EFFECTS OF PENTOXIFYLLINE ON ACTIVE OXYGEN-INDUCED SISTER-CHROMATID EXCHANGE

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ABSTRACT—Active oxygens cause major tissue damages in a number of distress syndromes. Pentoxifylline (POF) and its metabolites, a methylxanthine derivative, are inhibitors of superoxide anion production in stimulated human leukocytes. Examination was made of the effects of POF on the genotoxic actions of active oxygens. The frequency of sister-chromatid exchanges (SCEs) induced by paraquat (PQ), a generator of superoxide anions, was significantly reduced in cultured Chinese hamster lung cells (CHL/IU) pretreated with POF. POF metabolite samples were also effective. Superoxide anion scavenging effect of POF were found at 0.1 mM, and it was more potent than that of POF metabolite samples. POF and its metabolite sample inhibited xanthine oxidase (XOD) at 1 mM but not at 0.1 mM. POF would thus appear to protect cells from the toxic effects of active oxygens on chromosomes and tissue damage by not only inhibition of active oxygen production in leukocytes but also by scavenging activity of active oxygens.

KEY WORDS : Active oxygen, Pentoxifylline, Sister-chromatid exchange, Paraquat.

INTRODUCTION

In a previous paper, we reported the induction of sister-chromatid exchanges (SCEs) with active oxygens produced by paraquat (PQ) or diquat (Tanaka *et al.*, 1989). The frequency of SCE in cultured Chinese hamster lung cells (CHL/IU) increased at concentrations of these herbicides lower than chromosomal aberration-inducible concentrations and also at concentrations low enough to stimulate cell growth and DNA synthesis (Tanaka and Amano, 1985).

Pentoxifylline (POF), a methylxanthine derivative, is known to increase red blood cell ATP level, resulting in enhancement of cell deformability (Ehrly, 1979). Recently, inhibitable effects of POF on the generation of superoxide

anion and hydrogen peroxide were also reported in stimulated human leukocytes *in vitro* (Kumar *et al.*, 1992 ; Bessler *et al.*, 1986), and *in vivo* (Crouch and Fletcher, 1992). Bessler *et al.* suggested that these effects are due to increased intracellular levels of c-AMP. Crouch and Fletcher (1992) also demonstrated the POF effect by metabolite analysis.

In this paper, we examined the protective effects of POF on active oxygen-induced cell damage, and especially on genotoxicity.

MATERIALS AND METHODS

Sister-chromatid exchange (SCE) analysis *in vitro* : CHL/IU cells were obtained from Dainippon Pharmaceutical Co., Ltd. These cells were seeded at a density of 10^5 cells/ml into rectangular bottles, and then incubated for approximately 20 hr at 37°C in Eagle's minimum essential medium

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(MEM ; Nissui Pharmaceutical Co., Ltd.) supplemented with 10% calf serum (new calf serum ; Nacalai tesque, INC.).

To test the effect of POF (Sigma Chemical Co., St Louis, MO.), cells were preincubated with various concentrations of POF, POF metabolite samples or POF metabolite control samples for 1 hr. After washing with phosphate-buffered saline (PBS), cells were exposed to $4\text{ }\mu\text{M}$ PQ (1,1'-dimethyl-4,4'-bipyridylum dichloride, Wako Pure Chemical Industries Co. Ltd.), superoxide anion generator, and to $10\text{ }\mu\text{M}$ 5-bromo-2'-deoxyuridine (BrdU ; Sigma Chemical Co., St Louis, MO.) for 24 hr in complete darkness.

Colcemid ($0.2\text{ }\mu\text{g/ml}$, GIBCO Laboratories) was added to each bottle 4 hr before the end of the incubation period. At the end of the incubation period, the cells were harvested by the addition of trypsin and subsequently centrifuged ($112\times g$, 5 min). After treating the cells with 0.04 M KCl for 15 min at 37°C , the chromosomes were obtained by centrifugation ($175\times g$, 5 min) and fixed with methanol-acetic acid (3 : 1 v/v). These procedures were performed in the dark.

