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Inhibitory effects of ONO-3708 and S-145 on shape change and aggregation of rabbit platelets induced by STA_2

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KEY WORDS blood platelets; platelet aggregation; platelet aggregation inhibitors; thromboxane A_2; calcium; STA_2; ONO-3708; S-145

AIM: To study the mode of inhibition of ONO-3708 and S-145, 2 antagonists of thromboxane A_2 (TXA_2) receptors, against the rabbit platelet shape change and aggregation induced by stable analogue of TXA_2 (STA_2). **METHODS**: The platelet shape change and aggregation were quantified by the light transmission through platelet-rich plasma (PRP) and the intracellular calcium concentration ($[Ca^{2+}]_i$) was measured by fluorescence and imaging. **RESULTS**: (1) In PRP, STA_2 (3 $\mu mol \cdot L^{-1}$)-induced aggregation was inhibited by egtazic acid 3 $mmol \cdot L^{-1}$, ONO-3708 300 $\mu mol \cdot L^{-1}$, and S-145 1 $\mu mol \cdot L^{-1}$ ($P<0.01$), but not by indometacin (Ind) 3 $\mu mol \cdot L^{-1}$. The shape change induced by STA_2 was inhibited only by S-145 in a concentration-dependent manner. S-145 1 and 3 $\mu mol \cdot L^{-1}$ were required to inhibit the shape change and aggregation. (2) The inhibitory effect of S-145, but not ONO-3708, was increased along with the prolongation of preincubation. (3) ONO-3708 lost the inhibitory effect on STA_2-induced aggregation after washing, while the inhibitory effect of S-145 was enhanced by prolongation of preincubation and remained after washing. (4) STA_2 3 $\mu mol \cdot L^{-1}$-induced $[Ca^{2+}]_i$ mobilization was unaffected by Ind, partially reduced by ONO-3708 and egtazic acid 3 $mmol \cdot L^{-1}$ ($P<0.01$), but completely inhibited by S-145 ($P<0.01$). **CONCLUSION**: S-145 and ONO-3708 were bound to a different site of the TXA_2 receptor.

Platelet activation by thromboxane A_2 (TXA_2) results in a shape change in platelets or centralization of granules, the exposure of adhesive molecules, such as GP Ⅱb/Ⅲa, responsible for platelet aggregation and finally the release of dense granules and further aggregation. These sequential responses coincide with the activation of phospholipase C and the release of 1, 4, 5-inositol triphosphate (IP_3) and diaceglycerol (DG)[1] and are mediated by subsequent increase in cytosolic calcium and protein kinase C activation. The number of TXA_2 receptors in human was 1500 ~ 2500 sites per platelet[2,3]. In addition, the TXA_2/prostaglandin (PG) endoperoxide (PGH_2) receptor was purified by affinity chromatography using TXA_2 antagonist (±)-5 | *Z*-7-[3-indophenylsulfonylamino(2.2.1)bicyclohept-2-exo-yl]-heplanoic acid, sodium salt| (S-145)[4] and the presence of 2 TXA_2/PGH_2 receptors was reports in terms of reversible and irreversible binding sites of TXA_2 antagonists[5]. The present study was performed to investigate the possibility that 2 types of TXA_2 inhibitors may bind to the receptors in a different mode in terms of platelet shape change and aggregation.

MATERIALS AND METHODS

Agents S-145 was gifted by Shionogi Pharmaceutical Co Ltd, Osaka. ONO-3708 |7-[2α, 4α-(methylmethano)-6β-(2-cyclopentyl-2β-hydroxyactami-no)-1α-cyclohexyl]-5 (*Z*)-heptanoic acid, sodium salt (ONO Pharmaceutical Co, Osaka)| was dissolved in buffered saline. Indometacin (Ind, Merck, Sharp & Dohme Research Lab, Rahway NJ) was dissolved in absolute ethanol (10 $g \cdot L^{-1}$) and kept at 4 ℃, and diluted by buffered saline before use. STA_2 (9, 11-epithio-11, 12-methano-TXA_2, a stable analogue of TXA_2 kindly provided by ONO Pharmaceutical Co, Osaka) was dissolved in ethanol and stored at − 20 ℃. Egtazic acid (Sigma Co, St Louis) was dissolved in distilled water with NaOH 1 $mol \cdot L^{-1}$ and stored at room temperature after the pH was adjusted to 7.4 with HCl 1 $mol \cdot L^{-1}$. Fura-2 and Fura 2-AM (Wako Pure Chemicals, Osaka) were used for the Ca^{2+} calibration curve and platelet loading.

