Proapoptotic effect of a micropollutant (tris-(2-chloroethyl)-phosphate) at environmental level in primary cultured renal proximal tubule cells

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ABSTRACT

Being a typical micropollutant, tris-(2-chloroethyl)-phosphate (TCEP) is often found in aquatic environments. However, the potential effects of TCEP at environmental concentrations on apoptotic mechanisms are mostly unknown. Thus, the purpose of this study is to investigate the apoptotic regulatory protein expression of TCEP at environmental concentration in primary cultured renal proximal tubule cells (PTCs). The results show that TCEP at 0.01 and 1 mg L^{-1} significantly increased the phosphorylation of c-Jun-NH2-terminal kinase (JNK) (135.5 and 138.0% of the control, respectively), and significantly decreased the expression of Bcl-2 and cIAP-2 at all tested concentrations, except for a slight decrease of Bcl-2 at 0.01 mg L^{-1}. In addition, TCEP significantly increased the expression of caspase-3 at all three concentrations (132.6, 172.6 and 167.9% of the control, respectively) and caspase-9 at 1 and 10 mg L^{-1} (128.3 and 144.5% of the control, respectively). Furthermore, TCEP increased the apoptotic cell population in a flow cytometry analysis. In conclusion, environmental TCEP might have a dose-dependent proapoptotic effect with a decrease of DNA synthesis and cell number in primary cultured renal PTCs.

Key words | cell apoptotic regulatory protein, environmental concentration, fire retardant, micropollutant, primary cultured renal proximal tubule cells

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INTRODUCTION

As a typical environmental micropollutant (normally ranging between ng L^{-1} and µg L^{-1}), tris-(2-chloroethyl)-phosphate (TCEP), which is used as an organophosphate flame retardant (IPCS 1998), is often found in surface water, wastewater treatment plants, oceans and drinking water (Andresen et al. 2004; Stackelberg et al. 2004, 2007; Kim et al. 2007; Regnery & Püttmann 2010) due to its low elimination rate (Meyer & Bester 2004; Andresen & Bester

2006; Bernhard et al. 2006; Kim et al. 2007). Therefore, the effects of TCEP have been investigated through in vivo studies of its carcinogenic, neurotoxic, mutagenic, and tumoral effects (Simmon et al. 1977; Takada et al. 1989; Tilson et al. 1990; Zeiger et al. 1992; Matthews et al. 1993), and by in vitro studies of its cytotoxic, genotoxic, mutagenic and estrogenic effects (Föllmann & Wober 2006). However, rare effects of TCEP were found in the in vivo test, especially

in the *in vitro* study at environmental concentrations. Due to the subtle effects of micropollutants, including TCEP and other substances, many current cellular models might not be relevant in the *in vitro* study of environmental micropollutants. Thus, Ren *et al.* (2008, 2009) have developed new sensitive cellular models consisting of cell cyclic regulatory protein expression and primary cultured renal proximal tubule cells (PTCs), and of ion and non-ion transportation and the PTCs. In these studies, they reported on the subtle effects of TCEP at environmental levels on the growth and function of PTCs.

In this study, we develop a new cellular model consisting of cell apoptotic regulatory protein expression and PTCs in order to study the subtle effects of TCEP at environmental concentrations. In the cell apoptosis mechanism, a useful tool for studying cellular effects, the caspase pathway related to proteins plays a critical role because caspases, being a family of cysteine proteases, form a proteolytic cascade that cleaves distinct and vital proteins (Nuñez *et al.* 1998). Also, as mentioned in previous studies (Ren *et al.* 2008, 2009), primary cultured renal PTCs are a powerful tool for studying renal morphological and molecular effects of low level concentrations of target chemicals. Previous studies have shown that cells typically have a number of differentiated functions, pertaining to renal proximal tubules, including a polarized morphology, as well as a distinctive proximal tubule transport and hormone response (Chung *et al.* 1982; Han *et al.* 2005, 2007; Lee & Han 2006; Lee *et al.* 2006). Thus, the aim of this study is to investigate the proapoptotic effect of TCEP at environmental level via an examination of cell apoptotic regulatory protein expression, cell cycle phase distribution, and cell population of apoptosis in PTCs.

