Urate Synthesizing System and Its Relation to Inflammation

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INTRODUCTION

The generation of oxygen radicals in biological systems and their toxicities have been described by many authors¹⁻⁵. There are several lines of evidence suggesting that active species of oxygen like superoxide anion (\(\text{O}_2^-\)) or hydroxyl radical (\(\text{OH}'\)) may be involved in inflammatory processes⁶⁻⁸. In the biosynthesis of urate, xanthine oxidase (XOD) oxidizes hypoxanthine and xanthine to yield \(\text{O}_2^-\) primarily, that is disproportionated into \(\text{H}_2\text{O}_2\) and \(\text{O}_2\) (equation 1). Hydrogen peroxide will then react with \(\text{O}_2^-\) to form \(\text{OH}'\) and singlet oxygen (\(\text{O}_2\)) (equation 2)³⁻¹⁰.

\[
\begin{align*}
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad \text{---(1)} \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \rightarrow \text{OH}^- + \text{OH}' + \text{O}_2 \quad \text{---(2)}
\end{align*}
\]

Singlet oxygen, however, may be readily scavenged by hypoxanthine, xanthine or urate as reported by Kellogg and Fridovich.¹⁰ Xanthine oxidase/hypoxanthine system (XOD-HPX) is, therefore, an excellent source of \(\text{O}_2^-\), \(\text{H}_2\text{O}_2\) and \(\text{OH}'\).

In gout, attention has been focussed on urate crystals which are thought to be responsible for the inflammation.

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It is, however, interesting to note that active species of oxygen evolved during urate biosynthesis would play some role in the gout symptoms. We investigated whether these active species of oxygen could be stimuli of inflammation by employing XOD-HPX. The present paper describes several lines of evidence suggesting that active species of oxygen such as $O_2^-$, $H_2O_2$ and $OH^-$ are involved directly in the induction of inflammation.

**MATERIALS AND METHODS**

1) **XOD-HPX-induced foot edema.** Male rats of Sprague-Dawley strain weighing 140-160 g were used throughout this work (N=4-6). XOD-HPX system consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM hypoxanthine and 2 mg/ml of xanthine oxidase (Boehringer Mannheim; 0.4 U/mg). Where indicated, various agents were dissolved in this mixture. After injecting 0.1 ml of XOD-HPX into the hind foot paw of rats, an acute foot-edema was induced at the injected site. The foot volume was measured by water displacement method.

2) **Histamine release from rat mast cells.** Medium A and B with the following compositions were used. Medium A contained 0.16 M NaCl, 3 mM KCl, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, 7 mM KH$_2$PO$_4$, 9 mM Na$_2$HPO$_4$, 10 mM glucose and 0.5 % bovine serum albumin. In medium B, bovine serum albumin and glucose were omitted from medium A. Male rats of Sprague-Dawley strain weighing 350-400g were used. Mast cells were collected from the peritoneal cavity and were purified by Ficoll gradient centrifugation according to the procedure of Cooper and Stanworth. Complete system for the assay of histamine release from rat mast cells induced by XOD-HPX consisted of...
0.7 ml of medium A, 0.1 ml of XOD (0.05 mg), 0.1 ml of 5 mM hypoxanthine and 0.1 ml of mast cell suspension (ca, 10^6 cells/ml of medium A). XOD was previously passed through Sephadex G-25 column (1×25 cm) equilibrated with 70 mM phosphate buffer (pH 7.0) to remove EDTA and (NH₄)₂SO₄ in the commercial preparation. In some experiments, XOD-HPX was replaced by an appropriate amount of H₂O₂. The reactions were carried out at 37°C for 10 min and terminated by cooling in an ice-bath. Samples were usually treated with catalase (200 units) for 1 min to remove H₂O₂ because the compound disturbs fluorometric assay of histamine.

After removing mast cells by centrifugation, released histamine in the supernatant was determined fluorometrically according to the method of Shore et al. Histamine release was expressed as the per cent release of total histamine. All the data were presented as a mean value of duplicate experiments.