The chromosomal preparations described above were stained with 2% Giemsa's solution (MERCK) prepared in 0.3 M Na_2HPO_4 at pH 10.4 (adjusted with 1N NaOH) for 30 min (Ikushima and Wolff, 1974). With this technique, chromatids that had incorporated BrdU were darkly stained. The results were recorded as the frequency of SCE/cell for chromatids that had undergone two replication cycles (Tanaka and Amano, 1989).

Preparation of POF metabolite samples : Liver-microsome fraction (S9) was prepared from male Sprague-Dawley rats (8–10 weeks age). For induction of microsomal enzymes, rats were given 50 mg/dl sodium phenobarbital in drinking water for 1 week. The livers of these rats were carefully homogenated in ice-cold 1.15% KCl solution in a loosely fitting Teflon homogenizer. The homogenate was adjusted to 2 ml/g of wet liver weight and then centrifuged at 4°C for 20 min at $9000\times g$. Supernatant (S9) was stored at -80°C until used (Ames *et al.*, 1973).

The microsome reaction mixture (S9 mix) consisted of 3 ml of S9, 2 ml of 20 mM HEPES buffer (pH 7.2), 1 ml of 50 mM MgCl_2 , 1 ml of

330 mM KCl, 1 ml of 50 mM glucose 6-phosphate, and 1 ml of 40 mM NADP (Morita, 1989). Various concentrations of POF (1 ml) mixed with S9 mix were incubated for 60 min at 37°C . After cold centrifugation for 5 min at $2000\times g$, the resultant supernatant was used as the POF metabolite sample (POFM), which contains POF and its metabolites. As control to POFM, POF free samples were prepared (POF metabolite control samples, POFC).

Xanthine oxidase activity : To examine the effects of POF and POFM on superoxide anion generation system, xanthine oxidase (XOD) activity was determined spectrophotometrically by measuring the conversion of hypoxanthine to uric acid at 25°C . Reaction solution consisted of 2.5 ml of 50 mM K buffer (pH 7.4, contained 0.1 mM EDTA-2Na), 0.3 ml of POF or POF metabolite sample or POF metabolite control sample, 0.1 ml of XOD (0.4 U/ml , Sigma), and 0.1 ml of 3 mM hypoxanthine. After incubation for 10 min at room temperature, absorbance at 290 nm was determined against a blank sample which did not contain XOD (Robak and Gryglewski, 1988).

Superoxide anion scavenging activity : Scavenging effect of POF on superoxide anions generated from hypoxanthine/xanthine oxidase system by POF and its metabolite samples were examined by measurement of superoxide anions by NBT reduction method (Robak and Gryglewski, 1988).

Statistics : Data were expressed as mean $\pm$ S.E. Student's paired *t*-test or unpaired *t*-test were used for statistical analysis of the results.

RESULTS

Effect on the PQ-induced SCEs : In our previous study (Tanaka and Amano, 1989), a significant increase of SCE frequency was observed with $0.08\text{--}20.0\text{ }\mu\text{M}$ PQ in the 24 hr incubation period. Therefore, $4\text{ }\mu\text{M}$ PQ was selected as the superoxide anion generator in this study.

Fig.1 shows the effect of POF on the frequencies of SCEs induced by PQ, a generator of superoxide anion in cells. When cells were preincubated for 1 hr with various concentrations of POF, a significant decrease in SCE was observed with 1 mM and 0.1 mM POF, but not with 0.01 mM . A total of 50–60 cells were

Fig. 1. Effect of pentoxifylline (POF) on the frequency of sister-chromatid exchange (SCE) induced by paraquat (PQ).

CHL/IU cells were preincubated with POF for 1 hr, and after the washing with PBS, cells were cultured for 24 hr in the presence or in the absence of PQ.