MATERIALS AND METHODS

Chemical and antibodies

TCEP was purchased from Sigma-Aldrich (Carlsbad, CA, USA) and New Zealand White male rabbits (1.5–2.0 kg) were obtained from Dae Han Experimental Animal Co., Ltd (Chungju, Chungchongbuk-do, Korea). Class IV collagenase and soybean trypsin inhibitor were purchased

from Life Technologies (Gibco BRL, Grand Island, NY, USA). Antibodies of cIAP-2, caspase-9, and caspase-3 were acquired from Santa Cruz Biotechnology (Delaware, CA, USA). Bcl-2, phosphorylated c-Jun-NH2-terminal kinase (p-JNK), and total JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). In addition, β-actin antibody was obtained from Sigma-Aldrich, and goat anti-rabbit IgG was supplied by Jackson Immunoresearch (West Grove, PA, USA). Note that all reagents used were of the highest purity commercially available. Liquiscint was obtained from National Diagnostics (Parsippany, NY, USA).

Cell preparation and culture condition

Primary rabbit renal PTC cultures were prepared using the method reported by Chung *et al.* (1982). Initially, rabbit kidneys were perfused through the renal artery, first with phosphate buffered saline (PBS), and then with 0.5% iron oxide solution. Next, renal cortical slices were prepared and homogenized. The homogenate was sequentially poured through 253 and 83-μm mesh filters. Tubules and glomeruli were then recovered from the top of the 83-μm filter and transferred into a sterile medium. At this time, the glomeruli-containing portion (containing iron oxide) was removed using a magnetic stir bar, and the remaining proximal tubules were incubated briefly in a medium containing collagenase (0.125 mg mL^{-1}) and 0.025% soybean trypsin inhibitor. The tubules were then washed by centrifugation, resuspended in a medium containing the three supplements, and transferred into tissue culture dishes. During experimentation, the medium was changed 1 d after plating and every 2 d thereafter; the primary cultured rabbit kidney PTCs were maintained at 37 °C in a 5% CO$_2$ humidified environment in a serum-free basal medium (D-MEM/F-12 medium (Gibco-BRL, Gaithersburg, MD, USA) using 15 mM HEPES and 20 mM sodium bicarbonate (pH 7.4)), which was supplemented with the three growth supplements (5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, and 5×10^{-8} M hydrocortisone).

Cell count and lactate dehydrogenase assay

The cellular viability and damage of primary cultured renal PTCs were studied via cell count and lactate dehydrogenase

(LDH) assay. The total number of cells was counted using the following methodology. The cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was then mixed with a 0.4% (wt vol^{-1}) trypan blue solution and the number of live cells was determined using a hemocytometer; cells failing to exclude the dye were considered non-viable. Cell injury was assessed by LDH activity, with the LDH activity level in the medium measured using a LDH assay kit (TaKaRa, Japan). For measurement of LDH activity, the cells were treated with different concentrations of TCEP for 24 h, then with the LDH assay kit for 30 min. LDH activity was detected by microplate reader with 490 nm, then expressed as the fold-increase of control.

[³H] thymidine incorporation

The effect of DNA synthesis of TCEP on the PTCs was investigated via [³H] thymidine incorporation. The medium was changed for the last time when the cells reached 70–80% confluence. The thymidine incorporation experiments were then conducted according to the method described by Gabelman & Emerman (1992). The cells were incubated in the medium in the presence or absence of TCEP for 24 h and pulsed with 1 μCi of [*methyl*-³H] thymidine for 1 h at 37 °C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) (Sigma, USA) at room temperature for 15 min, and then washed twice in 5% TCA. Next, the acid-insoluble material was dissolved in 2 N NaOH at room temperature, and the level of radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA). All experiments were performed in three dishes in each of the three independent experiments, and values were converted from absolute counts to percentage of control to allow a comparison between the experiments. Again, the number of viable cells was determined by a hemocytometer using a 0.4% (wt vol^{-1}) trypan blue solution.

