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Antagonism of 5-hydroxykynurenamine against serotonin action on platelet aggregation

(indoleamine 2,3-dioxygenase/hemostasis/thrombogenesis)

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ABSTRACT **Serotonin induced an aggregation of human platelets, whereas 5-hydroxykynurenamine, produced from serotonin by the action of indoleamine 2,3-dioxygenase, did not cause any significant degree of platelet aggregation. 5-Hydroxykynurenamine specifically inhibited both a serotonin-induced aggregation of platelets and the potentiation of the ADP-induced platelet aggregation by serotonin. It did not, however, alter the profiles of the platelet aggregation induced by ADP, collagen, or adrenaline. The degree of inhibition was proportional to the time of preincubation of platelets with 5-hydroxykynurenamine, and to the concentration of 5-hydroxykynurenamine used. Available evidence indicated that 5-hydroxykynurenamine competed with serotonin for the same receptor sites. Studies with analogues of 5-hydroxykynurenamine indicated that the substitutions of *o*-aminobenzyl moiety with hydroxy or methoxy groups were somewhat tolerated, whereas the masking of alkylamine moiety with *N*-acetylation completely lost the inhibitory activity.**

5-Hydroxykynurenamine is produced from serotonin by the action of indoleamine 2,3-dioxygenase, an enzyme which is ubiquitously distributed in various organs of mammals (1, 2). The natural occurrence of 5-hydroxykynurenamine and its metabolite, 4,6-dihydroxyquinoline, was previously reported by Makino (3) and Kido *et al.* (4). The biological activity of 5-hydroxykynurenamine, however, has remained unclarified except for its effects on blood pressure (5). Recently, it has been reported by Toda *et al.* that 5-hydroxykynurenamine specifically antagonizes the effect of serotonin on the smooth muscle contraction of cerebral arteries (6). In this communication, we wish to report several lines of evidence that 5-hydroxykynurenamine inhibits the action of serotonin on the aggregation of human blood platelets.

METHODS AND MATERIALS

Blood from healthy donors, who had taken no medications for at least 1 week, was collected from the antecubital vein, mixed with 0.1 volume of 3.8% trisodium citrate solution and centrifuged at 190 × *g* for 10 min. The upper layer containing platelet-rich plasma was removed and kept at room temperature. The aggregation experiments were completed within 5 hr after the preparation of the plasma. The platelet-rich plasma was diluted with autologous plasma to give a platelet count of about 300,000 per μl (7). Samples, at a final volume of 1.0 ml, were warmed to 37° for 2 min, and the aggregation was monitored by continuous recording of light transmission (EEL-aggregometer, model 169, Evans Electroselenium Co. Ltd., Halstead, Essex, England). The light transmission of platelet-rich plasma and that of platelet-poor plasma from the same donor were taken as 0% and 100%, respectively.

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N^{α}-Acetyl-5-methoxykynurenamine and 5-hydroxykynurenamine were synthesized according to the methods described previously (6, 8). 4,6-Dihydroxyquinoline was enzymatically synthesized from 5-hydroxykynurenamine (1). 5-Methoxykynurenamine, 5-hydroxykynurenine, *N,N'*-dimethyl-5-hydroxykynurenamine, *N,N'*-dimethyl-3-hydroxykynurenamine, and 2-(3'-aminopropyl)aniline were generous gifts of Dr. T. Takei of Takeda Central Research Institute, Osaka, Japan. Serotonin creatinine sulfate, kynurenamine hydrobromide, kynurenine sulfate, collagen, adenosine diphosphate, and adrenaline were purchased from Sigma. All these reagents were dissolved in a solution containing 138.6 mM NaCl and 15.4 mM Tris·HCl buffer, pH 7.4.

