains in the legs, and impaired fertility and hypothyroidism secondary to endocrine disruption[3]. PCP is carcinogenic for mice[4], but it has no mutagenic activity in bacterial test systems with a weak mutagenicity being reported in other systems[2]. PCP is a known uncoupler of oxidative phosphorylation[5]. In addition, there are other mechanisms of PCP-induced toxicity in mammalian cells including lysosome destabilization and apoptotic death[6-7]. *E. coli* might have an adaptive response and become resistant to uncouplers, as they do to heat, oxidants, mutagens, and some other factors[8]. It has been shown that *E. coli* could become resistant to 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation[9].

Physiological systems contain significant concentrations of labile copper. Transition metals are

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Abbreviations: PCP, pentachlorophenol; PQ, paraquat; OP, 1,10-phenanthroline; DTPA, diethylenetriaminepentaacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; SOD, superoxide dismutase; BSA, bovine serum albumin; BCS, bathocuproinedisulfonic acid; DMSO, dimethyl sulfoxide.

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noted to enhance deleterious processes induced by tetrachlorohydroquinone[10-11]. Bis (1,10-phenanthroline)-Cu(I) is a known chemical that nicks the phosphodiester backbone of DNA under physiological pH and temperature by oxidatively degrading the deoxyribose moiety[12]. In eukaryocytes, 1, 10-phenanthroline-Cu^{2+} (CuOP) is known to form a $(OP)_2Cu^{2+}$ complex and promote production of reactive oxygen species (ROS), thus inducing apoptosis[7,13]. In prokaryotes, copper-mediated toxicity of PCP is markedly enhanced by 1,10-penanthroline (OP)[14]. Copper and OP could form three complexes with different stoichiometries and stabilities, namely CuOP, Cu $(OP)_2$, and $Cu(OP)_3$ with CuOP having the strongest effect on Copper/PCP toxicity in *E. coli* [10-11].

E. coli became resistant to PCP. The present study examined if CuOP and PCP had the synergistic toxicity in PCP-resistant *E. coli*., and the underlying mechanisms by which PCP and CuOP cause cytotoxicity.

MATERIALS AND METHODS

Chemicals

Pentachlorophenol, paraquat, 1, 10-o-phenanthroline, cytochrome c, SOD, catalase, firefly lantern extracts FLE250, diethylenetriaminepentaacetic acid, histidine, HEPES were purchased from Sigma (Sigma, St. Louis, MO, USA). Glucose, magnesium sulfate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Merck (Merck, Whitehouse Station, NJ, USA). Bathocuproinedisulfonic acid was obtained from Sigma-Aldrich (Sigma). Dimethyl sulfoxide was purchased from BDH (Poole, UK). Copper sulfate was purchased from Mallinckrodt (Hazelwood, MO, USA).

Bacterial Culture and Survival

Escherichia coli B [SR-9] was used throughout the experiments. Cells were grown in a 37℃ shaking incubator at 200 rpm in a medium as previously described[15]. For adaptation experiments, *E. coli* B grown overnight in the aforementioned medium was diluted to an initial OD 600 nm of 0.2 and incubated with 0.2 mmol/L PCP or 0.02 mmol/L Paraquat for 3 h before treatments. *E. coli* growth was monitored spectrophotometrically at 600 nm every 30 min. Inactivation of *E. coli* cells was determined from colony count on agar dishes in a mode analogous to the report by Korbashi *et al.*[15].

Preparation of Crude Extracts and Enzyme Assays

After 3 h growth at 37℃ with or without the test compound, the cells-free crude extracts were prepared as previously described[16]. Protein concentrations were analyzed with Bio-Rad reagents (Bio-Rad Laboratories, Richmond, USA) SOD[17] and catalase[18] was assayed as described previously.