Western blotting assay

The effect of cell apoptotic regulatory protein expression was studied via Western blotting assay. After confluent culturing of the cells under each specified condition, the medium was then removed. The cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and resuspended in Buffer A [137 mM NaCl, 8.1 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10-μg mL^{-1} leupeptin (pH 7.5)]. The resuspended cells were then mechanically lysed on ice via trituration with a 21.1-gauge needle. The cell lysates were initially centrifuged at 1,000 g for 10 min at 4 °C, and the supernatants were collected as a total cell fraction, and the protein was quantified using the Bradford procedure (1976). In the gel transfer chamber, the cell lysates (30 μg of protein) were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane at 100 V for 1 h. The blots were then washed with H$_2$O, blocked with 5% skimmed milk powder in TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 1 h and incubated with the primary polyclonal antibodies at the dilutions recommended by the supplier. Finally, the primary antibodies were detected using goat anti-rabbit-IgG conjugated to horseradish peroxidase, and the bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

Flow cytometry assay

The effect of cell cycle phase distribution was investigated via flow cytometry assay. The fluorescence activated cell sorting (FACS) propidium iodide (PI) staining protocol was used to detect the cell cycle distribution of primary cultured rabbit renal PTCs using a flow cytometry instrument. The PTCs treated by TCEP were detached from a 60-mm culture dish using a cell scraper, and placed into a 5-mL tube. The cells were then centrifuged in the tube at 2,000 rpm for 5 min at a constant temperature of 4 °C. The supernatants were removed, and centrifuged cells were washed using 4 mL of Dulbecco's PBS. The cells were again centrifuged, and the supernatants aspirated. Next, 200 μL PBS was added to the tube, and the cells were resuspended using a pipette. After making a single cell using pipetting, cold 70% ethanol (1.8 mL) was added to the cells with continuous vortexing. The cells were then stored in a −20 °C refrigerator for more than

2 h to ensure cell fixation. Next, the fixed cells were centrifuged at 2,000 rpm and 4 °C for 5 min. At this time, the supernatants were removed from the tube, and 3 mL PBS was added to the tube. The cells were resuspended using a pipette, and incubated at 37 °C for 60 s. The cells were again centrifuged at 2,000 rpm and 4 °C for 5 min, and the supernatants (PBS) were aspirated. The cells were then resuspended using a 1-mL PI solution consisting of 10% triton X-100, 20 mg mL^{-1} Rnase, 1 mg mL^{-1} PI, and PBS solution, and incubated at 37 °C for 15 min (or at room temperature for 30 min). Finally, the stained samples were detected using a flow cytometer (Beckman Coulter, USA).

Statistical analysis

The results were expressed as the mean ± standard error (SE). Note that all the experiments were analyzed by analysis of variance (ANOVA) and some experiments were examined by comparing the treatment means to controls using the Bonferroni–Dunn test, with a P-value < 0.05 and P-value < 0.01 being considered significant.