*** $P < 0.001$ vs # ($4 \mu\text{M}$ PQ in the absence of POF)

Table 1. Effect of SOD and catalase on the frequency of sister-chromatid exchange (SCE) induced by paraquat (PQ).

pretreatment (1 hr)	PQ (μM) (24 hr)	SCE/cell	n
0	0	2.70 ± 0.15	54
0	4	$4.07 \pm 0.19^{\#}$	55
SOD (250 U/ml)	4	3.85 ± 0.18	41
catalase (1000 U/ml)	4	$2.72 \pm 0.21^{***}$	46

CHL/IU cells were preincubated with SOD or catalase for 1 hr. After the washing with PBS, cells were cultured with PQ for 24 hr.

*** $P < 0.001$ vs # ($4 \mu\text{M}$ PQ), n : number of recorded cells

recorded.

Table 1 shows the effects of SOD, a scavenger of superoxide anion, and catalase, a scavenger of hydrogen peroxide, on the frequency of SCE induced by $4 \mu\text{M}$ PQ. Cells were preincubated with SOD (250 U/ml) or catalase (1000

U/ml) for 60 min, and then were washed out with PBS three times. The frequency of PQ-induced SCE, determined as described in methods, was significantly decreased ($P < 0.001$) by catalase but not by SOD, indicating that H_2O_2 derived from superoxide anions were scavenged with catalase, but intracellular superoxide anions were not scavenged with SOD.

Fig. 2 shows the numbers of cells (% of recorded cell number) incorporating respective frequencies of SCEs. Data are from one typical experiment, similar to that indicated in Fig. 1. At $4 \mu\text{M}$ PQ, 3–8 SCEs/cell were observed (mean = 4.20 ± 0.14 , highest SCE frequency was 5 SCEs/cell). When cells were preincubated with 1 mM POF, 1–5 SCEs/cell were observed (mean = 2.74 ± 0.15 , highest SCE frequency was 3 SCEs/cell). Similar effectiveness of POF was observed with 0.1 mM POF (3.39 ± 0.14 SCEs/cell), but not with 0.01 mM POF (4.18 ± 0.16 SCEs/cell). Similar results were obtained in four other experiment.

To examine the effect of POF metabolite, POFM samples were prepared as described in

Fig. 2. Effect of 0.01 mM, 0.1 mM and 1 mM pentoxifylline (POF) on the frequency of paraquat (PQ)-induced sister-chromatid exchange (SCE). CHL/IU cells were preincubated with POF for 1 hr, and then cultured with 4 μ M PQ for 24 hr in the absence of POF.

methods. When CHL/IU cells were preincubated with 1.0 mM and 0.1 mM POFM, the frequency of SCE decreased, but this effect was not observed with 0.01 mM POFM (Table 2). The effects of POFM were generally similar to POF itself.

Scavenging of superoxide anions: Effects of POF and POFM on xanthine oxidase samples (XOD) are indicated in Fig. 3. Conversion of hypoxanthine to uric acid was inhibited by 1 mM POF but not by 0.1 mM nor 0.01 mM. The same results were obtained with POFM. 0.1 mM and 0.01 mM POFM did not show apparent XOD inhibitable activity. Table 3 shows the scavenging properties of POF and POFM at concentrations which did not show XOD inhibition. Superoxide anions generated by hypoxanthine/xanthine oxidase system were determined by NBT method as described in methods. Significant scavenging activity was obtained with 0.1 mM POF, but not with 0.01 mM POF nor with both of 0.1 mM and 0.01 mM POFM. Native POF displayed a more potent scavenging activity than POFM.

DISCUSSION

Active oxygens have many toxic actions in human and animals. Toxic effects on cells, especially on DNA has been found to induce cell death and chromosomal abnormality (Tanaka and Amano, 1989).

In our previous experiments, POF weakly decreased the production and release of superoxide anions from cultured human endothelial cells (data not shown). However, intracellular superoxide anion production and release in CHL/IU cells was too small to detect. The SCE analysis method was therefore adopted for active oxygen determination.

