EFFECTS OF TINORIDINE ON LIPID PEROXIDATION AND RENIN RELEASE IN THE RAT RENIN GRANULE FRACTION

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This study was carried out to investigate the effect of tinoridine (2-amino-3-ethoxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine), a non-steroidal anti-inflammatory drug, on the lipid peroxidation and renin release in the renin granule fraction. The renin granule fraction was prepared from the kidney cortex homogenate by a discontinuous sucrose density gradient centrifugation. Incubation of this fraction at 37 °C resulted in an increase in lipid peroxide formation, accompanied by increased release of renin from the granules. When the renin granule fraction was incubated with 50 μM tinoridine at 37 °C, lipid peroxide formation in this fraction was completely inhibited. Simultaneously, the rate of renin release from the granules was significantly suppressed. Tinoridine, at concentrations from 5 μM up to 100 μM, produced a concentration-dependent inhibition on the simultaneous increases in lipid peroxide formation and renin release induced by 50 μM ascorbic acid in the renin granule fraction. On the other hand, indomethacin, hydrocortisone and prednisolone, which had no ability to inhibit the lipid peroxidation in the renin granule fraction, did not influence the release of renin from the granules. These results suggest that tinoridine suppresses renin release by inhibiting the oxidative disintegration of membranes of renin granules.

Keywords — rat kidney cortex; renin granule; renin release; lipid peroxidation; tinoridine

INTRODUCTION

The lipid peroxidation is the oxidative deterioration of unsaturated lipids. In living cells, the lipid peroxidation mainly occurs in membranes of subcellular organelles such as mitochondria and microsomes, since these membranes contain relatively large amounts of unsaturated lipids.1) Many studies2–6) have shown that the lipid peroxidation in subcellular organelles causes the impairment of their structure and functions. Recently, we demonstrated that the lipid peroxidation in the renin granule fraction caused a breakdown of renin granules, resulting in a remarkable stimulation of renin release from the granules.7) Tinoridine (2-amino-3-ethoxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine), a non-steroidal anti-inflammatory drug, was reported to have an anti-oxidative activity as well as a membrane stabilizing activity.8) The present study was undertaken to investigate the effects of tinoridine on the lipid peroxidation and renin release in the renin granule fraction.

MATERIALS AND METHODS

Chemicals — Tinoridine was kindly supplied by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). Indomethacin, hydrocortisone, and prednisolone were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of the Renin Granule Fraction — Male Wistar rats weighing 180–200 g were used. For at least 1 week before the study, the rats were fed a standard laboratory rat chow (Oriental Yeast Co., MF) and provided with tap water ad libitum. After the peritoneal cavity was
opened under pentobarbital anesthesia (35 mg/kg, i.p.), the kidneys were removed and immediately cooled in ice-cold physiological saline. The cortex was removed from the medulla, sectioned into thin slices, and homogenized with ice-cold 0.45 M sucrose (1:8, w/v). The renin granule fraction was prepared from the homogenate according to the method described previously. Briefly, the homogenate was centrifuged at 500 × g for 10 min to remove the unbroken cells, cell debris and nuclei. The supernatant was layered on a discontinuous sucrose density gradient solution (1.2 to 1.7 M with 0.1 M intervals) and centrifuged at 60000 × g for 90 min. Renin granules were mainly equilibrated in the fraction corresponding to 1.5 M sucrose. This fraction was used as the renin granule fraction in the following experiment.

**Incubation System of the Renin Granule Fraction** —One-half ml of the renin granule fraction (0.45—0.50 mg protein) was suspended in 0.5 ml of 0.1 M Tris–HCl buffer (pH 7.4) containing 0.15 M KCl. Ascorbic acid was dissolved in the same buffer. Tinoridine and other anti-inflammatory drugs were dissolved in ethanol and then diluted with the same buffer. These solutions were added to the above suspension prior to the incubation; the final concentration of ethanol was lower than 0.1%. Each of the mixtures was incubated at 37 °C for 0—180 min, and then it was separated into the supernatant and sediment by centrifugation at 105000 × g for 60 min. The sediment was resuspended in Tris–HCl buffer (pH 7.4) and analyzed for renin activity, lipid peroxides and protein content. The supernatant was analyzed for renin activity.

