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SHORT COMMUNICATION

REVERSAL OF INHIBITION OF REACTIVE OXYGEN SPECIES ON RESPIRATORY BURST OF MACROPHAGES BY POLYSACCHARIDE FROM *CORIOLUS VERSICOLOR*

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Abstract — Using a luminol-dependent, chemiluminescence assay we found tert-butylhydroperoxide to be a strong inhibitor of the respiratory burst of mouse peritoneal macrophages. However, the inhibition of respiratory burst induced by tert-butylhydroperoxide could be prevented after the interperitoneal injection of polysaccharide from *Coriolus versicolor* (PSK). Further investigation showed that glutathione peroxidase activity was markedly elevated in PSK-treated macrophages. After incubation with tert-butylhydroperoxide, higher activity of glutathione peroxidase was maintained in PSK-treated macrophages.

These results suggest that the immunological function of macrophages is related to the activity of glutathione peroxidase. The non-specific immunopolysaccharide might protect macrophages from the damage induced by reactive oxygen species by enhancing antioxidative capacity.

The effect of active oxygen species to cause tissue damage has been the focus of research in recent years (Suematsu *et al.*, 1987). PSK, a protein-bound polysaccharide derived from Basidiomycete *Coriolus versicolor*, not only enhances immunological function of murine peritoneal macrophages, but also inhibits tumor cell motility (Saito, Tomioka & Sato, 1988; Katano, Yamamoto & Torisu, 1987). However, to our knowledge it has not been shown that PSK protects macrophages against the inhibition of immunological function induced by active species. Thus, in order to clarify the effect of intraperitoneal injection of PSK on peritoneal macrophages in NIH mice, we, in this paper, studied the effect of tert-butylhydroperoxide (t-BuOOH) on respiratory burst of macrophages and the protective effect of PSK.

EXPERIMENTAL PROCEDURES

Reagent

Luminal and reduced glutathione were both purchased from Merck Co., PMA, glutathione reductase (type III, EC 1.6.4.2.), NADPH (type I) and t-BuOOH were from Sigma (U.S.A.). The PSK provided by the Nutrition Laboratory in our college was dissolved in physiological saline.

Preparation and culture of peritoneal macrophages

Male NIH mice (5 weeks old) were purchased from Guang Dong experimental animal center, Guang Dong, China. PSK was given to mice in a dose of 3 mg/mouse/day, and used for injection for 1, 3 or 9 days, respectively, for different groups. On the 4th day after PSK injection, peritoneal exudate cells were harvested in RPMI-1640 containing 10% (v/v) fetal bovine serum (FBS) and incubated in an incubator (5% CO_2, 95% humidified air) at 37°C for 12 h when t-BuOOH was added.

Assay of respiratory burst of macrophages

Adherent macrophages were resuspended in 600 μl RPMI-1640 containing 0.6 mmol/l luminol. After warming at 37°C and allowing samples to dark-adapt for 5 min, 0.6 μg PMA dissolved in 0.6 μl dimethyl sulfoxide were added, and chemiluminescence (CL) measured under constant agitation in 1251 Luminometer (LKB Wallac, Sweden). Data

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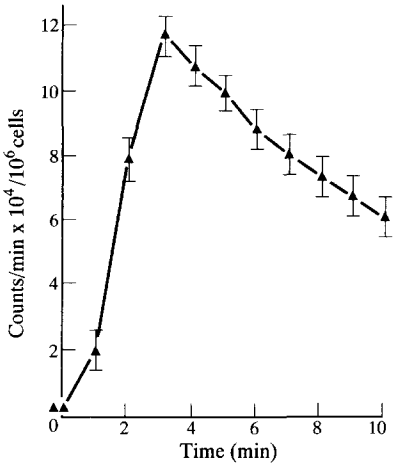

Fig. 1. Respiratory burst of the mouse peritoneal macrophages triggered by PMA. ▲-PMA stimulated.

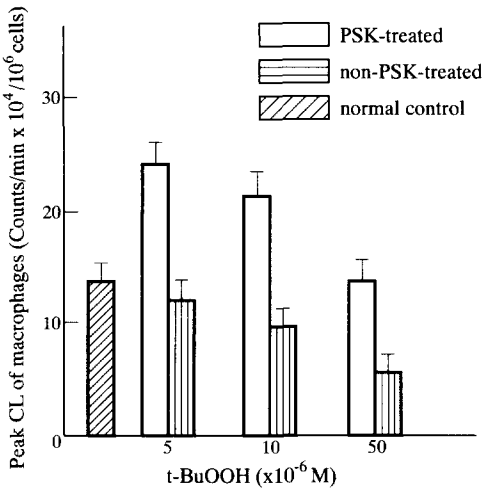

Fig. 2. Comparison of the effect of different concentrations of t-BuOOH on the peak of CL activity of the non-PSK-treated and PSK-treated peritoneal macrophages.

were expressed as counts/min. The maximal counts/min in the CL assay was considered as the peak of CL emission. Each determination was expressed in cell counts or content of cell protein. Cell protein was determined by the method of Lowry *et al.* (Lowry, Rosebrough, Farr & Randall, 1951), using bovine serum albumin as a standard.