Preparation of washed platelets[6]

Preincubation of platelets with TXA_2 antagonists The

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washed platelets ($4 \times 10^{11} \cdot L^{-1}$) were preincubated with S-145 (10 and 30 nmol·L^{-1}), ONO-3708 (3 and 10 μmol·L^{-1}), or buffer at 37 ℃ for 2, 5, or 10 min. The washed platelets were stimulated by STA_2 3 μmol·L^{-1} at 4 min after the addition of $CaCl_2$ 1 mmol·L^{-1} (Fig 1A).

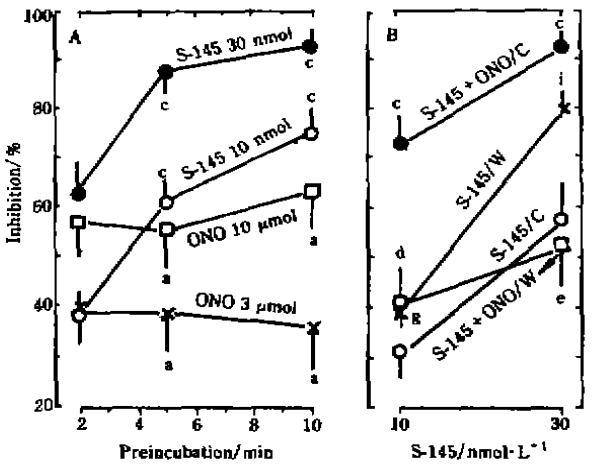

Fig 1. Aggregatory inhibition by preincubation with ONO-3708 and S-145 (A) and effects of washing (B). $n = 11 - 15$ samples from at least 6 rabbits, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$, $^dP > 0.05$ and $^fP < 0.01$ *vs* S-145/C, $^dP > 0.05$, $^eP < 0.05$ *vs* S-145/W. C: control (without washing); W: the addition of STA_2 after washing.

Washing experiments After the washed platelets were preincubated with S-145 10 or 30 nmol·L^{-1} plus ONO-3708 10 μmol·L^{-1} for 2 min, they were centrifuged at 700 × g for 20 min. The platelet pellets were suspended in HEPES-buffer and the final platelet counts were adjusted to 4×10^{11} ·L^{-1}. The rewashed platelets were aggregated by STA_2 3 μmol·L^{-1} at 4 min after the addition of $CaCl_2$ 1 mmol·L^{-1}. The control was run without washing.

Platelet shape change and aggregation[6,7]

Fura-2 loading and $[Ca^{2+}]_i$ measurement[6,8]

Data analysis Statistical analysis was performed by either paired or unpaired *t*-test.

RESULTS

Inhibitory effects of ONO-3708 and S-145 on STA_2-induced shape change and aggregation in PRP STA_2-induced aggregation was completely inhibited by ONO-3708 and S-145, and also inhibited by egtazic acid 3 mmol·L^{-1}, but not by Ind 3 μmol·L^{-1}. While the shape change was inhibited only by S-145. S-145 1 and 3 μmol·L^{-1} were required respectively to completely inhibit platelet shape change and aggregation (Tab 1).

Tab 1. Effects of indometacin (Ind), egtazic acid, S-145, and ONO-3708 on STA_2 (3 μmol·L^{-1})-induced platelet shape change and aggregation in rabbit PRP. $n = 7 - 11$ **samples from 6 rabbits,** $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* STA_2.