Luminometer Assays of Cellular ATP Content

An aliquot of *E. coli* culture (0.2 mL) was taken out at various time points and put into preheated 1.8 mL buffer containing 20 mmol/L Tris-HCl and 2 mmol/L EDTA at pH 7.75, and boiled for 5 min[19]. The assay was performed at room temperature by mixing 1.0 mL sample with 0.2 mL firefly lantern extracts FLE250, which was prepared and stored as previously described[20]. Chemiluminescence was determined immediately by a luminometer (Lumac/3M Biocounter M2010). The first 10-sec light intensity was recorded. Cellular ATP content was expressed as the percent of initial light intensity value.

UV-Visible Spectra of Copper Complexes

Kenton Uvikon 860 UV-visible spectrophotometer was used to measure the absorbance spectra of OP-copper-PCP complexes. Spectra (absorbance wavelength: 300-800 nm) of four groups were recorded 1 min after reagents were mixed in the spectrophotometer cuvette: (1) 40 μmol/L CuOP (40 μmol/L $CuSO_4$ and 40 μmol/L 1,10-o-phenanthroline) plus 0.4 mmol/L PCP and 20 μg/mL BSA; (2) CuOP plus PCP; (3) CuOP; (4) CuOP and BSA. Except for $CuSO_4$, the same concentrations of OP, PCP, and BSA were put into the reference cuvette to run baseline.

HPLC Assays of PCP Uptake in E. coli Cells

All reaction mixtures consisted of 0.3 mmol/L PCP, 3×10^9 cells/mL in HEPES buffer (10 mmol/L, pH 7.4) containing 0.5% glucose and 1 mmol/L $MgSO_4$. After 30 min, the mixtures were centrifuged, cell pallets were washed three times by HEPES buffer (10 mmol/L, pH 7.4) and digested by 1 mol/L NaOH at 100℃. PCP was extracted as ions[21]. After centrifuged and neutralized, PCP was assayed using HPLC. The concentration of PCP was decided by standard curves, and expressed as $\mu g/3\times10^9$ cells. The HPLC was consisted of a Varian 5000 liquid chromatography equipped with a Rheodyne 7125 sample injector (20 μL loop) connected to a linear UVIS 200 detector (Reno, USA). The column used for separation was a 25 cm × 4 mm LiChrosper 100 RP-18, 5 μm (E. Merck) provided with a guard column packed with the same matrix as the separate column. The signals from the detector were acquired

on a PC Microsoft MC-DOS version 5.0 data system and subsequently processed. The mobile phase contained 50% CH_3CN and 50% solution of 1.27 g H_3PO_4 and 0.4 g NaN_3 in 1 litre of water. The UV wavelength of detector is 254 nm.

Statistical Analysis

Statistical significance was evaluated by using Student *t*-test or one-way ANOVA, *P* values < 0.05 were considered significant and are indicated (*), *P* values <0.001 are indicated (**). All experiments were repeated at least three times. The results were expressed as $\bar{x} \pm s$.

RESULTS

Adaptation of E. coli to PCP Prevented Cytotoxicity of PCP

To test the adaptation to PCP, *E. coli* B cells were firstly grown for 3 h in a medium containing 0.2 mmol/L PCP, and then challenged with 0.4, 1.0, or 1.5 mmol/L of PCP. The results showed that 0.4 mmol/L PCP significantly inhibited the growth of uninduced *E. coli* cells, but it did not inhibited growth of PCP-induced cells (Fig. 1A), suggesting that *E. coli* hade become resistant to PCP.

Adaptation to PCP Altered Antioxidant Enzyme Activity and PCP Uptake in E. coli

To test whether PCP-resistant *E. coli* could alter their antioxidant enzyme activity, we assayed the superoxide dismutase (SOD) and catalase in cell-free extracts from uninduced, 0.2 mmol/L PCP-induced, or 0.02 mmol/L PQ-induced *E. coli*. The results showed that PCP as well as PQ, known to induce SOD and catalase, could also significantly induce *E. coli* cells to produce endogenous SOD and catalase (Table 1).

