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In Vitro Effect of Aspalatone on Platelet Aggregation and Thromboxane Production in Human Platelet Rich Plasma

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Abstract – *In vitro* inhibitory effect of aspalatone ([3-(2-methyl-4-pyronyl)]-2-acetyloxybenzoate) on collagen-, ADP-, and epinephrine-induced platelet aggregation in human platelet rich plasma (PRP) was compared with the effects of reference drugs (acetylsalicylic acid, cilostazol and ticlopidine). Aspalatone inhibited time and dose dependently human platelet aggregation induced by collagen; relative potency was in the order of cilostazol>acetylsalicylic acid>aspalatone>ticlopidine. Aspalatone, like acetylsalicylic acid, potently inhibited only the secondary phase of ADP- and epinephrine-induced aggregation. Thromboxane B_2 production evoked by collagen in human PRP was inhibited significantly and concentration-dependently by aspalatone and acetylsalicylic acid. These results were in agreement with the earlier studies in which the antiplatelet action of aspalatone was indicated to be due to the inhibition of platelet cyclooxygenase activity (Han *et al., Arzneim. Forsch./Drug Res.* 44(II), 1122, 1994; Suh and Han, *Yakhak Hoeji* 39, 565, 1995). In addition, the inhibitory activity of aspalatone on the platelet aggregation appears to be inversely related to the rate of nonspecific deacetylation of the drug in plasma.

Keywords □ aspalatone, platelet aggregation, thromboxane B_2

Acetylsalicylic acid (ASA) at low doses has been shown to possess significant potential for reducing mortality associated with myocardial infarction and thrombotic stroke (Underwood and More, 1994; Barnett, 1990). ASA, by virtue of cyclooxygenase inhibition, inhibits not only the production of proaggregatory thromboxane A_2 (TXA_2) in platelets but also the production of antiaggregatory prostacyclin (PGI_2) in blood vessel walls, known as "aspirin dilemma" (Braden *et al.*, 1991). Moreover, bolus ASA or chronic low-dose ASA increases the risk of gastric bleeding and/or erosions (The Dutch TIA Trial Study Group, 1991; Naschitz *et al.*, 1990) due to the inhibition of biosynthesis of "cytoprotective" PGI_2 in gastric mucosa and the topical toxic effect of its metabolite, salicylic acid (Kauffman, 1989). To circumvent these problems, much efforts have been made in designing different clinical regimen and formulations (Charman *et al.*, 1993; Keimowitz et al., 1993).

A different approach was sought in our previous study in which aspalatone was synthesized by esterification of ASA and maltol, an antioxidant (Han *et al*, 1994), based on the earlier findings that antioxidants might increase vascular PGI_2 synthesis by scavenging lipid peroxides (Brune *et al.*, 1991) and that ASA esters exhibited reduced ulcerogenicity (Rainsford and Whitehouse, 1980). Aspalatone was shown to possess a comparable antiplatelet effects to ASA *in vitro, ex vivo* and *in vivo* in rodents. Furthermore, it caused negligible gastric mucosal damage in rats (ulcer index=0.71 mm at 800 mg/kg *p.o.*) in a sharp contrast to ASA (ulcer index=29 mm at 200 mg/kg *p.o.*) (Han *et al.*, 1994). Metabolic fate of oral aspalatone in rats was also studied to show that aspalatone is not a prodrug of ASA (Suh *et al.*, 1995).

The present study was designed to compare the antiplatelet effects of aspalatone, ASA, cilostazol (Kimura *et al.*, 1985) and ticlopidine (Heptinstall *et al.*, 1995) using human platelet-rich plasma. In addition, a possible correlation between the *in vitro* antiplatelet aggregatory activity of aspalatone and the *in vitro* deacetylation rate of aspalatone in human plasma was investigated.

MATERIALS AND METHODS

Chemicals

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Aspalatone was supplied from the Bukwang Pharm. Co. (Seoul, Korea). Ticlopidine · HCl and cilostazol were obtained from respective manufacturers in tablets. Collagen, epinephrine and ADP were obtained from Chrono-Log (Havertown, PA, USA). Thromboxane B_2 (TXB_2) radioimmunoassay kit was purchased from Amersham (Buckinghamshire, UK).

Blood sampling

Venous blood was obtained from healthy volunteers (n =51, male=47, female=4, mean age=31±4.5) who had not taken any antiplatelet drugs for at least two weeks. Blood was collected from antecubital veins and nine volumes of blood were mixed with one volume of 3.8% trisodium citrate.

