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The Effect of Aspalatone, a New Antithrombotic Agent, on the Specific Activity of Antioxidant Enzyme in the Rat Blood

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The antioxidant efficacy of aspalatone, a new antithrombotic agent, has been recognized in the neurotoxic model and in the cardiotoxic model in preliminary studies. We examined the specific activity of antioxidant enzyme in the rat blood following administrations of aspirin, maltol, aspirin together with maltol, salicylmaltol (major metabolite of aspalatone) and aspalatone, respectively. Our assessment showed that salicylmaltol, maltol, aspalatone enhanced antiperoxidative activity. In addition, neither aspirin nor combination of aspirin and maltol, showed any significant effect on the activity of antioxidant enzyme. Because H_2O_2 accumulation may stimulate the thrombogenesis in blood, the result suggests that the induction of blood antiperoxidative activity produced by aspalatone may have beneficial effects on the thrombogenesis.

Key words : Aspalatone (acetylsalicylic acid maltol ester), New antithrombotic agent, Antioxidant efficacy, Rat blood

INTRODUCTION

A prudent clinical approach to management of thromboembolic disorder depends on a thorough understanding of pharmacology of antithrombotic drugs. The guideline on antithrombotic therapy has been underlined that it should be safe and have a wide therapeutic range (Sixma and Groot,1992). Recently, the primary prevention of thromboembolism with aspirin has been a topic of considerable interest for years (Jourdan *et al.*, 1995). Although there is no clear consensus, the useful effect of aspirin has been well-recognized in the thromboembolism induced by platelet aggregation (Jourdan *et al.*, 1995). However, gastrointestinal (GI) insult as manifested by peptic ulcer and upper GI bleeding, was evidenced in patient taking aspirin (Griffin *et al.*, 1988). Therefore, to relieve unpleasant adverse effect of aspirin in antithrombotic therapy, a new antithrombotic agent, aspalatone (acetylsalicylic acid maltol ester) has been developed by Han *et al.* (1994). Aspalatone showed a potent antithrombotic efficacy with negligible GI damage. In addition, Han *et al.* (1994) demonstrated that aspalatone possessed an antioxidant effect might be linked to maltol moiety in *in vitro* condition. Investigators have been described the beneficial effect of free radical scavengers during thrombogenesis (Kakishita *et al.*, 1990; Muruganandam *et al.*, 1992; Dikshit *et al.*, 1993; Kumari *et al.*, 1993; Kawabata and Hata, 1993; Jourdan *et al.*, 1995). In this study, as a first step for clinical approach, we evaluated the antioxidant capacity produced by aspalatone in *in vivo* as measured by the activity of antioxidant enzyme in both serum and erythrocyte.

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MATERIALS AND METHODS

Drugs and treatment of animals

Aspalatone was synthesized from Bukwang Pharm. Ind. Co. (Seoul, Korea) as described by Han *et al.* (1994). Salicylmaltol (as 97.5% purity) was supplied from Bukwang Pharm. Ind. Co. (Seoul, Korea). All other chemicals were of the highest commercial grade. All animals were handled in accordance with the NIH guideline for the humane care and use of laboratory animals. The male Sprague-Dawley rats (Animal Center, Korean FDA) weighing about 300 g were maintained on a 12 : 12 hr light : dark cycle and fed *ad libitum*. They were adapted for 2 weeks to the above conditions. The animals were received aspirin (15 mg/kg), maltol (9 mg/kg), aspirin (15 mg/

kg) together with maltol (9 mg/kg), salicylmaltol (20 mg/kg) and aspalatone (24 mg/kg). Each administration was done 5 times at every 12 hr. Rats were fasted for 12 hr before blood collection. Blood samples were drawn from heart puncture 12 hr after final treatment. After centrifuging at 750×g for 3 min at 4°C, the supernatant (serum) was saved at -70°C until enzyme assay. On the other hand, to monitor the specific activity of antioxidant enzyme in the whole blood, hemolysis was started by adding of 0.6 mM tert-butyl hydroperoxide on the remaining blood (Sinet *et al.*, 1975). After centrifuging at 15,600×g for 30 sec., collected supernatant was employed to measure the activity of antioxidant enzyme of erythrocyte.

The specific activity of antioxidant enzyme

Superoxide dismutase (SOD) acitivity was measured as inhibition superoxide-dependent reactions (Crapo *et al.*, 1978; Kim *et al.*, 1990). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 30 uM cytochrome C, 150 uM xanthine and enzyme extract in phosphate buffer, in final volume of 3 ml. The reaction was initiated by adding 0.16 unit of xanthine oxidase and the change in absorbance at 550 nm was recorded. One unit of SOD was defined as the quantity required to inhibit the rate of cytochrome C reduction by 50%.

For estimating catalase (CAT) activity, the assay medium contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and enzyme extract in a final volume of 1 ml. The change in absorbances at 240 nm was monitored at 25°C. One unit of catalase activity was defined as the quantity that decomposes 1 uM of H_2O_2 per minute (Aebi, 1974).