After 30 min exposure of uninduced and PCP-induced *E. coli* cells (3×10^9 cells/mL) to 0.3 mmol/L PCP, cellular PCP uptake was assayed by HPLC as described in Materials and Methods. The results showed that the cellular content of PCP in

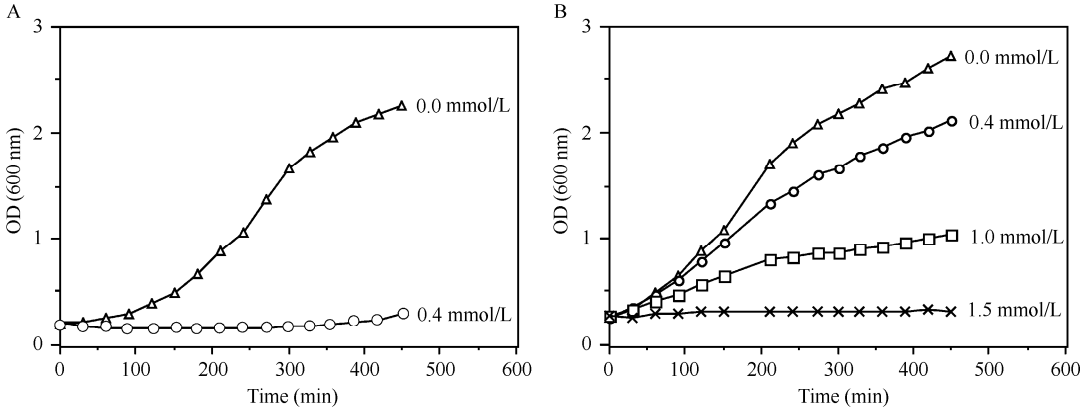

FIG. 1. Adaptation of *E. coli* to PCP. A: uninduced cells treated with or without 0.4 mmoL/L PCP; B: PCP-induced cells. After growth for 3 hours in minimal medium with 0.2 mmoL/L PCP, *E. coli* cells were washed three times with minimal medium and grown in the same medium containing different concentrations of PCP. *E. coli* growth was monitored spectrophotometerically at 600 nm every 30 min.

TABLE 1

Effects of Adaptation to Pentachlorophenol and Paraquat on the Activity of Superoxide Dismutase and Catalase, and the Accumulation of PCP in *E. coli* ($\bar{x} \pm s$)

	SOD (U/mg Protein)	Catalase[a] (U/mg Protein)	PCP[b] (μg/3×10 ^{9} Cells)
Control	16.7±2.6	2.40±0.44	3.7±1.0
Pentachlorophenol (0.2 mmol/L)	27.3±4.7*	4.44±0.74*	2.0±0.6*
Paraquat (0.02 mmol/L)	44.1±2.7*	6.41±0.59*	2.9±1.5

Note. [a] catalase activity was calculated by the difference in absorbance (ΔA240) of H2O2 per unit time (ε240 = 0.00394±0.0002 liters $mmol^{-1}$ mm^{-1}). [b] 0.3 mmol/L PCP was added in reactive mixture and the uptake of PCP in *E. coli* was assayed by HPLC assays as described in Materials and Methods. *P<0.05, vs. control (ANOVA).

PCP-induced cells was significantly declined, compared to uninduced cells (P<0.05). There was no significant difference between PQ-induced and uninduced cells in PCP uptake (Table 1).

Cytotoxicity of CuOP Plus PCP in PCP-Resistant E. coli

E. coli cells were firstly grown in a medium containing 0.2 mmol/L PCP (PCP-resistant cells) for 3 h. Subsequently, their sensitivity to PCP (1.4 mmol/L) and the combination of PCP (0.3 mmol/L) and CuOP (1.5 μmol/L, Cu (II):OP=1:1) was determined. All the reaction mixtures contained 4.8×10^6 cells/mL of PCP-resistant *E. coli*, glucose (0.5% w/v), and $MgSO_4$ (1 mmol/L) in HEPES buffer (10 mmol/L, pH7.4). The results showed that PCP and PCP+CuOP had *little no* toxicity to PCP-resistant *E. coli* (Fig. 2).