Groups	Shape change, %	Aggregation, %
STA_2	−8.4 ± 0.8	61 ± 6
Ind 3 μmol·L^{-1}	−8.3 ± 0.9[a]	65 ± 7[a]
Egtazic acid 3 mmol·L^{-1}	−10.4 ± 0.9[c]	1.1 ± 0.8[c]
S-145 1 μmol·L^{-1}	−5.7 ± 0.8[c]	1.9 ± 1.4[c]
3 μmol·L^{-1}	−1.0 ± 0.4[c]	0.0 ± 0.0
ONO-3708 100 μmol·L^{-1}	−8.1 ± 0.9	22 ± 4[c]
300 μmol·L^{-1}	−9.8 ± 0.9	0.0 ± 0.0

The dose-response curve of STA_2 (3 μmol·L^{-1})-induced aggregation was diminished by ONO-3708 and the maximal response was not affected, in case of S-145, the dose-response was diminished, but the maximal response was reduced markedly (data not shown).

Effect of washing and preincubation on STA_2-induced aggregation The inhibition of S-145 on STA_2-caused aggregation was increased ($P < 0.01$) along with the prolongation of preincubation time. In contrast, STA_2-induced aggregation was not affected by ONO-3708, even preincubating platelets with ONO-3708 for 10 min (Fig 1A).

S-145 concentration-dependently inhibited STA_2-induced aggregation. This inhibitory effect was enhanced by the administration of ONO-3708 together with S-145. The effect of S-145 remained after washing, but ONO-3708 lost its inhibitory effect (Fig 1B).

Intracellular calcium mobilization The cytosolic-Ca^{2+} concentration $[Ca^{2+}]_i$ was measured in single platelets. The STA_2 1 and 3 μmol·L^{-1}-induced elevation of $[Ca^{2+}]_i$ was not influenced by Ind. The calcium mobilization by STA_2 3 μmol·L^{-1} was partially depressed by ONO-3708 30 μmol·L^{-1} and egtazic acid 3 mmol·L^{-1}, and completely inhibited by S-145 100 nmol·L^{-1} ($P < 0.01$) (Fig 2).

DISCUSSION

It has been considered that platelets will undergo the morphological change in shape and then

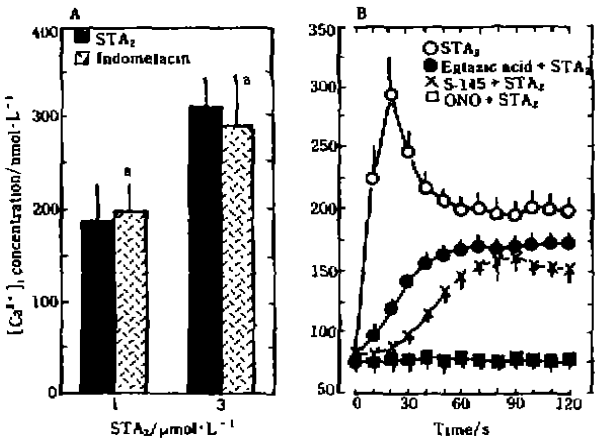

Fig 2. A) Effect of indometacin 3 μmol on STA_2-induced $[Ca^{2+}]_i$ mobilization in single platelets. B) Effects of egtazic acid 3 mmol, S-145 100 nmol, and ONO-3708 30 μmol on STA_2-stimulated $[Ca^{2+}]_i$ mobilization in single platelets. $n = 41 - 57$ cells from 6 rabbits, $\bar{x} \pm s$. $^aP > 0.05$ vs STA_2.

cell to cell aggregation once platelets are activated by aggregating agents, but, whether or not there are 2 different TXA_2 receptors or 2 different binding sites for TXA_2 antagonists to regulate the platelet shape change and aggregation, it is still unknown.

In this experiment, STA_2 (the specific agonist of TXA_2 receptor) was used to cause the platelet shape change and aggregation, which directly activated the TXA_2 receptors and its effects were not associated with the metabolic products of arachidonic acid because STA_2-induced platelet shape change and aggregation were not influenced by Ind, so the pharmacological effects of STA_2 and its antagonists on the shape change and aggregation of platelets were easily analysed.