RESULTS

The level of platelet aggregation induced by serotonin was always low and the aggregation was only transient, as shown in Fig. 1, left A. 5-Hydroxykynurenamine did not cause any significant degree of platelet aggregation itself, but rather

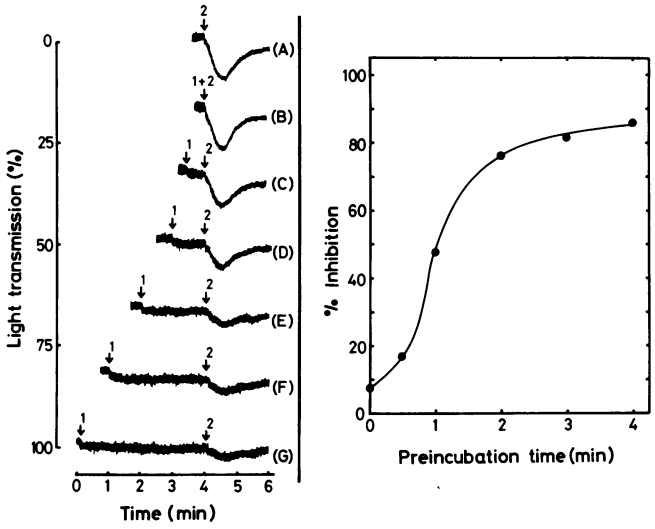

FIG. 1. (*Left*) Time course of inhibition by 5-hydroxykynurenamine on serotonin-induced platelet aggregation. The changes in light transmission were continuously recorded after the addition of 5-hydroxykynurenamine (5 μM final concentration) (arrow 1) and serotonin (5 μM final concentration) (arrow 2). (A) No 5-hydroxykynurenamine. The platelets were preincubated with 5-hydroxykynurenamine for 0 min (B), 30 sec (C), 1 min (D), 2 min (E), 3 min (F), and 4 min (G). (*Right*) Percent inhibition of the serotonin-induced aggregation plotted against the time of incubation with 5-hydroxykynurenamine. The maximal changes, obtained in the left figures (A–G), were measured and expressed as percent inhibition.

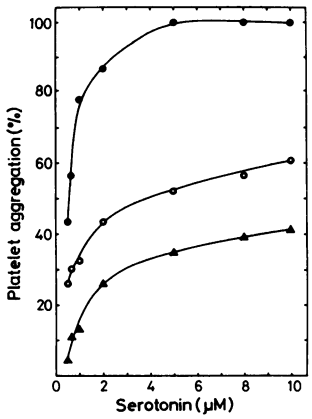

FIG. 2. Inhibitory effects of 5-hydroxykynurenamine on the platelet aggregation induced by serotonin at various concentrations. The maximal changes of platelet aggregation were measured in the absence of (●) or the presence of 1 μM (○) or 5 μM (▲) 5-hydroxykynurenamine at various concentrations of serotonin. The data were expressed as percentage of the platelet aggregation induced by 5 μM serotonin alone. Platelets were preincubated with 5-hydroxykynurenamine for 2 min.

inhibited the platelet aggregation brought about by serotonin (Fig. 1, left B–G). In the presence of inhibitory concentrations of 5-hydroxykynurenamine, the initial velocity of the platelet aggregation induced by serotonin decreased and the disaggregation seemed to occur sooner. Accordingly, the maximal change in the light transmission was less in the presence of 5-hydroxykynurenamine than that in its absence. The decrease in the maximal change was almost proportional to the time of preincubation of platelets with 5-hydroxykynurenamine over a range of 2 min (Fig. 1, right). Stirring the plasma at 37° for 4 min without 5-hydroxykynurenamine did not significantly diminish the effectiveness of serotonin, suggesting that the increased inhibition following the preincubation with 5-hydroxykynurenamine was not due to any changes in the fragility of the platelets. The preincubation period might be required, if metabolites from 5-hydroxykynurenamine were the effective agents. Although platelets contain monoamine oxidase, an enzyme which oxidizes 5-hydroxykynurenamine to 4,6-dihydroxyquinoline (1), addition of synthetic 4,6-dihydroxyquinoline to the platelets did not affect the serotonin-induced aggregation, suggesting that 5-hydroxykynurenamine itself was the inhibitor.