RESULTS

Effect of TCEP on cell damage and DNA synthesis

The effect of cellular damage and DNA synthesis of primary cultured renal PTCs treated with TCEP at varying concentrations were studied via a LDH assay and [^{3}H] thymidine incorporation (Figure 1). To investigate the effect of TCEP on cell damage, concentrations of TCEP ranging from 0.01 to 10 mg L^{-1} were added to the cell culture medium and allowed to incubate for 24 h, as shown in Figure 1(a). In the figure, TCEP at 0.01 mg L^{-1} did not incur cell damage (102.0% of the control), but significantly induced cell injury at 1 and 10 mg L^{-1}, to 158.0 and 184.0% of the control, respectively. Figure 1(b) then presents the effects of TCEP treatments on DNA synthesis, investigated via [^{3}H] thymidine incorporation; TCEP at 0.01 mg L^{-1} slightly decreased DNA synthesis (82.4% of the control), which significantly decreased at 1 and 10 mg L^{-1}, to 53.7 and 51.2% of the control, respectively.
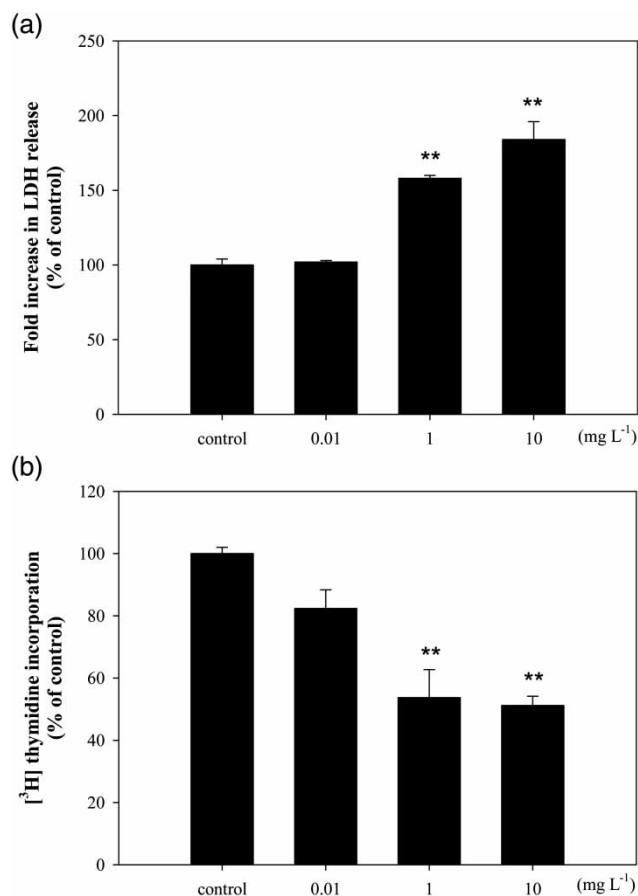

Figure 1 | Effect of TCEP on LDH release and DNA synthesis. (a) PTCs were treated with TCEP for 24 h. The LDH release (a) and [^{3}H] thymidine incorporation (b) were measured as described in Materials and methods. The values are the means ± S.E. of three independent experiments conducted in triplicate dishes. **$P < 0.01$ vs. control.

Effect of TCEP on cell number

The effect of TCEP on cell number was then studied based on a cell count assay, as shown in Table 1. TCEP treatment for 24 h slightly decreased the cell number at

Table 1 | Effect of TCEP on PTC cell number

TCEP (mg L^{-1})	Cell number ($\times 10^4$/dish)
Control	376 ± 9.3
0.01	324 ± 5.9
1	221 ± 11.2**
10	191 ± 7.7**

The PTCs were treated with 0.01, 1 and 10 mg L^{-1} TCEP for 24 h. Then, cell count assay was conducted. Values are the means ±S.E. of three independent experiments conducted in triplicate dishes. **$P < 0.01$ vs. control.

0.01 mg L^{-1} (86.2% of the control), though the count significantly decreased at 1 and 10 mg L^{-1}, to 58.8 and 50.8% of the control, respectively.

Effect of TCEP on cell apoptotic regulatory protein expression

In order to determine the effect of TCEP on apoptotic regulatory proteins, the PTCs were respectively treated with 0.01, 1, and 10 mg L^{-1} of TCEP for 24 h and the protein expressions were examined by Western blotting. Figure 2 shows the results of JNK phosphorylation and expression of total JNK, where TCEP did not affect the expression of total JNK at any concentration, but significantly increased the phosphorylation of JNK at 0.01 and 1 mg L^{-1} to 135.5 and 138.0% of the control, respectively. However, TCEP at 10 mg L^{-1} slightly increased the phosphorylation of JNK, to 109.9% of the control.

