Demonstration of Kinin-Release in the Peritoneal Exudate of Kaolin-Induced Writhing in Mice

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Abstract—Using a bradykinin enzyme immunoassay, we measured the amount of kinin in the peritoneal washings of mice with the kaolin-induced writhing reaction. Simultaneous treatment with captopril, a kininase II inhibitor, significantly increased the kinin level at 1 min after kaolin injection. Soybean trypsin inhibitor injected simultaneously with kaolin almost completely suppressed the kinin level at 1 min with or without treatment of captopril. These results suggest that kinin is released through activation of the plasma kallikrein-kinin system by kaolin, and that kinin could be a main mediator for the writhing reaction.

Recently, we have developed a new pain model in mice by using the kaolin-induced writhing reaction (1). A possible mechanism for the induction of writhing is that bradykinin could be released through the activation of factor XII by kaolin and causes the pain reaction. However direct evidence for the activation of the kallikrein-kinin system in this model has not yet demonstrated. Therefore, in this study, we measured the amount of kinin released in the peritoneal washings of mice after intraperitoneal (i.p.) injection of kaolin.

Male ICR mice (aged 5 weeks, weighing about 20 g) used were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka), and mice were intraperitoneally injected with kaolin suspension (Wako Pure Chemical Industries, Ltd.) (2.5 mg/0.5 ml saline/mouse). The writhing response was observed (early stage) as previously reported (1, 2). The writhing reaction of later stage was induced by intraperitoneal injection of captopril (Sankyo) (20 μg/0.2 ml saline/mouse) at 60 min after the kaolin injection as previously reported (1, 2). In some experiment on both stages, soybean trypsin inhibitor (SBTI, Sigma) (2.5 mg/mouse) was intraperitoneally injected simultaneously with kaolin (early stage) or with captopril (later stage).

For collection of peritoneal washings and assay of kinin, mice were sacrificed by exsanguination at 1 min after the kaolin injection (early stage) and at 5 min after the captopril injection in the later stage (60 min after kaolin injection). Mice were injected with 2 ml of phosphate-buffered saline (PBS)-ethanol (80:1) containing SBTI (62.5 μg), aprotinin (Sigma) (12.5 μg), polybrene (Sigma) (17.5 μg), disodium ethylenediamine tetraacetate (EDTA-2Na) (75 μg) and o-phenanthroline (Wako Pure Chemicals) (42.5 μg), intraperitoneally, just before the exsanguination. The peritoneal fluids were recovered with a polyethylene pipet and put into a polypropylene tube. Each recovered peritoneal wash was 1.8-1.9 ml. Measurement of kinin was carried out according to the modified methods of Minami et al. (3) and Ueno et al. (4).

All of the procedures for the extraction
and subsequent enzyme immunoassay of kinin were carried out with polypropylene tubes or siliconized glassware to protect the sample from artificial activation of the kinin generating systems. Collected washings from 5 mice were pooled in a tube and then immediately added with ethanol (at a final concentration of 80%). After standing at 0°C for 1 hr the mixture was centrifuged at 1,600×g for 20 min at 4°C. The supernatant was evaporated to dryness at 37°C in a rotating evaporator. The dried sample was dissolved in 200 μl of 0.05 M Tris-acetate buffer (pH 8.5) containing 0.1% gelatin (Merck), 0.05% Tween 20 (Sigma) and 0.02% NaN₃ (Sigma), as final concentrations; and then 50 μl of 1,1,2-trichloro-1,2,2-trifluoroethane (Wako) was added and the mixture shaken to remove extractable lipids. After centrifugation at 1,600×g for 20 min, the aqueous layer was transferred quantitatively to another polypropylene tube by decantation. The mixture was centrifuged at 1,500 g for 10 min to remove precipitated protein. A 100-μl aliquot of the supernatant was immunostained for kinin by using a Markit-A bradykinin kit (Dainippon Pharmaceuticals) (4, 5), and the amount of kinin was expressed as bradykinin equivalents. Data were expressed as the mean with standard errors. Evaluation of the difference in each treatment group was done by non-parametric statistical analysis (Mann-Whitney U-test).

As shown in Table 1, kinin was detected in the peritoneal exudate 1 min after injection of kaolin (2.5 mg/mouse, i.p.) (early stage). The amount of kinin measured at 1 min varied greatly in the different washes, but we chose this stage because the level of kinin at 5 min was markedly decreased, and in the washings obtained after 10 min, kinin could not be detected. The level of kinin at 1 min after kaolin injection was significantly suppressed by simultaneous injection of SBTI (2.5 mg/mouse, i.p.), which is known as a plasma kallikrein inhibitor. However the kinin amount was significantly augmented by simultaneous injection of captopril (50 μg/mouse, i.p.), as a kininase II inhibitor, and this augmentation was also suppressed to the detection limit by the simultaneous injection of SBTI (2.5 mg/mouse, i.p.) (Table 1). The fact that kinin release was detected at 1 min in the peritoneal washings, whereas the writhing reaction peaked at the stage of 5–10 min, could be explained by the follows: pain receptors in the peritoneal cavity could be sensitized by serially released PG1₂, after kinin production.