For analyzing glutathione peroxidase (GSHPx) activity, the assay medium contained 0.1 ml of blood sample, 0.1 ml of 1.4 units of glutathione reductase, 0.1 mM glutathione and 0. 5 ml of 0.1 mM potassium phosphate buffer (pH 7.0) with 1 mM EDTA and 2 mM sodium azide to inhibit CAT activity. The change in absorbance at 340 nm was recorded. The reaction was initiated by adding 0.1 ml of 0.5 mM NADPH followed by addition of 0.1 ml of 1.5 mM cumen hydroperoxide. GSHPx activity was expressed as the oxidation of NADPH/min at 25°C (Brannan *et al.*, 1980).

Protein was measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard.

Statistical analysis

All data were analyzed with an one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMR). Significance was attributed to $p<0.05$.

RESULTS

The specific activity of SOD

Each treatment did not have any influence on the specific activity of SOD in both serum and erythrocyte. All experimental groups remained as the control level (Fig. 1.).

The specific activity of CAT

The specific activity of CAT in the serum was significantly increased by treatment of either salicylmaltol ($p<0.01$) or aspalatone ($p<0.05$). The serum activities produced by aspirin, maltol and co-administration of aspirin and maltol were approximately equal to control level. Although erythrocytic CAT activities appeared to increase by administration of maltol, salicylmaltol and aspalatone, no significant difference among experimental groups was noted in CAT activity in the erythrocyte (Fig. 2.).

The specific activity of GSHPx

Although the activity of GSHPx in serum tended to increase in the presence of maltol, salicylmaltol or aspalatone, no significant difference was observed among experimental groups. The difference between the highest and the lowest activity of GSHPx was 26% in the serum. The erythrocytic activities of GSHPx induced by maltol ($p<0.05$), salicylmaltol ($p<0.01$) and aspalatone ($p<0.05$) significantly increased, respectively. Neither aspirin nor aspirin together with maltol, did show significant change in activity of GSHPx of control erythrocyte (Fig. 3.).

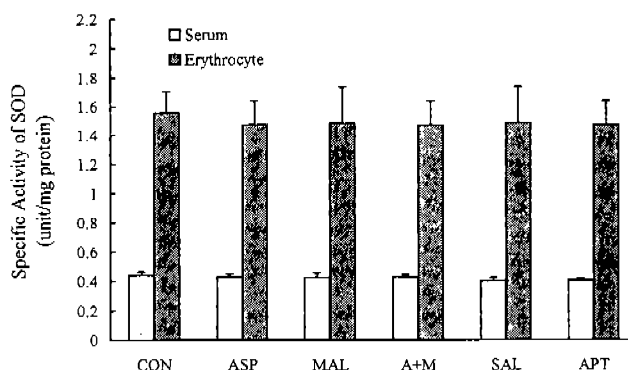

Fig. 1. The effects of multiple treatment of aspirin (ASP), maltol (MAL), aspirin+maltol (A+M), salicylmaltol (SAL) and aspalatone (APT) on the specific activity of superoxide dismutase (SOD) in the rat blood. There is no significant difference between each rat with medication and control rat (CON). Each value represents mean±SEM of 10 experiments.

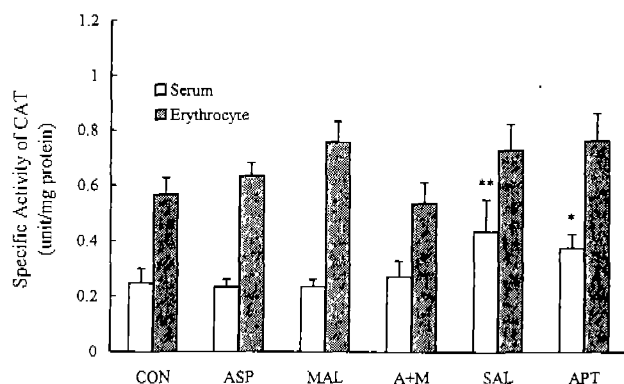

Fig. 2. The effects of multiple treatment of aspirin (ASP), maltol (MAL), aspirin+maltol (A+M), salicylmaltol (SAL) and aspalatone (APT) on the specific activity of catalase (CAT) in the rat blood. Each value represents mean±SEM of 10 experiments. *$p<0.05$, **$p<0.01$ compared to control level

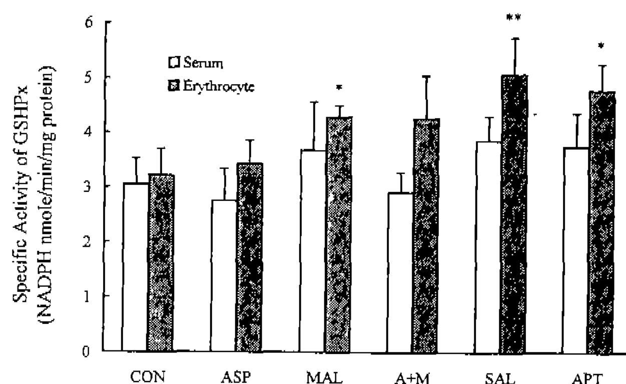

Fig. 3. The effects of multiple treatment of aspirin (ASP), maltol (MAL), aspirin+maltol (A+M), salicylmaltol (SAL) and aspalatone (APT) on the specific activity of glutathione peroxidase (GSHPx) in the rat blood. Each value represents mean±SEM of 10 experiments. *$p<0.05$, **$p<0.01$ compared to control level