The protein expressions of Bcl-2 and cIAP-2, as cellular inhibitors of apoptosis proteins, were also investigated using different TCEP concentrations, the results of which are shown in Figure 3. TCEP at 0.01 mg L^{-1} slightly decreased the expression of Bcl-2, to 95.1% of the control,
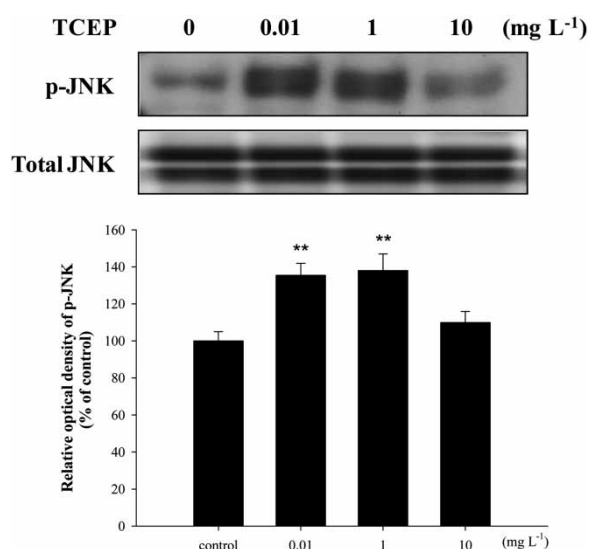

Figure 2 | Effect of TCEP at different concentrations on the activation of JNK. Each example shown is representative of three experiments. The panel denotes the mean means ± S.E. of three experiments for each condition as determined from densitometry relative to total JNK. **$P < 0.01$ vs. control.
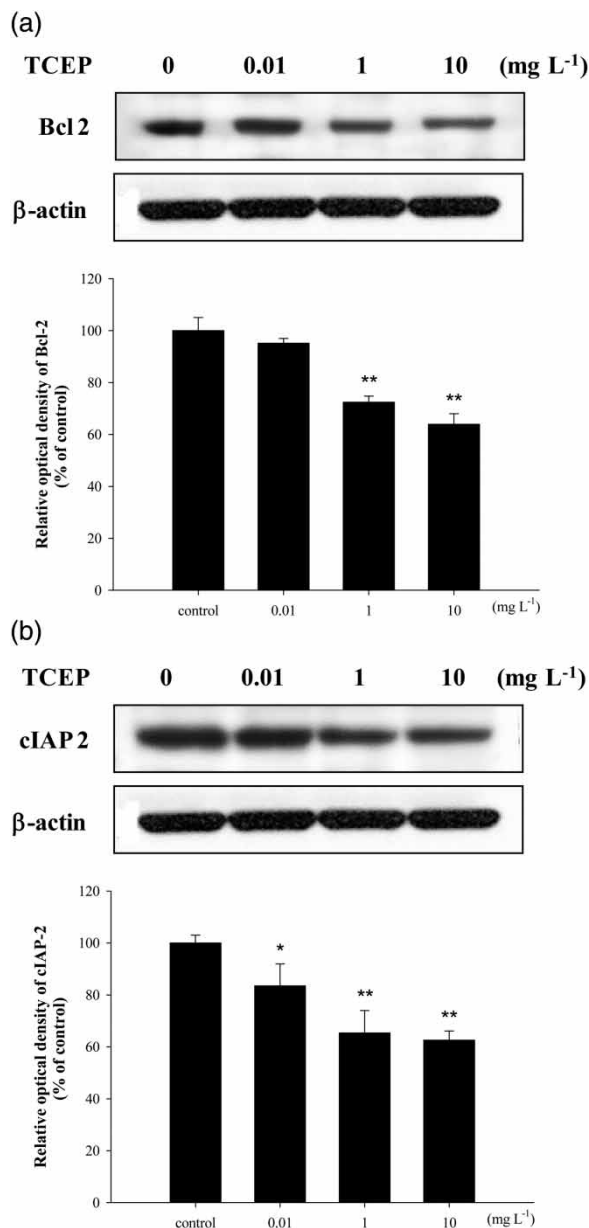

Figure 3 | Effect of TCEP on the expression of Bcl-2 (a) and cIAP-2 (b). Each example shown is representative of three experiments. The panel denotes the mean means ± S.E. of three experiments for each condition as determined from densitometry relative to β-actin. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

but significantly decreased the expression at 1 and 10 mg L^{-1}, to 72.4 and 63.9% of the control, respectively (Figure 3(a)). In the study of cIAP-2 expression (Figure 3(b)), TCEP significantly decreased the expression of cIAP-2 for all tested concentrations of TCEP (0.01, 1, and 10 mg L^{-1}), showing 83.5, 65.4, and 62.5% of the control, respectively.

