Salsolinol, an endogenous neurotoxin, enhances platelet aggregation and thrombus formation

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Summary
Salsolinol, an endogenous neurotoxin, is known to be involved in the neuropathy of Parkinson’s disease and chronic alcoholism. In these diseases, increased thrombotic events are also commonly reported, yet the mechanism underlying remains poorly understood. Here we report that salsolinol can enhance agonist-induced platelet aggregation and granular secretion, which is essential in the thrombus formation. In rat and human platelets, agonist-induced platelet aggregation was significantly increased by salsolinol in a concentration-dependent manner. Agonist-induced granular secretions of serotonin and concomitant P-selectin expression were also augmented by salsolinol. α2-adrenergic blockers attenuated the salsolinol-enhanced aggregation and the inhibition of cyclic AMP generation was found, suggesting the involvement of α2-adrenergic receptor-mediated pathways in these events. In accord with the in-vitro results, in an arterial and venous thrombosis model in vivo in the rat, salsolinol shortened vessel occlusion time and increased thrombus formation, respectively. In conclusion, we demonstrated that salsolinol can enhance agonist-induced aggregation and granular secretion in platelets through α2-adrenergic receptor activation, which resulted in the increased thrombus formation in vivo. These results suggest that salsolinol-enhanced platelet aggregation could be a possible contributing factor to the thrombotic events observed in Parkinson’s disease and alcoholism.

Keywords
Platelets, salsolinol, Parkinson’s disease, alcoholism, thrombosis

Introduction
Salsolinol, 1-methyl-5,6-dihydroxy-1,2,3,4-tetraisoquinoline, is an endogenous dopaminergic neurotoxin produced from the condensation reaction between dopamine and acetaldehydes (1, 2). Abnormally high levels of salsolinol have been found in the urine, plasma and the cerebrospinal fluid of Parkinson’s disease patients (4, 5) and alcoholics (6–8), reflecting its role in the pathological conditions of these diseases. Involvement of salsolinol in neuropathy has been demonstrated in various studies in vitro and in vivo, where salsolinol and its derivatives are shown to inflict cytotoxicities to dopaminergic neurons (9–12). Besides neuropathy, increased thrombotic events are another important complication commonly observed in these diseases (13, 14). Pulmonary embolism is one of the most frequent causes of death in Parkinson’s diseases (15, 16), and the incidence of stroke has been reported to increase in alcoholics (17, 18). Despite the abundant clinical evidence concerning the increased thrombotic risks in these diseases, mechanism underlying the altered thrombotic response has not been provided yet. In this context, attempts have been made to investigate the cardiovascular effect of salsolinol (19, 20), but its role in the increased thrombosis remains elusive.

Pathological thrombotic condition has been explained by abnormal haemostasis, which is mediated by excessive platelet aggregation (21, 22). Under normal conditions, platelet aggregations are elaborately controlled by the balance between pro-aggregatory agonists like thrombin or collagen, and anti-aggregatory prostaglandins through cyclic AMP dependent pathways (23, 24). If this haemostatic balance were perturbed by various pathological...
changes or chemicals, excessive aggregation and thrombosis could occur ultimately. Exemplifying this, in chronic diseases such as diabetes and hypertension, where platelets become hypersensitive to aggregatory stimuli, excessive platelet aggregation and thrombosis can be observed frequently (25, 26). Many xenobiotics like arsenic and oral contraceptives or endogenous mediators such as lysophosphatidic acid and platelet activating factor are also known to induce these hyper-aggregatory states, which could eventually contribute to the development of thrombotic diseases (27–30).

In the present study, we found that salsolinol could enhance agonist-induced platelet aggregation. The underlying mechanism was elucidated with freshly isolated platelets and its clinical significance was explored with in vivo rat thrombosis models. With this study, we demonstrated that salsolinol-enhanced platelet aggregation could be a possible contributing factor to the increased thrombotic events observed in Parkinson’s disease and chronic alcoholism.