PCP Had no Effect But CuOP Decreased Cellular ATP Cells in PCP-Resistant E. coli

When the *E. coli* cell membrane was damaged, it is expected that its cellular ATP level would decreased markedly compared to that of an intact cells[19]. Exposure of uninduced and PCP-induced *E. coli* cells (3×10^9 cells/mL) to 0.4 mmol/L PCP, the levels of cellular ATP were assayed by chemiluminescence method[20] (Fig. 3A). The ATP synthesis of PCP-resistant cells were not affected when treated with PCP. In contrast, both uninduced cells and PCP-resistant cells were susceptible to toxicity caused by CuOP (Fig. 3B).

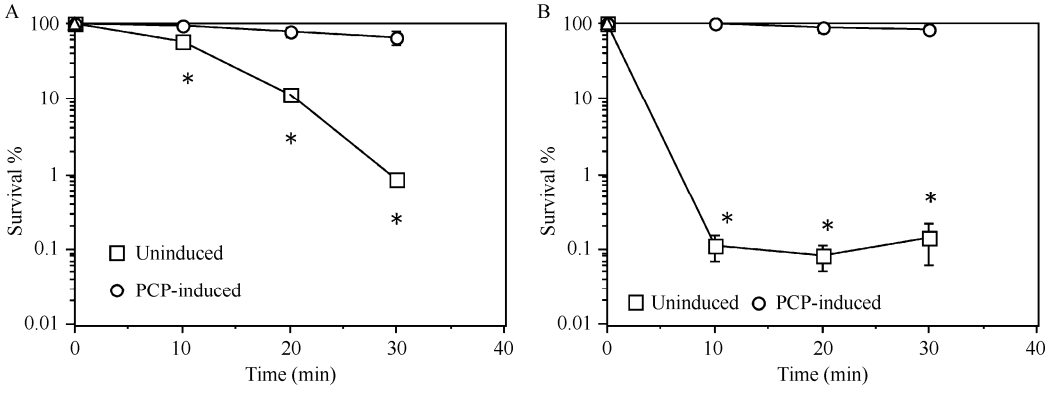

FIG. 2. Effects of adaptation on cytotoxicity of CuOP and/or PCP. All the reaction mixtures contained 4.8×10^6 *E. coli* cells/mL, glucose (0.5% w/v), and $MgSO_4$ (1 mmol/L) in HEPES buffer (10 mmol/L, pH7.4). A: Survival rate of uninduced or PCP-induced *E. coli* treated with 1.4 mmol/L PCP. B: Survival rate of *E. coli* treated with 1.5 μmol/L CuOP plus 0.3 mmol/L PCP. $^*P$<0.01 (Student's *t*-test), uninduced vs. PCP-induced cells.