Serotonin aggregated platelets in a concentration-dependent manner over a range of 0.2–5 μM, and the concentration required for half-maximal aggregation was around 0.6 μM (Fig. 2). When the aggregation of platelets was induced by 5 μM serotonin, the inhibition of the platelet aggregation was also proportional to the concentration of 5-hydroxykynurenamine used. The concentration of 5-hydroxykynurenamine required for half-maximal inhibition was around 25 μM without the preincubation, but was 1 μM after the preincubation for 2 min. As shown in Fig. 2, increasing amounts of serotonin were required to overcome the inhibitory effects of 5-hydroxykynurenamine, suggesting that 5-hydroxykynurenamine inhibits the action of serotonin possibly by binding at the same receptor sites. This possibility was further supported by the experimental results that 5-hydroxykynurenamine did not affect the platelet aggregation induced by other reagents such as collagen, ADP, and adrenaline (data not shown).

To obtain information on the structural requirements for the serotonin-antagonization, several analogues of 5-hydroxykynurenamine were tested (Fig. 3). Although kynurenamine also inhibited the platelet aggregation induced by serotonin, its effect was slightly less than those of 5-hydroxykynurenamine or 5-methoxykynurenamine, indicating that the 5-hydroxy group was not absolutely essential for the activity. The inhibition of serotonin-induced platelet aggregation by *N,N'*-dimethyl-5-hydroxykynurenamine was more pronounced than that by *N,N'*-dimethyl-3-hydroxykynurenamine. Therefore, the substitution at the 5 position with a hydroxy group might be the preferred structure, although the substitutions of benzene ring with hydroxy or methoxy groups might be tolerated with slight changes in their inhibitory effects. Since the inhibition by 2-(3'-aminopropyl)aniline was less than that by kynurenamine, the carbonyl group in the side chain might also play some role in the inhibition. 5-Hydroxykynurenine and kynurenine, amino acids that are the precursors of 5-hydroxykynurenamine and kynurenamine, respectively, had no inhibitory activities on the platelet aggregation by serotonin (data not shown). *N,N'*-Dimethyl-5-hydroxykynurenamine, which has properties of an amine, showed less inhibitory effects, compared with 5-hydroxykynurenamine. However, when the amine was masked by

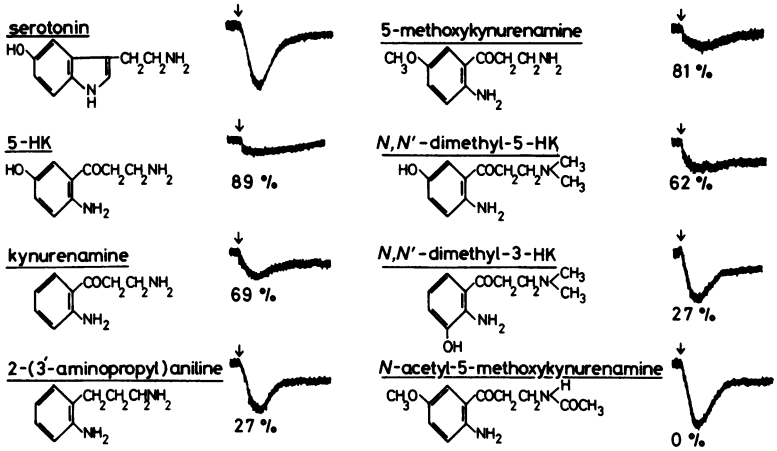

FIG. 3. Structural formulae and inhibitory degree of various analogues of 5-hydroxykynurenamine. The inhibitory effects of various analogues (50 μM final concentration) on the platelet aggregation induced by 5 μM serotonin were examined. The maximal changes in the presence of various analogues were measured and expressed as percent inhibition of the platelet aggregation. The preincubation of platelets with various analogues was carried out for 1 min. HK, hydroxykynurenamine.