Table 1. The kinin amounts in the peritoneal washes of mice that received intraperitoneal injection of kaolin with or without simultaneous injection of SBTI and/or captopril

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Treatment</th>
<th>N</th>
<th>Kinin (ng/5 mice)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>None</td>
<td>5</td>
<td>&lt;1.79</td>
</tr>
<tr>
<td>Kaolin</td>
<td>None</td>
<td>5</td>
<td>5.27±0.61b</td>
</tr>
<tr>
<td>(2.5 mg/mouse)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaolin</td>
<td>SBTI (2.5 mg/mouse)</td>
<td>5</td>
<td>&lt;1.79c</td>
</tr>
<tr>
<td>(2.5 mg/mouse)</td>
<td>Captopril (50 μg/mouse)</td>
<td>5</td>
<td>8.95±1.29d</td>
</tr>
<tr>
<td>Kaolin</td>
<td>Captopril (50 μg/mouse) + SBTI (2.5 mg/mouse)</td>
<td>5</td>
<td>&lt;1.79e</td>
</tr>
</tbody>
</table>

Peritoneal washes were obtained at 1 min after the kaolin treatment. Each value shows the mean level of kinin (bradykinin equivalent), from 5 experiments, in the pooled peritoneal washes from 5 mice with S.E. ¹Kinin amount is expressed as ng of bradykinin equivalents in the washes pooled from 5 mice. ²Indicates a significant difference from the intact group at P<0.005. ³and ⁴indicate significant differences from the kaolin-challenged group at P<0.005 and P<0.05, respectively. ⁵indicates a significant difference from the captopril-treated group at P<0.005.
so that a lower amount of kinin at 5–10 min could induce a further writhing reaction. Accordingly, it is suggested that there is some threshold of peritoneal kinin level for enhancing the writhing reaction, and the sensitizing action of PG12 is an indispensable factor in this reaction.

Furthermore, at 60 min after kaolin injection, the writhing reaction could be induced by intraperitoneal injection of captopril (later stage) as previously reported (1, 2). The kinin amount in the peritoneal washings 5 min after the captopril (20 μg/mouse) injection was also measured and shown in Table 2. A very small amount of kinin was found in the washings of mice that received saline only instead of captopril. The captopril-induced kinin amount was also suppressed to the assay limit by simultaneous injection of SBTI (1 mg/mouse). The above finding is consistent with the previous report in which the captopril-induced writhing reaction (later stage) was also completely suppressed by simultaneous injection of SBTI (1, 2).

It is well-known that kaolin induces a production of kinin by activation of the kallikrein-kinin system through activation of factor XII in mammalian plasma (6–8). Furthermore, we recently reported that the kaolin-induced writhing reaction (early stage) and the captopril-induced writhing reaction (later stage) were completely suppressed by pretreatment with bromelain (intravenous injection), and the mouse plasma kallikrein-kinin system was activated by incubation with kaolin in vitro (9). Our results demonstrated that the intraperitoneal injection of kaolin produced kinin in the peritoneal cavity of the mouse, and thus the produced kinin caused the pain reaction. In addition, these results suggest that kinin released with kaolin at the early stage was rapidly degraded by kininases, although the remaining kaolin could continue to produce kinin in the peritoneal cavity of the mouse, even 60 min after the kaolin injection.

We suggested previously that the mechanism of the kaolin-induced writhing reaction could be due to pain which may be caused by the released kinin and the sensitizing activity of PG12 that was simultaneously released by kinin, because we detected 6-keto-PGF1α in the peritoneal washings and the release of 6-keto-PGF1α was dependent on the activation of the kallikrein-kinin system (10).

Therefore, the above results give further proof for the mechanism of this writhing model, and this model could be useful for the evaluation of antiinflammatory and analgesic agents.

### Table 2. Effect of the simultaneously injected SBTI on the kinin amount in the peritoneal washes of captopril-treated mice 60 min after kaolin injection (later stage)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Kinin (ng/5 mice)</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>1.90±0.07</td>
<td></td>
</tr>
<tr>
<td>Captopril (20 μg/mouse)</td>
<td>5</td>
<td>4.68±0.64 b</td>
<td></td>
</tr>
<tr>
<td>Captopril (20 μg/mouse) + SBTI (1 mg/mouse)</td>
<td>5</td>
<td>&lt;1.79 c</td>
<td></td>
</tr>
</tbody>
</table>

Each value shows the level of kinin (bradykinin equivalent) in the peritoneal washes from 5 samples with the S.E. of the mean. Peritoneal washes from 5 mice 5 min after the captopril (or saline) injection were mixed and used for the bradykinin assay as one sample. a Kinin amount is expressed as ng of bradykinin equivalents in the washes pooled from 5 mice. b indicates a significant difference from the control (saline) at P<0.01. c indicates a significant difference from the captopril-treated group at P<0.005.

References


3 Minami, M., Togashi, H., Sano, H., Endoh, T.,