Antioxidative capacity of macrophages

Assay of glutathione peroxidase. Macrophages were washed twice at 120 **g** for 5 min in warm physiological saline, and then resuspended in 0.1 mol/l carbonate buffer at pH 9.9 for ultrasound (4°C). Activity of glutathione peroxidase (GSH-Px, EC 1.11.1.9.) was assayed employing a double beam spectrophotometer (Shimadzu UV-240; Shimadzu Co., Kyoto, Japan) by the method of Rokutan *et al.* (Rokutan, Kawai & Asada, 1987).

RESULTS

Respiratory burst of mouse peritoneal macrophages induced by PMA

After incubation for 12 h *in vitro*, the CL values of macrophages were 2000 – 4000 counts/min (per 10^6 cells) in the presence of luminol. As shown in Fig. 1, respiratory burst of macrophages in response to the stimulation of PMA was produced. The intensity of CL increased rapidly and reached maximal CL value at the third minute of measurement.

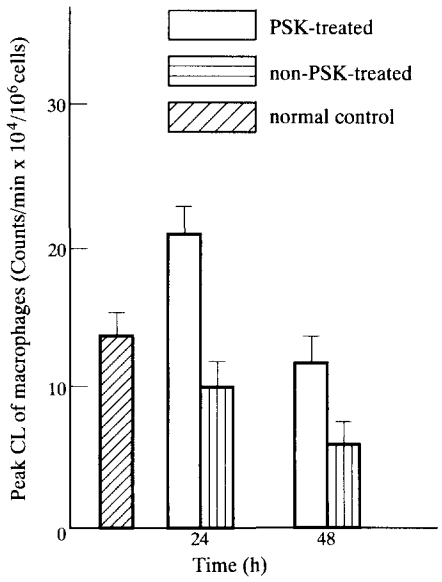

Fig. 3. Peak of CL activity of non-PSK-treated and PSK-treated peritoneal macrophages incubated with 5×10^{-6} mol/l t-BuOOH for 24 and 48 h.

Effect of t-BuOOH on the CL peak value and comparison of CL peak values of PSK-treated macrophages and non-PSK-treated ones

The peaks of CL of macrophages after incubation with t-BuOOH at 37°C for 12 h are indicated in Figs 2 and 3. As the concentration on t-BuOOH in

Table 1. The effect of i.p. injection of PSK on GSH-Px activity in peritoneal macrophages ($x \pm$ S.D.)

Injection	GSH-Px activities (nmol oxidized NADPH/min/mg protein)	
	Physiological saline-treated (non-PSK-treated)	PSK-treated
Once	38.13 ± 1.65	56.71 ± 7.68*
Three times	40.25 ± 2.01	55.39 ± 6.91*
Nine times	38.09 ± 2.14	51.53 ± 1.83*

*$P<0.001$ (compared with physiological saline-treated).

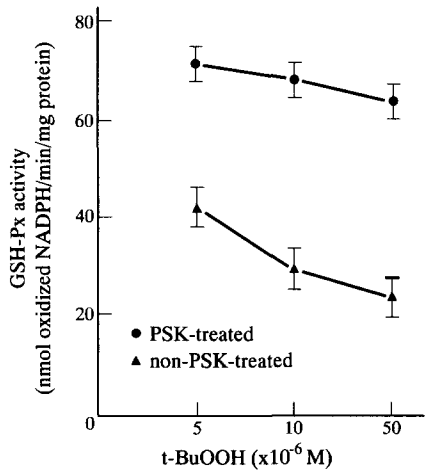

Fig. 4. Change of GSH-Px activity in non-PSK-treated and PSK-treated peritoneal macrophages incubated with different concentrations of t-BuOOH. ● — ● PSK-treated, ▲ — ▲ non-PSK-treated.

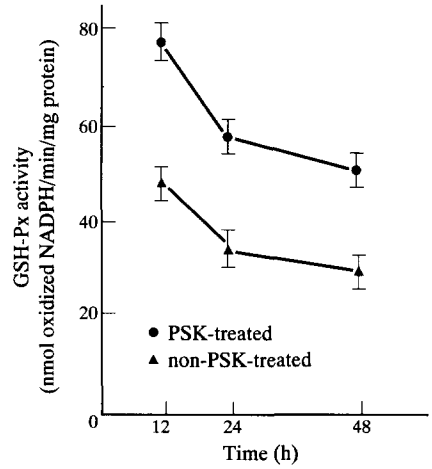

Fig. 5. Change of GSH-Px activity in non-PSK-treated and PSK-treated peritoneal macrophages incubated with t-BuOOH for different times. ● — ● PSK-treated, ▲ — ▲ non-PSK-treated.

the culture medium was elevated, the peak CL activity of macrophages decreased markedly, compared with that of normal control. The CL peak of PSK-treated macrophages (3 mg/mouse/injection in daily injection for 9 days) was significantly higher than that of non-PSK-treated macrophages ($P<0.001$). In the presence of 5×10^{-6} mol/l or 1×10^{-5} mol/l t-BuOOH, the peak of CL of PSK-treated macrophages was also higher than that of normal control. On the other hand, a significant decrease in CL activity of macrophages appeared by lengthening the time of incubation with t-BuOOH. All the CL peaks of the PSK-treated group were much higher than that of non-PSK-treated ones ($P<0.001$). Furthermore, after incubation with t-BuOOH for 24 h, the peak of CL of PSK-treated macrophages was still higher than that of normal control.