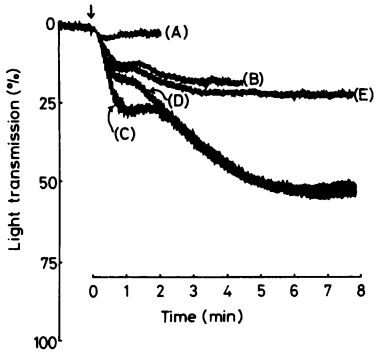

FIG. 4. Effects of 5-hydroxykynurenamine on the potentiation of serotonin in the ADP-induced platelet aggregation. The aggregation profiles were measured in the presence of (A) 5 μM serotonin, (B) 1 μM ADP, and (C) 1 μM ADP and 5 μM serotonin. The platelets preincubated with 50 μM 5-hydroxykynurenamine for 1 min (D) or 2 min (E) were exposed to 5 μM serotonin and 1 μM ADP at the time indicated by the vertical arrow.

N-acetylation, the inhibitory activity was completely lost. Therefore, the alkylamine in the side chain might be essential for the activity.

Another important role of serotonin in the platelet aggregation is its potentiating effect on the aggregation induced by ADP or adrenaline (9). Serotonin (5 μM) produced a slight aggregation of platelets as described above (Fig. 4A). ADP (1 μM) also caused the aggregation of platelets, but this aggregation was quite different from that induced by serotonin and was not reversed upon continued incubation (Fig. 4B). When 1 μM ADP and 5 μM serotonin were added to the plasma simultaneously, the observed aggregation exceeded the additive maximal changes induced by ADP and by serotonin (Fig. 4C). The profile of the platelet aggregation induced by the addition of both ADP and serotonin appeared to consist of two different phases. When the platelets were preincubated with 5-hydroxykynurenamine for one minute prior to the addition of both ADP and serotonin, only the first phase of aggregation was partially inhibited (Fig. 4D). However, when the preincubation time was increased to 2 min, the potentiation of serotonin in the aggregation was almost completely blocked and the aggregation profile under these conditions was essentially similar to that induced by ADP alone (Fig. 4E). This suggests that 5-hydroxykynurenamine specifically inhibited the potentiating effects of serotonin on the ADP-induced platelet aggregation.

DISCUSSION

The experimental results presented in this communication clearly demonstrated that 5-hydroxykynurenamine antagonized the action of serotonin in human platelet aggregation. 5-Hydroxykynurenamine is produced directly from serotonin by the action of indoleamine 2,3-dioxygenase (1). Alternatively, it was also formed by the action of aromatic amino acid decarboxylase on 5-hydroxykynurenine, which in turn is produced from 5-hydroxytryptophan by the action of indoleamine 2,3-dioxygenase (1). Although the natural occurrence of 5-hydroxykynurenamine has been reported (3), it is still unknown by which metabolic pathway 5-hydroxykynurenamine is formed *in vivo*. Indoleamine 2,3-dioxygenase has not yet been detected in platelets, but is found in choroidal plexus, pia mater, aorta, heart, and other organs that belong to the circulatory system of blood (unpublished data). Therefore, under certain conditions, platelets might be exposed to a sufficient concentration of 5-hydroxykynurenamine to antagonize the action of serotonin.

Platelets contribute to the arrest of bleeding by adhering to the edges of a break in the vessel and to each other to form a hemostatic plug. They react in a similar way to nonhemorrhagic intimal lesions by forming thrombus within the lumen (10). It was suggested that serotonin may initially enhance the formation of the hemostatic plug and later, by saturating the platelet serotonin receptors, limit the increase of intravascular aggregation (9). Further, serotonin may have a secondary and potentiating role in thrombosis (11). Therefore, the present investigation suggests a potentially important role for 5-hydroxykynurenamine in hemostasis and thrombogenesis through modulation of the effect of serotonin on the platelets' function. Since evaluation of serotonin for its contribution to hemostasis and thrombogenesis has produced some conflicting results, depending on the experimental conditions used, a more definitive answer to this question might be obtained by a more direct experimental approach which utilizes both serotonin and its antagonist, 5-hydroxykynurenamine.

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