**Renin Assay and Measurements of Lipid Peroxides and Protein Contents** —Renin activity was measured by radioimmunoassay of angiotensin I produced after incubation with semipurified rat renin substrate, which was prepared from the plasma of nephrectomized rats according to the procedure described previously. Total renin was taken to be the sum of renin contents in the supernatant and sediment, and the amount of renin release during incubation was expressed as a percentage of total renin. Lipid peroxides were measured in terms of the formation of 2-thiobarbituric acid-reacting substances, presumed to be malondialdehyde (MDA), by means of a fluorometric assay described by Ohkawa et al. Lipid peroxide formation in the renin granule fraction was expressed in terms of MDA (nmol per mg of protein). The protein contents were determined by the method of Lowry et al. with modifications as described by Bensadoun and Weinstein to eliminate the interference by Tris.

**Statistical Analysis** —All data were presented as the mean ± S.E. of the values obtained in five separate experiments. Statistical significance was determined using Student’s t-test. Differences were not considered significant if p > 0.05.

**RESULTS**

**Effects of Tinoridine on Lipid Peroxide Formation and Renin Release in the Renin Granule Fraction**

The renin granule fraction prepared from rat kidney cortex contained a small amount of

![Graph](image)

**FIG. 1. Effects of Tinoridine on Lipid Peroxide Formation during Incubation of the Renin Granule Fraction**

The renin granule fraction was incubated with various concentrations of tinoridine at 37 °C for 30 min. The level of lipid peroxides in the renin granule fraction before incubation was 0.58 ± 0.12 nmol MDA/mg protein. Columns and bars represent the mean ± S.E. of five separate experiments. a, b) Significantly different from the value in the absence of tinoridine (a) p < 0.05, (b) p < 0.01.
endogenous lipid peroxides (0.58 ± 0.12 nmol MDA/mg protein). When the renin granule fraction was incubated at 37 °C for 30 min, approximately 3-fold increase in lipid peroxide formation was observed. The addition of tinoridine, at concentrations of 5 to 100 μM, produced a dose-related inhibitory action on the lipid peroxidation due to the incubation at 37 °C. At concentrations over 50 μM, this agent decreased the lipid peroxidation to the level before incubation (Fig. 1). When the incubation of the renin granule fraction was carried out at 0 °C, a significant increase in lipid peroxide formation was not observed.

Figure 2 shows the time course of the lipid peroxidation during 180 min incubation of the renin granule fraction, in the absence or presence of 50 μM tinoridine. In the absence of tinoridine, lipid peroxide formation rapidly increased and reached a maximum level (1.49 ± 0.18 nmol MDA/mg protein) at 30 min after the start of incubation. In contrast, no significant change in lipid peroxide formation as observed during incubation of the renin granule fraction in the presence of 50 μM tinoridine.

Renin release from the granules increased during incubation of the renin granule fraction at 37 °C, and the amount of renin release was 31.3 ± 1.61% at 180 min after the start of incubation. When the renin granule fraction was incubated at 37 °C in the presence of 50 μM tinoridine, renin release significantly decreased, compared with control incubations containing no tinoridine. At 180 min after the start of incubation, the amount of renin release was 25.4 ± 1.43% (p < 0.05, Fig. 3). On the other hand, the release of renin did not occur throughout the incubation for 180 min, when the renin granule fraction was incubated at 0 °C in the presence or absence of 50 μM tinoridine.

**Effects of Tinoridine on Lipid Peroxide Formation and Renin Release Induced by Ascorbic Acid in the**

![Graph](image)

**FIG. 2. Time Course of Lipid Peroxide Formation during Incubation of the Renin Granule Fraction with or without Tinoridine**

The renin granule fraction was incubated in the absence (○) or presence (●) of 50 μM tinoridine at 37 °C for 0–180 min. Points and bars represent the mean ± S.E. of five separate experiments. a, b) Significantly different from the value in the absence of tinoridine (a) p < 0.05, b) p < 0.01).

![Graph](image)

**FIG. 3. Time Course of Renin Release during Incubation of the Renin Granule Fraction with or without Tinoridine**

The renin granule fraction was incubated in the absence (○) or presence (●) of 50 μM tinoridine at 37 °C for 0–180 min. Points and bars represent the mean ± S.E. of five separate experiments. a) Significantly different from the value in the absence of tinoridine (p < 0.05).
Renin Granule Fraction