In the study of caspase-9 and caspase-3 protein expressions (Figure 4), TCEP slightly decreased the expression of caspase-9 protein, to 93.6% of the control, but significantly increased the expression at 1 and 10 mg L^{-1}, to 128.3 and 144.5% of the control, respectively (Figure 4(a)). Similarly, Figure 4(b) shows that the TCEP significantly increased the expression of caspase-3 protein for all tested concentrations (132.6, 172.6, and 167.9% of the control, respectively).
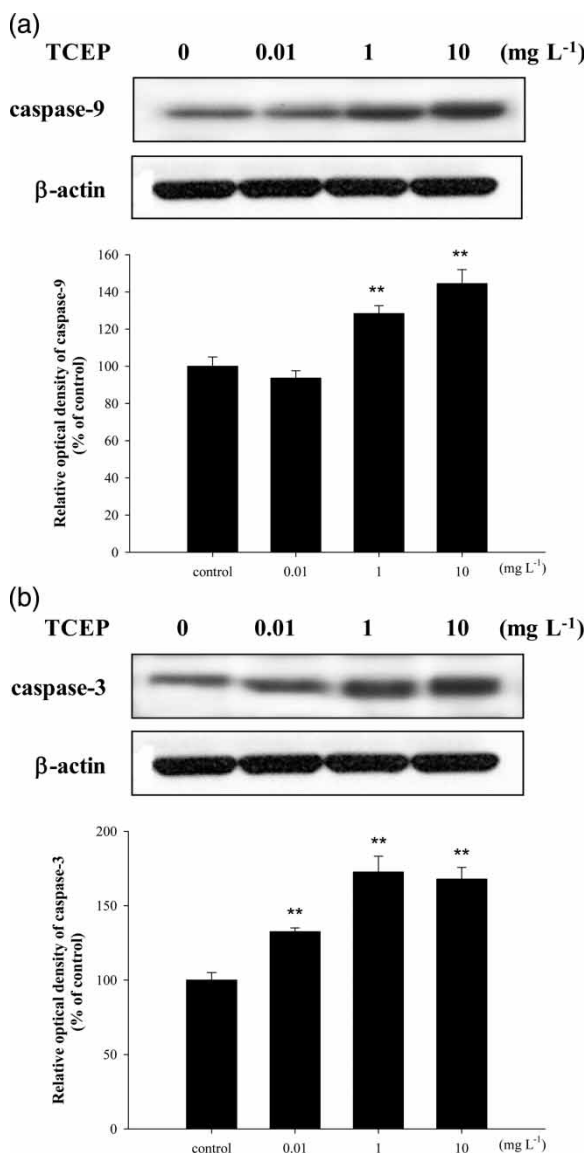

Figure 4 │ Effect of TCEP on the expression of caspase-9 (a) and caspase-3 (b). Each example shown is representative of three experiments. The panel denotes the mean means ± S.E. of three experiments for each condition as determined from densitometry relative to β-actin. **$P < 0.01$ vs. control.

Effects of TCEP on cell cycle phase distribution

In order to study apoptotic effects of TCEP on the PTCs, the cell cycle phase distribution was investigated via a flow cytometric assay, as shown in Table 2. Most PTCs were in G0/G1 (41.1–48.4%) and G2/M phases (47.8–55.9%), but only around 1.5% cell number percentage at DNA synthesis phase (S phase). TCEP did not affect cell cycle phases (G0/G1, S, and G2/M phases) with dose-dependence. However, the DNA flow cytometric analysis (Figure 5) showed that TCEP significantly induced cellular apoptosis at 1 and 10 mg L^{-1}, with values of 31.3 and 58.0%, respectively. At the concentration 0.01 mg L^{-1}, TCEP only slightly increased the apoptosis cell population (by 10.5%).