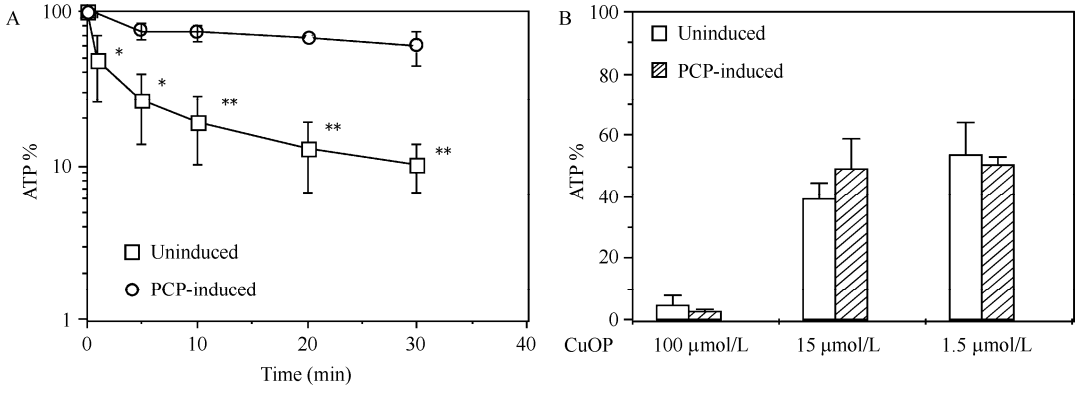

FIG. 3. Effects of adaptation on PCP- or CuOP-declined cellular ATP contents. A: The incubation mixture contained 3×10^9 *E. coli* cells/mL of uninduced or PCP-induced cells in phosphate buffer (1 mmol/L, pH7.4), glucose (0.5%) and $MgSO_4$ (1 mmol/L), treated with 0.4 mmol/L PCP. The ATP levels in PCP-induced cells was significantly different from that in uninduced cells, $^*P$<0.05, $^{**}P$ <0.01 (Student's *t*-test). B: 3×10^9 *E. coli* cells/mL were treated for 30 minutes with 100 μmol/L, 15 μmol/L or 1.5 μmol/L CuOP. There were no significant differences between uninduced and PCP-induced cells in the effects of CuOP on ATP contents. Cellular ATP was determined by chemiluminescence method and expressed as the percent of initial light intensity value.

Mechanism Underlying CuOP-Enhanced Cytotoxicity of PCP in E. coli

To detect the mechanism of the synergistic toxicity of CuOP and PCP in *E. coli*, we exposed *E. coli* B for 30 min to 0.3 mmol/L PCP in the presence of 1.5 μmol/L CuOP. It showed that 0.3 mmol/L PCP alone only caused 2% cell death (P<0.001 vs. CuOP/PCP) whereas 1.5 μmol/L CuOP alone caused only 9% cell death (P<0.001 vs. CuOP/PCP) (Table 2). In contrast, combination of PCP and CuOP had almost caused death of all *E. coli cells*, demonstrating that CuOP and PCP had a synergistic cytotoxicity in *E. coli*.

Although PCP induced an increase in endogenous SOD and catalase activity (Table 1), the present results showed that low concentrations (10 μg/mL) of catalase and SOD had little protection against the damage induced by PCP plus CuOP while 100 μg/mL catalase and SOD could partly protect against the damage (Table 2). In order to find out whether their ability to halt the inactivation of the cells resulted from their enzymatic activity, heat-inactivated catalase and BSA were added to the reaction mixture. The inactivated catalase and BSA were also efficient in reducing cell death (Table 2). The present results also showed that a free radical scavenger mannitol had a little protection (96% of cell deaths) against the damage induced by PCP and CuOP, while 2-mercaptoethanol, histidine, bathocuproinedisulfonic acid (BCS) or DTPA decreased the cell death induced by PCP and CuOP (Table 2).

TABLE 2

Cytotoxicity of PCP in the Presence of CuOP and the Effects of Free Radical Scavengers, Proteins, and Copper Chelators ($\bar{x} \pm s$)

CuOP (1.5 μmol/L)	PCP (0.3 mmol/L)	Additive	Survival% (n=3)
+	+	-	0.21±0.12
+	-	-	91±19.00
-	+	-	98±9.00
+	+	10 μg/mL Catalase	3.2±0.60
+	+	10 μg/mL SOD	3.0±1.00
+	+	10 μg/mL BSA	5.7±3.40
+	+	100 μg/mL Catalase	74±8.00
+	+	100 μg/mL Heat-inactive Catalase	74±10.00
+	+	100 μg/mL SOD	25±7.00
+	+	100 μg/mL BSA	100±4.00
+	+	1 mmol/L Mannitol	4.0±3.40
+	+	1 mmol/L 2-mercatoethanol	88±8.00
+	+	1 mmol/L Histidine	92±10.00
+	+	0.1 mmol/L BCS	85±17.00
+	+	0.1 mmol/L DTPA	98±4.00