Platelet aggregation

Platelet rich plasma (PRP) was prepared by centrifugation at 200 g for 15 min, then blood was centrifuged further for 15 min at 1200 g to yield platelet poor plasma (PPP). PRP was diluted with autologous PPP to adjust the platelet counts in the PRP to 3×10^8 cells/ml using a Coulter counter (Coulter Electronics, Hialeah, FL, USA). Platelet aggregation was measured by Born's turbidimetric method (Born and Cross, 1968). To 490 μl of PRP was added 5 μl of the drug solution and the PRP sample was preincubated for 10 min at 37℃. The drug solutions were prepared in 75% dimethyl sulfoxide (DMSO) for collagen-induced aggregation and 50% DMSO for ADP-and epinephrine-induced aggregation experiments to give the final DMSO concentration of 0.75% and 0.5%, respectively. Then, 5 μl of inducer (collagen at a final concentration of 2 μg/ml, ADP at 10 μM, epinephrine at 5 μM) was added to effect aggregation. The extent of aggregation was expressed taking the maximum change in light transmission for PRP against PPP. The percent inhibition of aggregation by the test drug was calculated by dividing the difference in maximum aggregation between the vehicle run and the test run by maximum aggregation observed in vehicle run, and then multiplying by 100. The IC_{50} value was calculated by the least square method. In ADP-and epinephrin-induced aggregation where biphasic response with two (primary and secondary) waves of aggregation was observed, the inhibitory effects of aspalatone and ASA on each phase of aggregation were assessed (Gerrard, 1982).

Measurement of thromboxane B_2 production

Aspalatone or ASA at fixed concentration was preincubated in PRP for 10 min. At the end of 6 min aggregation with collagen (2 μg/ml), ethanol and formic acid (60 μl each, 9.7%) were added. Thromboxane B_2 was extracted with ethyl acetate (2×500 μl) and the organic phase was evaporated to dryness using a centrifugation vacuum system (Spin-Vac, Hanil, Korea). Thromboxane B_2 level was measured with radioimmunoassay kit (Biotrak system, Amersham) according to the procedure described by the manufacturer; the values were expressed as ng/ml.

***In vitro* hydrolysis of aspalatone in PPP**

The rate of nonspecific deacetylation of aspalatone in plasma was determined to assess a possible correlation with the antiaggregatory potency of the drug. The mixture of 80 μl of the drug solution in 10 mM phosphate-buffered saline (PBS, pH 7.4) and 20 μl of PPP was incubated for 5 min at 37℃. The final concentration of aspalatone was 0.17 mM. The reaction was stopped by addition of 500 μl of CH_3CN. Following centrifugation and filtration, aliquots of the supernatant was analyzed by HPLC according to the previously described method (Suh *et al*, 1995). *In vitro* aspalatone hydrolysis rate was expressed in nmol aspalatone hydrolyzed/min · μl PPP.

Data analysis

The experimental results are expressed as the means ± SEM. Statistical significance was assessed by Student's t-test and p values less than 0.05 were considered significant.

RESULTS

Effects of drugs on platelet aggregation in human platelet rich plasma

Due to the low solubilities of cilostazol and aspalatone, all drugs tested were first solubilized in 75% DMSO for collagen-induced aggregation experiments. The final concentration of DMSO was 0.75% and DMSO at this concentration did not affect the collagen-induced aggregation in human PRP.

IC_{50} values of aspalatone and reference drugs (ASA, cilostazol and ticlopidine) against platelet aggregation in human PRP induced by collagen (2 μg/ml), ADP (10 μM) and epinephrine (5 μM) are shown in Table 1. Aspalatone showed a concentration-dependent inhibition of platelet aggregation induced by collagen in human platelet rich plasma. Under the reaction conditions employed, relative potency against collagen-induced aggre-

gation was cilostazol>ASA>aspalatone> ticlopidine. When the aggregation was induced by epinephrine, aspalatone and ASA did not strongly inhibit the primary aggregation. Aspalatone at 80 M inhibited the epinephrine-induced primary aggregation by 20±6.6% (n=6) and ASA at 40 μM inhibited by 17±5.5% (n=5). However, aspalatone and ASA potently inhibited the secondary phase of the aggregation (Table I). For ADP-induced aggregation, only cilostazol potently inhibited the aggregation and the rest failed to inhibit by 50% at 400 μM. ASA and aspalatone concentration-dependently inhibited the secondary phase of ADP-induced aggregation and IC_{50} of aspalatone against the secondary aggregation was 98±28 μM (n=4).