DISCUSSION

The main finding of this study is that maltol, salicylmaltol (major metabolite of aspalatone) and aspalatone produced profound increases of the activity of H_2O_2 scavenger enzyme without any induction of SOD level in blood. However, aspirin administration did not show any significant effect on the activity of antioxidant enzyme in blood. This study demonstrates that antioxidant mechanism of aspalatone is different from that of aspirin, suggesting that this phenomenon supports our preliminary observations as demonstrated by abstract form (Kim *et al.*, 1995 a; Kim *et al.*, 1995 b; Choi *et al.*, 1995). The generation of free radicals in thromboembolism in rodent is well documented (Kakishita *et al.*, 1990; Muruganandam *et al.*, 1992; De la Cruz *et al.*, 1992; Dikshit *et al.*, 1993; Kawabata and Hata, 1993; Kumari *et al.*, 1993; Jourdan *et al.*, 1995). There is an enhanced cellular lipid peroxidation during thromboembolism (Kumari *et al.*, 1993). It has been suggested that H_2O_2 could stimulate the aggregation of platelets through calcium mobilization (Principe *et al.*, 1991). Similarly, GSHPx impairment leads to hyperaggregability of the diabetic platelet (Muruganandam *et al.*, 1992). The CAT and deferoxamine, an iron chelator, which inhibited the formation of hydroxyl radical by Fenton reaction, were more effective than other scavengers against thrombosis suggesting that H_2O_2 play an active role in thrombogenesis (Dikshit *et al.*, 1993; Kumari *et al.*, 1993). The significant inductions of GSHPx and CAT produced by either salicylmaltol or aspalatone might lead to better clearance H_2O_2 formed by SOD in the blood. The salicylmaltol, the major metabolite of aspalatone, was maximally efficacious to induce the activity of H_2O_2 scavenger. Thus, the present results indicate that metabolism to salicylmaltol was a prerequisite for antioxidant capacity. No change in the enzyme activity of H_2O_2 scavenger was evidenced following treatment of aspirin together with maltol, which suggests that this combined administration may not be helpful to induce the antioxidant efficacy. On the other hand, the novel antioxidant effect of maltol has been well-characterized (Han *et al.*, 1985; Shin *et al.*, 1990; Suh *et al.*, 1996). The antioxidant activity of aspalatone may be related to that of maltol (Han *et al.*, 1994). However, detailed mechanism of aspalatone as an antioxidant remains to be further delineated.

Recently, we showed that aspalatone treatment attenuated the imbalance of endogenous oxyradical scavenger system following seizure behavior (Kim *et al.*, 1995a), and that aspalatone clearly reduced cardiotoxicity produced by doxorubicin (Kim *et al.*, 1995b). These beneficial effect of aspalatone may attributable to H_2O_2 scavenging capacity. In addition, we employed electron spin resonance (ESR) technique and the spin trap 5,5-dimethyl-1- pyrrolidine-N-oxide (DMPO) to monitor the scavenging effect of hydroxyl radical of aspalatone in the presence of cardiac fraction of mouse. Aspalatone was found to be the most effective quencher of the signal of OH/DMPO among various substances. Briefly, a descending order of scavenging efficacy was as follows; aspalatone > maltol > vitamin E > aspirin > vehicle (Choi *et al.*, 1995). Therefore, scavenging activity of H_2O_2 induced by aspalatone might have, at least in part, contributed to the scavenging efficacy of toxic hydroxyl radical.

On the basis of current knowledge, it is difficult to explain why aspirin challenge shows a lack of significant alteration in the level of antioxidant enzyme in the present experimental condition. But it is pos-

sible that an undefined pharmacologic interaction between aspirin and various blood components exists *in vivo* (Spanos, 1993). Although it is accepted that aspirin possesses a potent antithrombotic effect, the efficacy of aspirin in certain conditions has been disputed up to now (Doutremepuich *et al.*, 1994; Spanos, 1993). Further experiments are required to characterize full effect of aspirin on the antioxidant system of blood. Twenty four hr after final treatments, the increased activities of H_2O_2 scavenging enzyme induced by salicylmaltol, maltol, and aspalatone appeared to decrease (data did not show). Thus, further studies might be conducted (over longer periods of time) how to optimize the advantage of aspalatone for achieving a wanted degree of antioxidant efficacy.

In summary, the antiperoxidative efficacy of aspalatone may be beneficial to peroxidative stress mediated by oxidation of blood arachidonate/undefined metabolite during the thrombogenesis. The findings of the present study can be applied to human situation, but need further investigation to achieve better understanding of therapeutic benefit of aspalatone.

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