Note. All the reaction mixtures contained 4.8×10^6 *E. coli* cells/mL, glucose (0.5% w/v) and $MgSO_4$ (1 mmol/L) in HEPES buffer (10 mmol/L. pH7.4). CuOP was prepared in DMSO. PCP was dissolved in 0.1 mol/L NaOH. Survival was determined 30 minutes after treatment.

When PCP (0.4 mmol/L) was mixed with CuOP (40 μmol/L, Cu:OP=1:1) in HEPES buffer (10 mmol/L, pH7.4), the solution turned pale purplish red. Analysis of the solution with UV-visible spectra (300-800 nm) showed a broad peak (maximum absorbance wavelength around 335 nm), and there was no absorbance of copper plus OP, CuOP plus BSA and copper plus PCP or PCP alone, suggesting that a PCP-Cu-OP complex was formed. Our data confirmed the result of Zhu *et al.*[14] that the PCP-Cu-OP complex was tentatively assumed to be $[PCP]_2[Cu^{II}(OP)_2]$. After BSA was put into the mixture of CuOP and PCP, the absorbance was enhanced, suggesting that BSA might have a weak interaction with PCP-Cu-OP complex.

In addition, the present results showed that cationic uncoupler CuOP had no synergetic effect on the decline of cellular ATP content with PCP (Fig. 4), suggesting that the synergetic cytotoxicity demonstrated by CuOP and PCP in *E. coli* was not caused by their effects on oxidative phosphorylation.

DISCUSSION

E. coli might have an adaptive response to uncoupler of oxidative phosphorylation 2,4-dinitrophenol[9], as

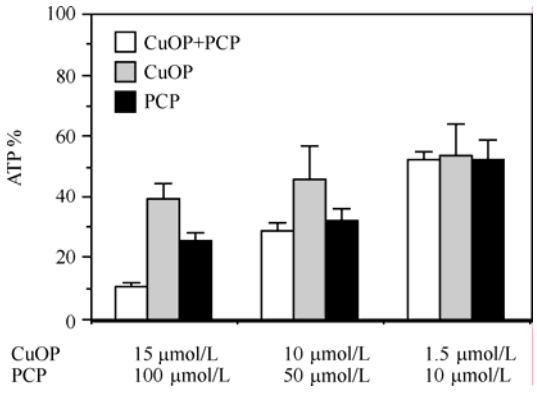

FIG. 4. Effects of CuOP and/or PCP on cellular ATP levels. Uninduced *E. coli* B cells were exposed to different concentrations of CuOP and/or PCP for 30 minutes. Cellular ATP levels were measured as described in Materials and Methods. CuOP had no synergetic effect on the decline of cellular ATP content by PCP.