To further investigate whether the suppressive effect of tinoridine on renin release would be associated with the inhibition of the lipid peroxidation, the following experiments were performed. Various concentrations of tinoridine were added to the renin granule fraction, and incubated with 50 μM ascorbic acid at 37 °C for 30 min. As shown in Fig. 4, lipid peroxide formation in the renin granule fraction was greatly stimulated by the addition of 50 μM ascorbic acid (from 1.56 ± 0.29 to 11.28 ± 0.80 nmol MDA/mg protein). However, tinoridine, at concentrations over 5 μM, produced a potent inhibitory action on ascorbic acid-induced lipid peroxidation. In the presence of 50 or 100 μM tinoridine lipid peroxide formation decreased below the level observed during incubation of the renin granule fraction alone, i.e., the values of lipid peroxide formation were 0.72 ± 0.03 and 0.50 ± 0.02 nmol MDA/mg protein, respectively. Simultaneously, the increase in renin release from the granules due to 50 μM ascorbic acid (from 23.6 ± 1.52 to 97.7 ± 1.26%) was effectively suppressed by tinoridine at concentrations over 5 μM. In the presence of 50 or 100 μM tinoridine, renin release decreased below the level observed during incubation of the renin granule fraction alone, i.e., the amounts of renin release were 20.7 ± 3.01 and 19.6 ± 1.36%, respectively (Fig. 5).

**Effects of Indomethacin, Hydrocortisone and Prednisolone on Lipid Peroxide Formation and Renin Release Induced by Ascorbic Acid in the Renin Granule Fraction**

![Graph](image-url)

**FIG. 4. Effects of Tinoridine on Lipid Peroxide Formation Induced by Ascorbic Acid in the Renin Granule Fraction**

The renin granule fraction was incubated with various concentrations of tinoridine in the presence of 50 μM ascorbic acid at 37 °C for 30 min. Columns and bars represent the mean ± S.E. of five separate experiments. a) Significantly different from the value in the absence of ascorbic acid (p < 0.001). b, c) Significantly different from the value in the presence of ascorbic acid alone (b) p < 0.01, c) p < 0.001). □: no addition, □□: 50 μM ascorbic acid, □□□: 50 μM ascorbic acid + tinoridine.

![Graph](image-url)

**FIG. 5. Effects of Tinoridine on Renin Release Induced by Ascorbic Acid in the Renin Granule Fraction**

The renin granule fraction was incubated with various concentrations of tinoridine in the presence of 50 μM ascorbic acid at 37 °C for 30 min. Columns and bars represent the mean ± S.E. of five separate experiments. a) Significantly different from the value in the absence of ascorbic acid (p < 0.001). b) Significantly different from the value in the presence of ascorbic acid alone (p < 0.001). □: no addition, □□: 50 μM ascorbic acid, □□□: 50 μM ascorbic acid + tinoridine.
The renin granule fraction was incubated with indomethacin, hydrocortisone or prednisolone at various concentrations (5, 50 or 500 μM) in the presence of 50 μM ascorbic acid, and effects of these agents on the lipid peroxidation and renin release were compared with those of tinoridine. As shown in Table I, indomethacin, hydrocortisone and prednisolone did not cause significant changes in lipid peroxide formation and renin release induced by ascorbic acid, in contrast to tinoridine.

DISCUSSION

Tinoridine has been reported to exert a potent inhibitory action on the lipid peroxidation in microsomes and mitochondria.15-17) In these studies, the formation of lipid peroxides was significantly suppressed by 10 to 100 μM tinoridine. The present study also indicated that tinoridine, at concentrations of 5 to 100 μM, exerted a dose-related inhibitory action on the lipid peroxidation during the incubation of the renin granule fraction alone. On the other hand, a significant decrease in renin release from the granules was observed, when the lipid peroxidation was suppressed by 50 μM tinoridine. Our previous study18) demonstrated that the renin granule fraction contained a small amount of endogenous iron (12.1 ± 0.77 nmol iron per mg protein) and that the lipid peroxidation and renin release during the incubation of the renin granule fraction alone were significantly suppressed by the addition of ethylenediaminetetraacetic acid. The importance of inorganic iron as a catalyst for the lipid peroxidation has been indicated in microsomes.19) Furthermore, it has been reported that the peroxidative activity of ferrous ion was much more potent than that of other metal ions.20,21) Thus, it is suggested that endogenous inorganic iron ions are involved in the stimulation of the lipid peroxidation and renin release in the renin granule fraction. In this study, a gradual increase in renin release was observed during incubation of the renin granule fraction at 37 °C with 50 μM tinoridine, in spite of the complete suppression of lipid peroxide formation. But, the increased release of renin was not detected during the incubation at 0 °C. Accordingly, it seems that