RESULTS AND DISCUSSION

By injecting 0.1 ml of XOD-HPX into hind foot paw, an acute foot edema was induced at the injected site. The edema reached maximum in about 20 min and decreased gradually thereafter, as shown in Fig.1. This acute paw swelling was dependent on hypoxanthine, in particular, in the initial phase. Omission or boiling of XOD resulted in a marked reduction of the paw swelling. Uric acid which is an oxidation product of hypoxanthine by XOD was not inflammatory. As indicated in Table 1, urate did not cause foot edema, nor had any effect on XOD-HPX-induced paw swelling. Other miscellaneous proteins like bovine serum albumin, lactate dehydrogenase or horse radish peroxidase showed slight inflam-
matory activities compared with XOD (Data not shown). These results suggest that the foot edema was induced specifically by XOD-HPX and that $O_2^-$ or other active oxygen species derived from $O_2^-$ would play a major role in this inflammation.

![Graph showing time-course of XOD-HPX-induced foot edema in rats.](image)

**Fig. 1** Time-course of XOD-HPX-induced foot edema in rats.

(-○-) XOD-HPX, (—□—) minus hypoxanthine, (—△—) minus XOD, (—●—) XOD-HPX (XOD was boiled at 100°C for 5 min).

**Table 1** Effect of uric acid on XOD-HPX-induced foot edema in rats.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>increase in foot volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min</td>
</tr>
<tr>
<td>buffer*</td>
<td>12.8 ± 1.3</td>
</tr>
<tr>
<td>XOD-HPX</td>
<td>63.6 ± 4.1</td>
</tr>
<tr>
<td>XOD-HPX + uric acid**</td>
<td>62.2 ± 2.8</td>
</tr>
<tr>
<td>uric acid</td>
<td>15.1 ± 2.9</td>
</tr>
</tbody>
</table>

* Sodium phosphate buffer 50 mM (pH 7.0).
** Uric acid (1 mM) was dissolved in XOD-HPX.
In order to assure this assumption, effect of allopurinol, superoxide dismutase (SOD) and catalase on XOD-HPX-induced foot edema was investigated as illustrated in Fig. 2. These agents were injected directly into the foot paw by mixing with XOD-HPX. Allopurinol, an inhibitor of \( \text{XOD} \)\(^{13}\) considerably suppressed the paw swelling, thus indicating that turnover of XOD is essential for the inflammation. That a low but significant paw swelling was still observed in the presence of allopurinol suggests that XOD or allopurinol itself is somewhat inflammatory. In order to determine which of these active species of oxygen are really responsible for the foot edema, the effect of scavengers of active oxygens was examined. These agents were injected directly into the

![Fig. 2 Effect of allopurinol, SOD and catalase on XOD-HPX-induced foot edema in rats. All the agents were injected into the foot paw by mixing with XOD-HPX.](image)

\((-\bigcirc-)\) control (XOD-HPX), \((-\bigtriangleup-)\) + allopurinol(1 mM), \((-\bigtriangledown-)\) + SOD(200 units), \((-\bigbullet-)\) + catalase(250 units), \((-\bigstar-)\) + SOD(200 units) + catalase(250 units).
foot paw by mixing with XOD-HPX. As shown in Fig.2, both SOD and catalase slightly inhibited foot-edema. The inhibition, however, was enhanced when both enzymes were used in combination. The extent of inhibition did not exceed 50% since these enzyme proteins themselves were somewhat inflammatory. Superoxide dismutase catalyzes the disproportionation of \( O_2^- \) to form \( H_2O_2 \) and \( O_2 \). The reaction is faster by \( 10^4 \)-fold than the corresponding nonenzymic reaction (equation 1). Therefore, it is likely that SOD promotes the formation of \( OH' \) according to equation 2 through supplying \( H_2O_2 \) as proposed by Fong et al. On the other hand, p-benzoquinone that reduced \( H_2O_2 \) formation by oxidizing \( O_2^- \) to \( O_2 \) and would, therefore, decrease the production \( OH' \), inhibited the paw swelling markedly as indicated in Table 2.

Table 2. Effect of oxygen radical scavengers on XOD-HPX-induced foot edema in rats.