Materials and methods

Materials
Salsolinol (1-methyl-5,6-dihydroxy-1,2,3,4-tetraisoquinoline), thrombin, citric acid, trisodium citrate, bovine serum albumin (BSA), phenolamine, yohimbine, luminol, menadione, digitonin, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagen was obtained from Chrono-log Corp. (Havertown, PA, USA). 14C-serotonin was from GE Healthcare (Piscataway, NJ, USA). Anti-CD62P (P-selectin) antibodies were purchased from BD Biosciences (San Jose, CA, USA). All other reagents used were of the highest purity available.

Animals
All the protocols were approved by the Ethics Committee of Animal Service Center at Seoul National University. Male Sprague-Dawley rats (Samtako Co., Korea) weighing 300–400 g were used in all experiments. Before the experiments, animals were acclimated for one week. Food and water were provided ad libitum.

Preparation of platelets
After blood was collected from the abdominal aorta of ether-anesthetized rats using acid-citrate-dextrose (1:6), platelets were prepared as described previously (31). Briefly, after isolation of platelet-rich plasma (PRP) by centrifugation of blood at 150 g for 15 minutes (min), and platelets were pelleted by centrifugation at 500 g for 10 min. Pellet was resuspended with Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 1.0 mM MgCl2, 10.0 mM HEPES,
Measurement of platelet aggregation
Aggregation experiments were performed using a lumi-aggregometer (Chrono-log Corp., USA). Platelets were incubated with various concentration of salsolinol or vehicle (DW) for 30 min in washed platelets or for 5 min in PRP at 37°C and aggregation was induced by sub-threshold concentration of thrombin (0.065–0.08 U/ml in washed platelets or 0.2–0.4 U/ml in PRP) or collagen (1.0 µg/ml), which do not significantly increase light transmission in the lumi-aggregometer. In experiments using α2-blockers, platelets were preincubated with phentolamine (final 30 µM) for 1 min or yohimbine (final 2 µM) for 5 min and exposed to salsolinol. Platelet aggregation was initiated by thrombin addition.

Measurement of serotonin secretion
PRP was pre-incubated with 0.5 µCi/ml 14C-serotonin (55 mCi/mmol) for 45 min at 37°C, and 14C-serotonin loaded platelets were prepared from PRP as described above. After incubation with salsolinol for 30 min at 37°C, sub-threshold concentration of thrombin (0.025 U/ml) was added to platelets. The reaction was terminated by the addition of EDTA (final 5 mM). The resultant platelet suspensions were centrifuged at 12,000 g for 1 min, and the supernatant was obtained for determination of 14C-serotonin secretion. Radioactivity in each sample was measured in Wallac 1409 liquid scintillation counter (Perkin Elmer, Boston, MA, USA), after dilution with ACSII scintillation cocktail (GE Healthcare). Serotonin secretion was expressed as the percentage of maximum secretion obtained by stimulating platelets with thrombin (0.2 U/ml) for 5 min.

Measurement of P-selectin expression
After incubation with salsolinol at 37°C for 30 min, sub-threshold concentration of thrombin (0.05 U/ml) was added to platelets. Activated platelet suspension was added to anti-CD62P (P-selectin) antibodies. The rabbit IgG was used as isotype control antibody to determine non-specific binding. Binding of anti-CD62P antibody was revealed by fluorescein isothiocyanate-conjugated secondary antibody. Flow cytometric analysis was carried out in a FACSCaliber (BD Biosciences) equipped with argon laser (λex 488 nm). Data from 3,000 events were collected and analyzed using CellQuest (BD Biosciences) software. Platelets were identified as being within the platelet window defined by forward and side light scatter characteristics.