Time-dependency of aspalatone inhibition on collagen-induced platelet aggregation

The inhibitory effect of aspalatone on collagen-induced aggregation was time-dependent (Fig. 1). Percent inhibition of the aggregation by aspalatone increased as the preincubation time of the drug in PRP increased, and inhibition reached the maximum after 10 min preincubation. IC_{50} of aspalatone, therefore, decreased with increasing preincubation time; IC_{50} values at 4 min and 10 min preincubation were 320±79 μM (n=5) and 180±15 μM (n=6), respectively. This time-dependency was also observed with ASA; IC_{50} values decreased from 86±4.9 μM (n=6) to 47±6.7 μM (n=6) as the preincubation time increased from 4 to 10 min.

Table I. IC_{50} of Aspalatone and Reference Drugs against Human Platelet Aggregation Induced by Collagen, Epinephrine and ADP.

Aggregation inducer	IC_{50} (mean ± SEM, μM)			
	Aspalatone	ASA	Cilostazol	Ti-
Collagen (2 μg/ml)	180±15	47±6.7	4.9±0.67	440±52
Epinephrine (5 μM)	42±13[a]	14±4.9[a]	5.3±1.4	60±14
ADP (10 μM)	>400[b]	>400[b]	12±1.6	>400[b]

[a]IC_{50} values for the inhibition of secondary aggregation. [b]Less than 50% inhibition at 400 μM. n=6, preincubation time=10 min.

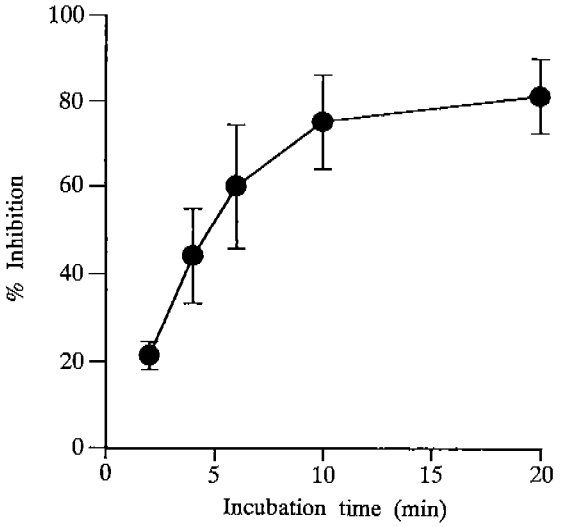

Fig. 1. Time-dependent inhibition by aspalatone of collagen-induced aggregation in human platelet rich plasma. Aspalatone (400 μM) was preincubated in PRP for different time interval and the aggregation was induced by collagen (2 μg/ml). Values represent means±SEM (n=4-7).

Inhibition of thromboxane B_2 production in PRP

The effects of aspalatone and ASA on TXA_2 production by platelets were also tested by measuring the levels of TXB_2, a stable metabolite of TXA_2, in PRP after stimulation with collagen. TXB_2 levels in collagen-stimulated PRP were markedly elevated over those in samples incubated in the absence of collagen (basal value in Table II). Preincubation of PRP for 10 min with 200 μM aspalatone resulted in a significant inhibition of TXB_2 production compared to control ($p<0.05$, Student's t-test), whereas incubation of PRP with ASA at 40 μM resulted in a significant inhibition of TXB_2 accumulation.

In vitro hydrolysis of aspalatone in PPP

The effect of *in vitro* hydrolysis rate of aspalatone in plasma on the inhibitory activity of aspalatone against the collagen-induced aggregation was investigated by measuring the *in vitro* hydrolysis rate of the drug in individual PPP samples. Aspalatone was deacetylated to yield salicylic acid maltol ester which remained intact after a longer incubation up to 10 min. An inverse linear relationship (r=-0.86) was found in the hydrolysis rate and

Table II. Effect of aspalatone and ASA on collagen-induced thromboxane B_2 production in human platelet rich plasma.

	Concentration	Thromboxane B_2
Collagen (2 μg/ml)		33.3 ±13.2
Basal (no collagen)		0.00609±0.0149
Aspalatone	100	7.75 ±4.63
+ Collagen (2 μg/ml)	200	2.29 ±1.26[a]
	375	2.24 ±0.816[a]
ASA	20	9.18 ±4.26
+ Collagen (2 μg/ml)	40	1.66 ±0.934[a]
	100	0.179±0.089[b]

Values are presented as means ± SEM (n=5-8).
[a]$p<0.01$ compared with the control by Student's t-test.