In a study employing POF in peripheral vascular disease (Ciuffetti, 1991), POF was found to inhibit the generation of leucocyte-derived active oxygens (superoxide dismutase-inhibitable reduction of ferricytochrome) *in vivo*. Crouch and Fletcher (1992) reported superoxide anion production by polymorphonuclear cells (PMN), and a strong correlation between reduced PMN

Table 2. Effect of POF metabolite samples on the frequency of sister-chromatid exchange (SCE) induced by paraquat (PQ).

POFM (mM) (1 hr)	PQ (μ M) (24 hr)	SCE/cell	n
0	0	3.73 ± 0.19	50
0	4	4.33 ± 0.20	43
1.0	4	$3.36 \pm 0.17^{***}$	39
control	4	4.38 ± 0.15	48
0.1	4	$3.49 \pm 0.16^{***}$	38
control	4	4.63 ± 0.21	30
0.01	4	4.20 ± 0.28	30
control	4	4.57 ± 0.17	46

CHL/IU cells were preincubated with POF metabolites or its control samples for 1 hr. After the washing with PBS, cells were cultured with PQ for 24 hr.

*** $P < 0.001$ vs control, n : number of recorded cells

Table 3. Superoxide anion scavenging activity of POF and POF metabolite samples.

	Concentration (mM)	Reduced NBT (%)
POF	0	100.0 ± 0.4
	0.1	$66.3 \pm 1.0^{***}$
	0.01	101.5 ± 1.8
POFM	0.1	102.0 ± 1.0
	control	100.0 ± 2.3
POFM	0.01	101.0 ± 1.1
	control	100.0 ± 2.1

Superoxide anions were generated by hypoxanthine/xanthine oxidase system and measured by NBT reduction method.

POF : Pentoxifylline, POFM : POF metabolite sample.

Values are mean $\pm$ S.E. for three experiments.

*** $P < 0.001$ vs 0 mM POF

Fig. 3. Effect of POF and POF metabolite samples on XOD.

Values are mean $\pm$ S.E. for 4 experiments (% of control; C, POFMC).

POF : pentoxifylline, POFMC : POF metabolite control sample, POFM : POF metabolite sample, XOD : xanthine oxidase.

*** $P < 0.001$ vs C or POFMC

response to activated complement and plasma concentration of POF metabolites. The same study also suggested that POF reduced oxygen radical production and protected against unwanted tissue damage *in vivo* via the action of its metabolites. Thus, in addition to POF alone, we also examined the effects of POF metabolite samples.

In the present study, 0.1 mM POF decreased the frequency of PQ-induced SCEs (Figs. 1, 2). The frequency of SCEs was also decreased with 0.1 mM POF metabolite samples, but not with 0.01 mM POF metabolite samples. Difference in SCE decreasing activity between POF and POF metabolite samples were not observed.

To determine the POF mechanism of protective action against SCE induction, superoxide anion scavenging activity of POF and POF metabolite samples were investigated. In our experiment using thin layer chromatography, POF metabolite samples were separated to POF (about 70% of total) and two major metabolites (Fuzimoto, 1976; Burrows, 1987). One (about 20% of total) is presumed to be carboxylic acid metabolite, and another (about 10% of total) is hydroxylated metabolite (data not shown).

0.1 mM POF significantly decreased NBT reduction by superoxide anions, generated by hypoxanthine/XOD system, indicating a superoxide anion scavenging effect. However the 0.1 mM POF metabolite sample did not show a scavenging effect (Table 3): neither 0.01 mM POF nor 0.1 mM and 0.01 mM POF metabolite samples showed inhibition of xanthine oxidase (Fig. 3). The scavenging activity of POF was more potent with native POF than with POF metabolite samples. The present experimental procedures possibly include the production (by PQ) and scavenging (by POF) of active oxygens in cells over the 24 hr incubation periods. Therefore, it was assumed that POF effects a decrease in active oxygen-induced SCEs by superoxide anion scavenging.

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