Determination of cytotoxicity
Platelet cytotoxicity was determined by the leakage of lactate dehydrogenase (LDH) from platelets. After platelets (3×10^8/ml) were incubated with salsolinol for 2 hours (h), aliquots were collected at various time points and supernatant was separated by centrifugation (12,000 g, 2 min) for LDH assay. The reduction in absorbance at 340 nm by the conversion of NADH to NAD* was measured for the evaluation of LDH activity in the aliquots. The extent of LDH leakage was expressed as % of total enzyme activity measured in platelets completely lysed with 0.3% Triton X-100.

5.0 mM glucose, 12.0 mM NaHCO3, 0.34 mM Na2HPO4, and 0.3 % BSA, pH 7.4) containing 10% ACD. After centrifugation at 500 g for 10 min, platelets were resuspended in Tyrode buffer to a cell concentration of 3×10^8 cells/ml and final CaCl2 concentration was adjusted to 2 mM prior to use. Human blood was isolated from healthy male volunteers (18–25 years old), with approval from the Ethics Committee of Health Service Center at Seoul National University. Preparation of platelets from human blood was conducted as described above.

Figure 2: Potentiation of serotonin secretion and P-selectin expression in isolated rat platelets by salsolinol. A) 14C-serotonin-loaded platelets were pretreated with salsolinol for 30 min at 37°C and then a sub-threshold concentration of thrombin was treated for 5 min. Serotonin secretion from platelets was determined by measuring the radioactivity of 14C-serotonin in medium. Values are expressed as % of a maximum serotonin secretion induced by thrombin (0.2 U/ml). B) After platelets were treated with salsolinol for 30 min at 37°C, thrombin was added for 5 min. P-selectin expression on the platelet surface was determined by flow cytometric analysis using anti-P-selectin antibody. Cells were considered to be positive for P-selectin expression when fluorescence intensity was > 95% of the signal from the isotype control. Inset shows representative histograms of fluorescence signal for antibody binding. Values are mean ± SEM of 3–5 independent experiments.

* represent significant differences from corresponding control (p < 0.05)
Measurement of generated reactive oxygen species (ROS)

Generation of ROS was measured by means of lucigenin-induced chemiluminescence. After luminol (40 µM) was added to platelets, and the reaction was initiated by adding salsolinol. Chemiluminescence was monitored continuously up to 30 min using a luminometer (Berthold, Germany). The amount of chemiluminescence was calculated by integrating the area under the curve.

Measurement of cyclic AMP

After incubation with salsolinol for 30 min at 37ºC, sub-threshold concentration of thrombin (0.05 U/ml) was added to platelets. After additional incubation for 5 min, cells were lysed by the addition of HCl (final 0.1 M) and centrifuged at 12,000 g for 2 min. The amount of cyclic AMP was determined using an enzyme-linked immunoassay kit (Biomol, Plymouth Meeting, PA, USA), according to the procedure provided by the manufacturer.

Arterial thrombosis animal model

Two h after intraperitoneal (i.p.) administration of salsolinol, rats were anesthetized with urethane (1.25 g/kg i.p.), and approximately 15 mm of the right carotid artery was exposed and dissected free of nerve and connective tissue. Thrombosis was induced by the FeCl₃ application method as previously described (28). Briefly, a filter paper (1 x 2 mm, Whatman No. 1) was soaked in FeCl₃ (35% w/v in saline) and applied to the external surface of the carotid artery segment for 10 min. An ultrasonic flow-probe was placed around the arterial segment proximal to the injured site. The flow-probe was connected to a Doppler flowmeter (Transonic Systems, Ithaca, NY, USA) to monitor blood flow. The time needed for occlusion to occur was measured for up to 60 min, and occlusion time was assigned a value of 60 min for vessels that did not occlude within 60 min.