DISCUSSION

The present experiment investigated the effect of TCEP on the apoptotic mechanism of PTCs. The investigation was conducted after a study of cell cytotoxicity and cell cyclic regulator protein expression (Ren *et al.* 2008) and ion and non-ion transportation in primary cultured renal PTCs (Ren *et al.* 2009). In this study, the environmental concentration was designed to 0.01 mg L^{-1} based on the presence of TCEP in an aquatic environment, from 2.0×10^{-5} to 2.6×10^{-3} mg L^{-1} (Andresen *et al.* 2004; Stackelberg *et al.* 2004, 2007; Kim *et al.* 2007; Regnery & Püttmann 2010). The results from this study demonstrated that TCEP had slight or no effect at environmental levels on DNA synthesis, cell number, and cell damage, but significant effect at higher concentrations; the results showed a similar trend to previous research by Ren *et al.* (2008, 2009).

Table 2 │ Effects of TCEP on the cell cycle distribution of PTCs

	Cell cycle phase		
TCEP (mg L^{-1})	G0/G1	S	G2/M
Control	48.4 ± 6.5	1.6 ± 0.00	47.8 ± 6.1
0.01	46.0 ± 3.7	1.5 ± 0.13	51.4 ± 3.9
1	41.1 ± 0.66	1.6 ± 0.15	55.9 ± 0.78
10	42.3 ± 0.07	1.5 ± 0.19	55.0 ± 0.33

Primary cultured renal PTCs were treated with 0.01, 1, and 10 mg L^{-1} TCEP for 24 h. Values are the means ± S.E. of three independent experiments conducted in triplicate dishes.
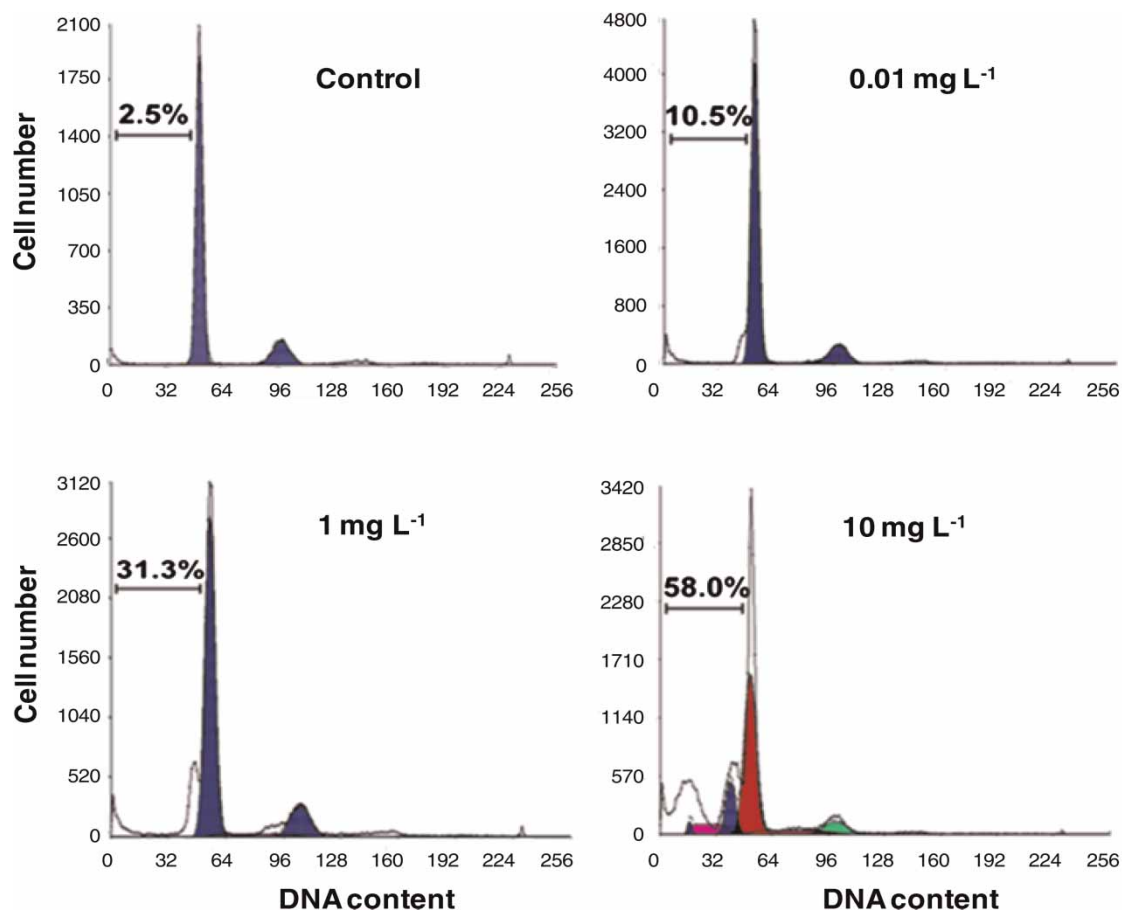
Figure 5 │ Effect of TCEP on cell apoptosis in a flow cytometry assay. The values are the means ± S.E. of three independent experiments conducted in triplicate dishes. **$P < 0.01$ vs. control.