<table>
<thead>
<tr>
<th>Oxygen radical scavengers</th>
<th>% swelling</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>48.5 ± 1.6</td>
<td>-</td>
</tr>
<tr>
<td>+ p-benzoquinone (1 mM)</td>
<td>25.9 ± 1.5</td>
<td>46.6</td>
</tr>
<tr>
<td>+ benzoic acid (1 mM)</td>
<td>21.2 ± 2.2</td>
<td>56.5</td>
</tr>
<tr>
<td>+ D-mannitol (1 mM)</td>
<td>29.2 ± 4.5</td>
<td>39.8</td>
</tr>
</tbody>
</table>

All the agents were injected into foot paw by mixing with XOD-HPX. Paw swelling was measured at 20 min after injecting the stimulus.

Furthermore, \( OH' \) scavengers like benzoic acid and D-mannitol prevented the foot edema markedly (Table 2). These results suggest that \( OH' \) plays a major role in XOD-HPX-induced foot edema in rats. This would explain why SOD could not suppress the paw swelling to a considerable extent. The involvement of \( OH' \) in inflammation has been predicted by some authors.
In the presence of catalase, $H_2O_2$ derived from $O_2^-$ will be decomposed into $H_2O$ and $O_2$ immediately, thus leading to the decrease in the formation of $OH'$. Therefore, $O_2^-$ would be a major species of active oxygen under this condition. The observation that the foot edema was suppressed only partially in the presence of catalase suggests that $O_2^-$ itself is also involved in the foot edema. The combination of SOD and catalase that leads to the reduction in the levels of both $O_2^-$ and $OH'$ would result in an enhanced suppression of the paw swelling induced by XOD-HPX. And this was the case as illustrated in Fig.2.

Injection of 2 mM $H_2O_2$ into the foot paw did not cause a significant paw swelling. This does not, however, rule out the possibility that $H_2O_2$ may participate in the potentiation of the foot edema induced by $O_2^-$ and $OH'$ because it is difficult to remove $H_2O_2$ specifically without altering the level of $O_2^-$ and $OH'$.

The results described above showed that active species of oxygen like $O_2^-$, $H_2O_2$ and $OH'$ could be stimuli of inflammation. Of antiinflammatory agents tested, diphenhydramine

| Table 3. Effect of various drugs on XOD-HPX-induced foot edema in rats |
|---------------------------------|--------|--------|
| **Drugs** | **Dose (mg/kg)** | **% inhibition** |
| indomethacin* | 10 | 31.4 |
| dexamethazone* | 1 | 3.0 |
| diphenhydramine** | 25 | 35.3 |
| cromoglycate** | 50 | 57.3 |
| BHT* | 50 | 49.0 |

* given p.o. 1 hr before XOD-HPX challenge.

** given i.p. 30 min before XOD-HPX challenge.
and cromoglycate strongly suppressed XOD-HPX-induced foot edema, thus indicating that histamine is one of the most important mediator in this inflammation. (Table 3)

In order to clarify the mechanism of XOD-HPX-induced foot edema, we investigated whether XOD-HPX could cause histamine release from rat mast cells. Incubation of XOD-HPX with rat peritoneal mast cells resulted in a marked release of histamine as indicated in Table 4. The histamine release was reduced when either XOD or hypoxanthine was omitted, or when boiled XOD was used. Thus, it was indicated that the histamine release was dependent on the enzymic activity of XOD. It, therefore, was expected that $O_2^-$ evolved from XOD-HPX, or active species of oxygen derived from $O_2^-$ according to equation 1 and 2 would be involved in the release of histamine.

**Table 4. XOD-HPX-induced histamine release from rat mast cells: Cofactor requirements and effect of SOD, catalase and D-mannitol.**

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>Histamine release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1.</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>40.0 (100)</td>
</tr>
<tr>
<td>- hypoxanthine</td>
<td>14.1 (37)</td>
</tr>
<tr>
<td>- XOD</td>
<td>1.4 (4)</td>
</tr>
<tr>
<td>Complete system (boiled XOD)</td>
<td>5.6 (14)</td>
</tr>
<tr>
<td>Experiment 2.</td>
<td></td>
</tr>
<tr>
<td>Complete system + SOD (50 μg)</td>
<td>43.0 (100)</td>
</tr>
<tr>
<td>+ catalase (50 μg)</td>
<td>43.6 (101)</td>
</tr>
<tr>
<td>+ SOD + catalase</td>
<td>19.8 (46)</td>
</tr>
<tr>
<td>+ D-mannitol (1 mM)</td>
<td>41.8 (97)</td>
</tr>
</tbody>
</table>