Venous thrombosis animal model

Thrombus formation was induced by stasis combined with hypercoagulability. Male Sprague-Dawley rats (180–250 g) were anesthetized with urethane (1.25 g/kg i.p.). The abdomen was surgically opened, and the vena cava was exposed after careful dissection. Two loose cotton threads were prepared 16 mm apart around the vena cava. All side branches were ligated tightly with cotton threads. One h after the intravenous infusion of saline or salsolinol into a left femoral vein for 3 min, 500-fold diluted thromboplastin was infused for 1 min to induce thrombus formation. Stasis was initiated by tightening the two threads, first the proximal and the distal thereafter. The abdominal cavity was provisionally closed, and blood stasis was maintained for 15 min. After the abdomen was reopened, the ligated venous segment was excised and opened longitudinally to remove the thrombus. The isolated thrombus was blotted of excess blood and immediately weighed.

Figure 3: Involvement of α2-adrenergic receptor in salsolinol-enhanced platelet aggregation. A) Non-specific cytotoxicities of salsolinol were investigated in isolated rat platelets by lactate dehydrogenase (LDH) assay. Menadione (MEN; 250 µM) was used as a positive control. B) Reactive oxygen species (ROS) generation by salsolinol was examined in isolated rat platelets using luminol-enhanced chemiluminescence. Menadione (100 µM) was used as a positive control. C) After pretreatment of phentolamine (Phen; 30 µM), an α2-adrenergic receptor antagonist, the effect of salsolinol on thrombin-induced platelet aggregation was examined. Values are mean ± SEM of 3–5 independent experiments. * represent significant differences from corresponding control (p < 0.05), # represents significant difference from the group treated with salsolinol in the absence of phentolamine (p < 0.05).
Statistics
The means and standard errors of mean (SEM) were calculated for all treatment groups. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test to determine which means were significantly different from the control. Statistical analysis was performed using SPSS software (Chicago, IL, USA). In all cases, a p-value of < 0.05 was used to determine significance.

Results
To examine the effect of salsolinol on platelet function, isolated rat platelets were incubated with salsolinol (10–250 µM) for 30 min, and then, were challenged with sub-threshold level of thrombin, a physiological pro-aggregatory agonist. While platelet aggregation was not affected by salsolinol alone (data not shown), thrombin-induced platelet aggregation was potentiated by salsolinol in a concentration-dependent manner (Fig. 1A). Enhancing effects of salsolinol on platelet aggregation were also observed with collagen, another physiological agonist (Fig. 1B), with similar patterns to thrombin.

During platelet activation, various mediators such as serotonin or P-selectin could be released from intracellular granules, which can further exacerbate cardiovascular diseases by enhancing blood vessel contraction and cell adhesion. To investigate the effect of salsolinol on the granular secretion, serotonin release from dense granules was examined using 3H-serotonin in the presence of sub-threshold level of thrombin. As a result, salsolinol was shown to augment thrombin-induced serotonin secretion in platelets (Fig. 2A). In addition, the pretreatment with salsolinol significantly enhanced thrombin-induced expression of P-selectin, an adhesion molecule in platelet α granules (Fig. 2B). These results are in a good agreement with platelet aggregation in Figure 1, indicating that the enhancement of platelet aggregation by salsolinol is accompanied by the release of serotonin from dense granules and the expression of the adhesion molecule, P-selectin, on platelet plasma membranes.

To examine whether the non-specific cytotoxicity might be involved in these effects, lactate dehydrogenase (LDH), a marker for membrane disturbance, was measured. Treatment of salsolinol did not induce LDH leakage (Fig. 3A), showing that salsolinol specifically rendered platelets to enhanced aggregatory states. We also examined whether salsolinol could induce ROS generation, through which salsolinol is known to mediate dopaminergic cell degeneration (10, 12). In platelets, however, salsolinol did not generate ROS (Fig. 3B), suggesting that ROS was not involved in the salsolinol-enhanced platelet aggregation.