Effect of PSK on GSH-Px activity in macrophages and changes of GSH-Px activity in PSK-treated as well as non-PSK-treated macrophages after incubation with t-BuOOH

Table 1 shows a marked increase in GSH-Px activities in peritoneal macrophages on the third day after i.p. daily injection of PSK for 9 days. The activities of GSH-Px in PSK-treated peritoneal macrophages reached a level of 51.53 ± 1.83 after 9 injections; this was 0.35-fold higher than that of the control group. Single or three injections of PSK enhanced GSH-Px activity in a similar fashion. The differences in GSH-Px activity between PSK-treated and non-PSK-treated macrophages became greater after incubation with t-BuOOH as shown in Fig. 4. Although GSH-Px activity of the two groups decreased with increasing concentrations of t-BuOOH, the decrease of GSH-Px activity in PSK-treated macrophages was less than that of the non-PSK-treated group. The activities of GSH-Px in the non-PSK-treated group were 1.82-fold less than that of the PSK-treated group, while the concentration of t-BuOOH in the medium was 5×10^{-5} mol/l.

Further comparison of the effect of t-BuOOH incubated for different times is shown in Fig. 5. A similar change in GSH-Px activity in the two groups was observed.

DISCUSSION

PMA, as a non-particulate stimulus, can induce macrophage respiratory burst with generation of active oxygen species (O^-_2, H_2O_2, $^1O_2$ and $\cdot OH$) when peritoneal macrophages were activated. As indicated in Fig. 1, the peak of the respiratory burst could reach 119,312 ± 1006 counts/min/10^{-6} cells (x ± S.D.) in the CL assay. This reflects increased phagocytosis and microbicidal activities and is related to the action of membrane-bound NADPH oxidase (EC 1.11.1.2.) (Robertson, Cross, Jones & Andrew, 1990).

The fundamental structure of the biomembrane is a lipid bilayer which contains many polyunsaturated fatty acids, which are easily attacked by active oxygen species, resulting in peroxidation (Hoepelman, Bezamer, van Doormalen, Verhoef & Marx, 1989). Thus, it is most likely that lipid peroxidation mediated by active oxygen species may be the main factor of biomembrane injury. Our previous study showed that the content of lipoperoxide in the U937 cells incubated with t-BuOOH was increased and the activity of SOD (EC.1.15.1.1.) and fluidity of the plasma membrane were decreased (Li, Zhou & Chen, 1991). The mechanism by which lipid peroxidation induced by peroxides inhibits the activity of NADPH oxidase remains to be determined. One possibility may be a decrease in the content of unsaturated fatty acids in lipid bilayer of the membrane, such that an alteration in the micro-environment of NADPH oxidase is induced. Second, aldehyde products of lipid peroxidation induced by peroxides (Esterbauer, 1982) may cause destruction of membrane-bound NADPH oxidase.

In the present study, to further elucidate the effect of t-BuOOH on the immunological function of macrophages, we determined the respiratory burst of peritoneal macrophages incubated with t-BuOOH. The results showed that the respiratory burst of macrophages was inhibited, and the inhibition was dose and time dependent. Thus, the lipoperoxidation induced by t-BuOOH might play an important role in the inhibition of immunological function of macrophages. In addition, we found that PSK enhanced the peritoneal macrophages' resistance to the damaging effect of t-BuOOH. The intensity of respiratory burst of PSK-treated macrophages was significantly higher than that of non-PSK-treated ones. This suggested that PSK, an immunopotentiator, could also protect macrophages against the damaging effect of active oxygen species. Furthermore, we observed that, regardless of the number of PSK injections, GSH-Px activities in PSK-treated macrophages were markedly elevated, compared with non-PSK-treated macrophages. A high level of GSH-Px activity in PSK-treated macrophages persisted after incubation with t-BuOOH. It has been established that hydroperoxides alter the intracellular thiol homeostasis, producing a variety of metabolic disturbances and cell damage (Slater & Cheeseman, 1988). GSH-Px may play an important role in hydroperoxide catabolism (Flohe, 1982) and macrophage functions (Rokutan *et al.*, 1988). Our results indicate that peritoneal macrophages from PSK-treated mice had higher GSH-Px activity after incubation with t-BuOOH corresponding with the higher level of respiratory burst. Therefore, the increase in GSH-Px activity of macrophages may be responsible for the antioxidative protection by PSK.

In conclusion, the immunological phagocytic function of macrophages was related to the activity of GSH-Px. Non-specific immunopolysaccharide might prevent macrophages from damage induced by active oxygen species by enhancing antioxidative capacity. These results provide a new clue to investigating the mechanism of immunological damage and the immunomodulation of PSK.

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