Some evidences from present experiments supported the fact that TXA_2 receptors exist 2 binding sites for ONO-3708 and S-145, and these 2 binding portions are considered to be relative to the regulation of platelet shape change and aggregation. Firstly, the effect of ONO-3708 and S-145 on platelet shape change was exactly different from each other even though the aggregation was completely inhibited by both ONO-3708 and S-145. The dose-response curve of STA_2-induced aggregation was shifted parallely by ONO-3708 and unparallely by S-145 with the decrease in the maximal response. Secondly, the inhibitory effect of S-145 was not affected by the procedure of washing platelets, but the procedure rendered ONO-3708 lost its effect. In addition, the effect of S-145 was enhanced by the prolongation of preincubation time, the effect of ONO-3708 under the same condition was not changed. These results suggested that S-145 tightly and irreversibly bound to its binding portion and the combination of ONO-3708 with its binding site was untight and reversible. Finally, we compared the action of both antagonists on STA_2-induced $[Ca^{2+}]_i$ mobilization, the results indicated that S-145 abolished both calcium influx and intracellular calcium release and ONO-3708 only inhibited STA_2-induced calcium influx without the effect on the intracellular calcium release, this effect of ONO-3708 was similar to that of egtazic acid but due to the different mechanisms. All together it can easily be explained that why S-145 completely inhibited STA_2-induced platelet shape change and aggregation and that the reason why ONO-3708 and egtazic acid only inhibited STA_2-induced aggregation without the effect on the platelet shape change. In other words, the platelet shape change is dependent on the intracellular calcium release while the aggregation is extracellular calcium related. So far, the relationship between intracellular calcium release and extracellular calcium influx during platelet shape change and aggregation and the molecular mechanisms connected these 2 cellular events are still unclear.

In summary, according to the evidences mentioned above and the biochemical data from other reports[4,9], it has been suggested that S-145 may be tightly and irreversibly bound to its binding portion different from that for ONO-3708 in quite opposite way on the same TXA_2 receptor, therefore they regulated the different cellular event. But the possibility of more than one TXA_2 receptors on the platelets is still remained although the single cDNA cloned was detected[10], which did not exclude a single protein moiety with different glycoprotein.

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ONO-3708 和 S-145 对 STA_2 介导的家兔血小板变形和聚集反应的抑制作用

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关键词 血小板；血小板聚集；血小板聚集抑制剂；血栓素 A_2；钙；STA_2；ONO-3708；S-145

目的：评价 ONO-3708 和 S-145 对血小板变形和聚集反应的不同抑制模式. **方法**：以透光度法测量血小板变形和聚集反应，荧光图像分析法测量单细胞内游离钙的变化. **结果**：(1) STA_2 的聚集反应可被依他酸，ONO-3708 和 S-145 抑制($P<0.01$)，血小板变形仅被 S-145 抑制. (2) S-145 的抑制作用随孵育时间延长而增强，ONO-3708 不变. (3) 洗脱后 ONO-3708 的作用消失，而 S-145 抑制作用依然存在. (4) STA_2 的细胞内游离钙动员部分被 ONO-3708 和依他酸取消($P<0.01$)，但可被 S-145 完全抑制. **结论**：S-145 和 ONO-3708 分别作用于血小板 TXA_2 受体的不同结合位点.

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Protective effects of tetrandrine on CCl_4-injured hepatocytes

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KEY WORDS tetrandrine; liver; cultured cells; carbon tetrachloride poisoning; malondialdehyde; calcium; membrane fluidity; lactate dehydrogenase

AIM: To study the protective effects of tetrandrine (Tet) on CCl_4-injured hepatocytes. **METHODS**: The cultured rat liver cells were poisoned by CCl_4 (10 mmol · L^{-1}). The membrane fluidity was detected by 1,6-diphenyl-1,3,5-hexatriene (DPH), a lipid probe. The Ca^{2+} concentration was assayed with Fura 2-AM, a sensitive calcium indicator. **RESULTS**: Tet (1 - 1000 nmol · L^{-1}) increased viability of liver cell (from 71 % to 72 % - 89 %), reduced lactate dehydrogenase (LDH) release, and malondialdehyde (MDA) formation. Tet prevented the heightening of the intracellular Ca^{2+} concentration and the attenuation of the membrane fluidity of liver cells ($P<0.05$). **CONCLUSION**: Tet had a protective effect on CCl_4-injured hepatocytes by inhibiting the lipid peroxidation,

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