<table>
<thead>
<tr>
<th>Drug (μM)</th>
<th>Lipid peroxide formation (nmol MDA/mg protein)</th>
<th>Renin release (%)</th>
</tr>
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<tbody>
<tr>
<td>No addition</td>
<td>11.43 ± 1.23</td>
<td>94.3 ± 1.76</td>
</tr>
<tr>
<td>Tinoridine</td>
<td>6.81 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.3 ± 2.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>0.86 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.1 ± 3.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td>0.46 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.3 ± 3.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>11.67 ± 0.98</td>
<td>95.1 ± 1.67</td>
</tr>
<tr>
<td>5</td>
<td>11.01 ± 1.42</td>
<td>94.8 ± 1.19</td>
</tr>
<tr>
<td>50</td>
<td>10.79 ± 2.31</td>
<td>91.3 ± 2.67</td>
</tr>
<tr>
<td>500</td>
<td>12.01 ± 1.21</td>
<td>93.6 ± 0.89</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>11.23 ± 2.38</td>
<td>95.7 ± 2.16</td>
</tr>
<tr>
<td>5</td>
<td>11.48 ± 2.67</td>
<td>95.0 ± 1.73</td>
</tr>
<tr>
<td>50</td>
<td>11.49 ± 0.91</td>
<td>92.6 ± 2.01</td>
</tr>
<tr>
<td>500</td>
<td>11.16 ± 2.31</td>
<td>95.6 ± 1.69</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>11.89 ± 2.48</td>
<td>94.9 ± 1.23</td>
</tr>
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</table>

The renin granule fraction was incubated with indicated concentration of anti-inflammatory drugs in the presence of 50 μM ascorbic acid at 37 °C for 30 min. Values are the mean ± S.E. of five separate experiments. a, b) Significantly different from the value in the absence of drugs (a) p < 0.01, (b) p < 0.001).
Tinoridine and Renin Release

the auto-oxidation of granular membrane lipids, initiated by endogenous iron ions under the incubation at 37 °C, are associated with the rate of basal renin release from isolated renin granules.

Ascorbic acid has been reported to cause the lipid peroxidation in the presence of iron ions in various tissues. The role of ascorbic acid is thought to maintain iron ions in their reduced form. In the present study, the effects of tinoridine on the lipid peroxidation and renin release in the renin granule fraction due to ascorbic acid were also examined. Results clearly indicated that tinoridine inhibited the lipid peroxidation induced by ascorbic acid in a concentration-dependent manner. In addition, the stimulative effect of ascorbic acid on renin release was effectively suppressed by tinoridine. The addition of tinoridine at concentration over 50 μM produced a complete inhibition on lipid peroxide formation and renin release due to ascorbic acid. On the other hand, indomethacin, hydrocortisone and prednisolone, which had no ability to inhibit the lipid peroxidation, did not suppress the release of renin from the granules. These steroidal and non-steroidal anti-inflammatory drugs are reported to have no inhibitory action on the lipid peroxidation in microsomal membranes. It is assumed that the anti-oxidative characteristic of tinoridine is contributory to the suppressive action on renin release from renin granules.

It has been reported that active oxygen species such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical are not involved in the peroxidative reaction induced by iron ions and ascorbic acid. Fukuzawa et al. indicated that the anti-oxidative activity of α-tocopherol on the lipid peroxidation catalyzed by ferrous ion and ascorbic acid is due to the interaction of the anti-oxidant with peroxy and/or alkoxyl radicals generated in the peroxidation process. Although the mechanisms by which tinoridine exhibits the anti-oxidative characteristic can not be explained from our results, this agent may interact with lipid free radicals formed in the peroxidative reaction, in the same way as α-tocopherol.

We recently reported that vitamin E-deficiency for 4 weeks caused simultaneous increases in the lipid peroxidation and renin release in the renin granule fraction and that the increased levels of the lipid peroxidation and renin release due to vitamin E-deficiency were restored to each control level by dietary supplementation of α-tocopheryl acetate. Tinoridine has been reported to have the same protective effect as α-tocopherol on changes of fatty acid composition in the lipids of liver lysosomal and microsomal membranes obtained from vitamin-E-deficient rats. On the basis of these findings and the present results, there is a possibility that tinoridine exerts a protective action on the stimulation of renin release from the granules due to vitamin E-deficiency. Further studies are required to clarify the above possibility.

The present study indicated that the increase of lipid peroxide formation in the renin granule fraction accelerated the rate of renin release from the granules. Tinoridine potently suppressed the release of renin by inhibiting the lipid peroxidation in this fraction. In our previous study, anti-oxidants, such as N,N'-diphenyl-p-phenylenediamine and hydroquinone have been reported to cause the simultaneous decreases in the lipid peroxidation and renin release in the renin granule fraction. From these findings, it is strongly suggested that there is a positive correlation between the lipid peroxidation and renin release in the renin granule fraction.

REFERENCES
4) E. D. Wills and A. E. Wilkinson: Release of enzymes from lysosomes by irradiation and the relation of lipid