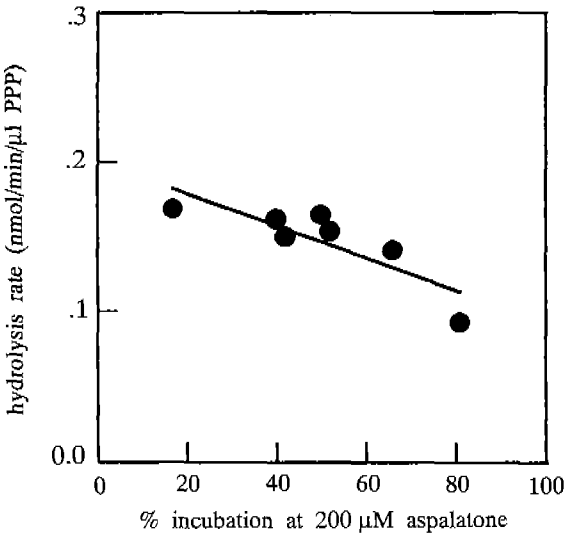

Fig. 2. Correlation between the in vitro inhibitory activity of aspalatone (200 μ M) on collagen (2 μg/ml)-induced aggregation in human PRP and the *in vitro* hydrolysis rate of aspalatone in autologous PPP. Data are the means of the duplicate determinations and the curve was fitted by linear regression analysis (r = -0.86).

% inhibition of collagen-induced aggregation by 200 μM aspalatone (Fig. 2). The hydrolysis of aspalatone in buffer alone was not detected under the experimental conditions.

DISCUSSION

The results of the present study indicate that aspalatone time-and concentration-dependently inhibited *in vitro* human platelet aggregation induced by collagen and the secondary phase of the aggregation induced by ADP and epinephrine. Relative potency of reference drugs tested in this study was in agreement with other studies. The inhibitory activity of cilostazol, a platelet cyclic AMP phosphodiesterase inhibitor, was more potent than that of ASA (Kimura *et al.*, 1985) and ticlopidine was shown to be a weak inhibitor of platelet aggregation *in vitro* (Schror, 1993). In epinephrine and ADP-induced human platelet aggregation, aspalatone and ASA inhibited the secondary aggregation. This secondary phase was known to be due to the TXA_2 formation and ADP release (Weiss, 1983), and was affected by the cyclooxygenase inhibitor, ASA (Zucker and Peterson, 1968). These results, together with the finding that aspalatone (200 μM) as well as ASA (40 μM) significantly inhibited the TXA_2 production by collagen-treated platelets, indicate that the antiplatelet action of aspalatone is due to the inhibition of platelet cyclooxygenase. Aspalatone was demonstrated to inhibit the production of cyclic endoperoxides from arachidonate catalyzed by partially purified bovine seminal vesicle cyclooxygenase *in vitro* (Suh and Han, 1995).

The *in vitro* inhibitory activity of aspalatone on human platelet aggregation varied widely in individual PRP samples (Fig 2). Under the experimental conditions used, aspalatone (200 M) inhibited collagen (2 μg/ml)-induced platelet aggregation by 17 to 81% in seven different human PRP samples. A similar variance in inhibitory effect was also observed in ASA. The acetyl group in aspalatone and ASA are readily hydrolyzed by nonspecific esterases in biological samples including tissue homogenates and serum (Suh *et al.*, 1995; Pedersen and Fitzgerald, 1984). When the percent inhibition of aggregation by aspalatone in individual PRP samples was plotted against the deacetylation rate of aspalatone to salicylic acid maltol ester in autologous PPP samples, an inverse correlation was found. This result suggested that there exists a competition between inhibition of cyclooxygenase by aspalatone, likely through transacetylation, in platelets and nonspecific deacetylation of aspalatone in plasma. This nonspecific deacetylation represents the metabolic fate of aspalatone (Suh *et al.*, 1995) as well as that of ASA (Morris *et al.*, 1973). In fact, this underlies the proposed platelet selectivity of ASA at low doses in that ASA inhibits platelet cyclooxygenase in portal vein and then undergoes almost complete deacetylation in the liver not to affect PGI_2 production in systemic endothelium (Pedersen and Fitzgerald, 1984). Further investigations are warranted to assess whether aspalatone exhibits the similar presystemic acetylation of platelet cyclooxygenase.

In conclusion, aspalatone, acetylsalicylic acid maltol ester, inhibited in a time-and concentration-dependent fashion the *in vitro* platelet aggregation induced by collagen and the secondary phase of ADP- and epinephrine-induced aggregation in human PRP. Aspalatone also inhibited collagen-evoked TXA_2 production by human platelets. The inhibitory activity of aspalatone on the platelet aggregation appears inversely related to the rate of nonspecific deacetylation of the drug in plasma.

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