It is reported that salsolinol could bind to α2-adrenergic receptor in platelets (32), and therefore, the involvement of the α2-adrenergic receptor in salsolinol-enhanced aggregation was investigated. Pretreatment of phentolamine, an antagonists for α2-adrenergic receptor, attenuated the enhancing effect of salsolinol significantly, reflecting that the activation of α2-adrenergic receptor might play a key role in salsolinol effect in platelets (Fig. 3C). When the α2-adrenergic receptor was activated in platelets, agonist-induced cAMP increase is attenuated by inhibitory G protein (GI) activation, leading to increased susceptibility of platelets to activation (33). As shown in Table 1, salsolinol could decrease platelet cAMP level in the presence of thrombin indeed, suggesting that enhancing effect of salsolinol is possibly due to α2-adrenergic receptor binding and subsequent decrease of cAMP in platelets.

In an attempt to explore the in-vivo significance of these in-vitro results, effect of salsolinol on thrombus formation was investigated in rat arterial and venous thrombosis models, where platelets play a major role in thrombus formation (34, 35). As shown in Figure 4, salsolinol increased both arterial and venous thrombus formation in rats in a dose-dependent manner. These results imply that salsolinol can enhance the platelet aggregation, leading to increased thrombus formation.

To examine if salsolinol effect could also be observed with human platelets, sub-threshold concentration of thrombin were added to salsolinol-treated human platelets. As a result, salsolinol enhanced thrombin-induced aggregation in human washed platelets with a similar pattern to rat platelets (Fig. 5A), indicating that these events could be reproduced in human patients. Enhanced aggregation in human platelets by salsolinol was also attenuated by pretreatment of phentolamine and yohimbine, an-

Figure 4: Increased thrombus formation by salsolinol in rat in-vivo arterial and venous thrombosis model. A) After intraperitoneal administration of salsolinol, FeCl3 was applied to carotid artery to initiate thrombus formation. Disturbance of blood flow caused by arterial thrombosis was monitored using flow-probe connected to a Doppler flowmeter. The time needed for occlusion was measured for up to 60 min, and occlusion time was regarded to be 60 min if occlusion had not occurred up to 60 min. B) After the i.v. infusion of saline (vehicle) or salsolinol (1.5 and 3 mg/ kg/min/0.5ml) for 3 min, thrombus formation was induced by the infusion of thromboplastin in a rat venous thrombosis model. Values are from 3–4 animals in each group. Bar represents mean value of occlusion times.
other α2-adrenergic receptor antagonist (Fig. 5B), suggesting that salsolinol affects human platelet through α2-adrenergic receptor activation, similar to rat platelets. These effects could also be reproduced in human PRP where platelets are suspended in biological fluid (Fig. 5C).

**Discussion**

In the present study, we demonstrated that salsolinol can increase agonist-induced platelet aggregation and secretion through attenuation of anti-aggregatory cAMP generation by α2-adrenergic receptor mediated pathways. These in-vitro results were confirmed in in-vivo animal models, showing that both the arterial and the venous thrombosis can be significantly activated by salsolinol. To our knowledge, this is the first study to demonstrate that salsolinol can result in increased thrombus formation, suggesting that salsolinol might contribute to increased thrombotic events, commonly observed in Parkinson’s disease and chronic alcoholism.

Several clinical reports have been published on the increased risk of thrombotic events in the patients with Parkinson’s diseases and alcoholism (15, 18). Despite the frequent reports on the abnormally high levels of salsolinol in these diseases (4, 8), there have been few studies focusing on the effects of salsolinol on cardiovascular systems. Melzig and Zipper (20) reported the cytotoxic effects of salsolinol on endothelial cells, but they only dealt with the disturbance of blood brain barrier, leaving the effects on the cardiovascular system unknown. Of note, however, considering that damaged endothelial cells could alter blood vessel tone, exacerbating cardiovascular diseases further (36, 37), these cytotoxic effects of salsolinol on endothelial cells might be synergistic with the enhanced platelet aggregation as shown in the present study.