The findings of the present study also demonstrated that TCEP affected apoptosis-related regulatory protein expression levels. Caspases are cysteine proteases that are activated by apoptotic stimuli, and caspase-3 is a crucial enzyme in caspase-dependent apoptosis (Shastry *et al.* 2007). Moreover, Bcl-2 and IAP family proteins are involved in the inhibition of apoptosis upstream of caspase-3 activation (Kim *et al.* 2008; Lee *et al.* 2008; Moon *et al.* 2008). In this study, the higher concentrations of TCEP (1 and 10 mg L^{-1}) significantly decreased the expressions of Bcl-2 and cIAP-2 and increased the expressions of caspase-9 and caspase-3. However, these responses were not distinct in PTCs treated with 0.01 mg L^{-1} TCEP. Indeed, even though the target chemical was different from the present study, Pomati *et al.* (2006) also attempted an investigation at environmental levels to determine the apoptotic effects of

a complex mixture of pharmaceuticals in HEK293 cells due to the activation of caspase-3, caspase-9 and other apoptosis-related regulatory proteins, but failed to detect any effects – although morphological changes were found.

Furthermore, the present study showed that TCEP stimulated JNK phosphorylation; evidence was obtained that shows that JNK can promote or suppress apoptosis. The activation of JNK is involved in cell proliferation or cell survival in various cells (Potapova *et al.* 2002; Ventura *et al.* 2006; Kim *et al.* 2010). However, many studies showed the role of JNK as an apoptosis inducer (Lei *et al.* 2002; Deng *et al.* 2003; Brnjic *et al.* 2010). The results of the present investigation on the expression levels of apoptotic regulatory proteins strongly supported previous studies that the increase of p-JNK might decrease the expression of Bcl-2 (Lei *et al.* 2002; Weston & Davis 2002), and that the decreased Bcl-2 and cIAP-2 might

increase the expression of caspase-9 (Luo *et al.* 1998; Jönsson *et al.* 2003; Yang *et al.* 2009). In addition, the increased caspase-9 might increase the expression of caspase-3 (Nuñez *et al.* 1998; Denault *et al.* 2007; Twiddy & Cain 2007), and that the increased caspase-3 levels might subsequently induce cell apoptosis (Woo *et al.* 1998; Zhivotosky & Orrenius 2001; Yang *et al.* 2009). Practically, we found that rising levels of TCEP decreased DNA synthesis and cell number but increased cell population of apoptosis in flow cytometry analysis.

In conclusion, TCEP might induce apoptosis of the PTCs dose dependently due to a significant decrease in anti-apoptotic regulatory protein (Bcl-2 and cIAP-2) expressions, and a significant increase in proapoptotic protein (JNK, caspase-3, and caspase-9) activation in the PTCs. Therefore, these results not only warn of the potential risks of environmental TCEP on the ecosystem and human health, but also show the potential use of PTCs as a cellular model for subsequent molecular toxic studies of other environmental micropollutants.

ACKNOWLEDGEMENTS

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