they respond to heat, oxidants, mutagens, and some other factors[8]. The present results showed that PCP treatment could lead *E. coli* to become PCP-resistant. It was observed that compared with that of uninduced cells, the cellular content of PCP significantly declined in PCP-resistant cells as shown by HPLC analysis, suggesting that *E. coli* can develop an adaptive system to detoxify and remove PCP from the cells. The levels of SOD and catalase were higher in PCP-induced cells than in wild type cells, suggesting that PCP might induce production of reactive oxygen species (ROS)[22] because the restoration of oxidative phosphorylation from uncoupling might contribute to enhanced reactive oxygen species production[23]. The present results showed that PCP caused extensive biological damage in the presence of CuOP in *E. coli*. PCP-induced cells were more resistant to toxicity caused by PCP or by CuOP plus PCP than PQ-induced cells (data not shown). But the levels of endogenous SOD and catalase in PCP-induced cells were less than PQ-induced cells (Table 1), suggesting that the toxicity of PCP plus CuOP and PCP alone did not have the same mechanism as PQ plus copper which produces ROS[24-25]. Neither •OH scavenger (mannitol) nor SOD and catalase (10 μg/mL) inhibited bacterial killing but high concentration (100 μg/mL) catalase and SOD could partly prevent cell killing of PCP plus CuOP. However, the same concentration of heat-inactive catalase and BSA also had protective effects, suggesting that the protective effects of catalase and SOD are attributed to their general properties as proteins, rather than their specific enzymatic activities. Under the same experimental conditions, Zhu *et al.*[14] showed that neither pentachlorophenoxyl radical nor tetrachlorosemiquinone radical could be detected by ESR technique. Thus, under this experimental condition, it seems that pentachlorophenoxyl radical does not play an important role in the synergistic cytotoxicity of PCP and $Cu(II)OP_2$.

The mechanism by which proteins protect against biological damage may be due to the interaction among proteins, CuOP, and PCP. In our study, PCP interacted with CuOP to form a complex of PCP-Cu-OP as demonstrated in UV-visible spectra, The data were in agreement with those of Zhu and Chevion[14]. We showed that BSA had a weak interaction with PCP-Cu-OP complex, while histidine and 2-mercaptoethanol reduced cytotoxicity induced by PCP and CuOP, suggesting that the PCP-Cu-OP complex interacted with cell proteins, and binding sites might be histidine residues and/or sulfhydryl groups. PCP targets mainly on the cell membrane level[26] whereas cytoplasmic membrane is the target organelle by transition metals[19]. It is believed that cell membrane is the major target for damage caused by CuOP and PCP. The interaction of PCP-Cu-OP complex with cell membrane proteins could disrupt the protein functions, and then led to further biological damage. At neutral pH, the net charge of the surface of *E. coli* B cells is negative[27], the negative charge layer might be involved in binding CuOP cation ions and excludsion of PCP anion ions. Lipid bilayers of outer membrane are usually quite permeable to uncharged lipophilic molecules[28]. The PCP-Cu-OP complex is easier to cross the bacterial outer membrane than CuOP cation ions or PCP anion ions alone, which might be the reason why CuOP had a synergetic toxicity with PCP. Formation of a lipophilic ternary complex of $Cu(OP)_2$/PCP could enhance its penetration through the cytoplasmic membrane, and consequently increase the cellular uptake of copper ions by bacterial cells compared to $Cu(II)(OP)_2$ complex alone. In fact, about 20-fold copper had been found inside the cells in the presence of PCP[14].

ATP is formed by oxidative phosphorylation and the proton-translocating ATPase (H^+-ATPase) plays the central role in ATP synthesis[29-30]. PCP is a known uncoupler of oxidative phosphorylation and inhibits amino acid transport by binding to specific sites on proteins and changes membrane permeability[31-32]. It has been postulated that the uncoupling of oxidative phosphorylation by PCP might account, at least in part, for its cytotoxicity. There is evidence that the uncoupler PCP interacts with H^+-ATPase[33]. The $(o\text{-phenanthroline})_2\text{-}Cu^{2+}$ complex is a cationic uncoupler of oxidative phosphorylation, its uncoupling is due to its

modification of sulfhydryl groups in the H^+-ATPase[34-35]. Our results indicate that CuOP has a mechanism of uncoupling oxidative phosphorylation different from that of PCP. CuOP and PCP had a synergistic cytotoxicity in *E. coli* but it had nothing to do with uncoupling of oxidative phosphorylation.

CONCLUSION

Adaptation of *E. coli* to PCP decreaseed the synergistic effects of 1,10-phenanthroline-copper and pentachlorophenol on prokaryotic cell death, which is due to the formation of lipophilic PCP-Cu-OP complex.

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