In the previous report, salsolinol was shown to attenuate epinephrine-induced platelet aggregation (19), which is contradictory to the enhancing effect in the present observation. Platelet aggregation by epinephrine was mediated mainly through α2-adrenergic receptor activation (38), in which salsolinol could also function as a partial agonist. The inhibitory effect of salsolinol observed in the previous study might be from the competitive binding to α2-adrenergic receptor, as the authors have discussed. When platelets are activated by other physiological agonists like thrombin or collagen, however, binding to a α2-adrenergic receptor by a partial agonist like salsolinol could enhance platelet aggregation (38, 39). In our results, the enhancing effect of salsolinol was indeed mediated by the activation of α2-adrenergic receptor (Fig. 3C). Exemplifying the proaggregatory effect of a α2-adrenergic partial agonist, morphine was shown to potentiate collagen- or U46619-induced platelet aggregation and secretion through α2-adrenergic receptor binding, resulting in the increased platelet plug formation in an in-vivo study (40, 41).

In the present study, we demonstrated that decreased cAMP by α2-adrenergic receptor activation could play a key role in salsolinol-enhanced platelet aggregation (Fig. 3C and Table 1). It is well known that cAMP regulates platelet function through the modulation of agonist-induced platelet activating processes such as calcium increase (38). Interestingly, potentiation of agonist-
Table 1: The level of cyclic AMP (cAMP) in isolated rat platelets. The level of cAMP was measured after thrombin stimulation (0.05 U/ml) following salsolinol treatment (250 µM, 30 min), using enzyme-immunoassay. Basal level of cAMP was from platelets without thrombin addition. * represent significant differences from thrombin alone (p < 0.05).

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<th>Basal (pmol/3 x 10⁸ cells)</th>
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<tr>
<td>Control</td>
<td>8.03 ± 0.91</td>
<td>8.06 ± 0.51</td>
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<tr>
<td>Salsolinol</td>
<td>6.16* ± 0.38</td>
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induced calcium increase was not observed by salsolinol treatment (data not shown), implying that other cAMP-mediated pathways are involved. Other than calcium increase, several pathways like aggregation and phosphorylation by protein kinase A could be directly affected by cAMP in platelets (42), suggesting the possible involvement of these pathways in the effects of salsolinol. As shown in Figure 2, salsolinol also enhanced P-selectin expression and serotonin secretion along with platelet aggregation. P-selectin is known to be responsible for the interactions between platelets and other blood cells, playing an important role in coagulation and thrombosis (43). In addition, serotonin secreted from platelets can cause vasocstriction or proliferation of vascular smooth muscle cells (44, 45), suggesting that the enhancement of these subsequent responses as well as platelet aggregation by salsolinol could potentiate further the development of cardiovascular diseases in Parkinson’s diseases and chronic alcoholism.

Although the presence of plasma protein attenuated the response of salsolinol in PRP up to 100 µM (Fig. 5C), the enhancement of platelet aggregation was observed at as low as 50 µM of salsolinol in human washed platelets (Fig. 5A), which is within similar ranges to those employed in previous in-vitro studies on salsolinol (9, 10). Despite the technical limitation in preservation of the fresh platelets for long time, the significant effects of salsolinol were shown in a relatively short incubation time. Moreover, considering that salsolinol could be actively taken up and accumulated in platelet dense granules (19, 46), local concentration of salsolinol around platelets could be substantially elevated during platelet granular secretion. In this regard, it is possible that salsolinol might induce meaningful effects at nanomolar concentrations (47, 48) in the real clinical conditions where patients are chronically exposed to salsolinol, although further clinical studies are required to fully understand the role of salsolinol in the increased thrombus formation.

In summary, we demonstrated that salsolinol enhanced agonist-induced aggregation and secretion in platelets through the α2-adrenergic receptor and decrease of cAMP, leading to increased thrombus formation. This salsolinol-enhanced activation of platelets could be a possible contributing factor in thrombotic events observed in patients with Parkinson’s diseases and chronic alcoholism.

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