In experiment 1, indicated cofactors were omitted from the complete system described in Methods. In experiment 2, SOD, catalase or D-mannitol was added to the complete system.
Effects of SOD, catalase and D-mannitol on XOD-HPX-induced histamine release were then examined to study which active species are really responsible for the releasing reaction. As shown in Table 4, the histamine release was inhibited markedly by catalase, whereas SOD or D-mannitol had no effects. These results strongly suggest that $\text{H}_2\text{O}_2$ derived from $\text{O}_2^-$ according to equation 1 would play a major role in the histamine release from the mast cells. This was further confirmed by the result that histamine was similarly released when the mast cells were incubated with $\text{H}_2\text{O}_2$ as illustrated in Fig.3. Maximal release was observed with 0.1 mM $\text{H}_2\text{O}_2$, but higher concentrations of the stimulus were inhibitory. This is presumably due to the damage to the process

![Fig. 3](image)

**Fig. 3** Effect of hydrogen peroxide concentrations on the histamine release from rat mast cells.

Ca. $10^5$ mast cells were incubated with the indicated concentrations of $\text{H}_2\text{O}_2$ as described in Methods.
of histamine release by higher concentrations of H₂O₂. The histamine release induced by XOD-HPX or by H₂O₂ is not due to nonspecific lysis of the mast cells because these releasing stimuli did not liberate lactate dehydrogenase, a cytoplasmic enzyme during histamine release (Table 5).

Table 5. Release of histamine and lactate dehydrogenase (LDH) from mast cells incubated with XOD-HPX or with H₂O₂.

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>% release</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>histamine</td>
<td>LDH</td>
</tr>
<tr>
<td>control</td>
<td>5.1</td>
<td>2.0</td>
</tr>
<tr>
<td>XOD-HPX</td>
<td>42.0</td>
<td>1.4</td>
</tr>
<tr>
<td>H₂O₂ (0.1 mM)</td>
<td>44.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Effects of various agents that are known to inhibit histamine release induced by antigens or by compound 48/80 were studied in order to examine the properties of histamine release induced by XOD-HPX or by H₂O₂. Neither dibutyryl cAMP nor prostaglandin E₁ affected the release (Fig.4A). Colchicine, however, strongly inhibited the release induced by XOD-HPX as well as that by H₂O₂. Both XOD-HPX- and H₂O₂-induced histamine release were found to be dependent on Ca²⁺ and glucose in a similar manner as shown in Fig.4B.

The results described above showed that H₂O₂ was a real stimulus in the histamine release induced by XOD-HPX. These results and our previous observation that diphenhydramine strongly suppressed the foot edema induced by XOD-HPX appear to support the idea that H₂O₂-induced histamine release may play a vital role in causing the paw swelling. Histamine is
not, however, a sole mediator in this inflammation because
the injection of H₂O₂ or histamine alone did not cause a
significant edema. XOD-HPX-induced foot-edema would be a,
more complicated process in which active species of oxygen
may act not only upon mast cells but also upon other tissue
cells, thus leading to the edema.

Histamine release induced by antigens or compound 48/80 is
known to be inhibited by theophylline, PGE₁, dibutyryl cAMP
or colchicine.¹⁵⁻¹⁷) On the other hand, A-23187 (a calcium
ionophore)-induced histamine release was suppressed by col-
chicine and deoxyglucose, but not by dibutyryl cAMP.

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Figure 4 A) Effect of dibutyryl cAMP, PGE₁ and colchicine on
histamine release from rat mast cells induced by
XOD-HPX or by H₂O₂ (0.05 mM)

Histamine release was expressed as the per cent
of control. (a) control (no addition), (b) +
dibutyryl cAMP (1 mM), (c) + PGE₁ (0.05 mM),
(d) + colchicine (1 mM)

B) Dependence of histamine release from rat
mast cells induced by XOD-HPX or by H₂O₂
upon Ca²⁺ and glucose.

Mast cells were prepared in medium A in
which Ca²⁺ and glucose were omitted.
(a) complete system, (b) minus glucose
(8 mM), (c) minus CaCl₂ (0.72 mM).
Lichtenstein proposed that A-23187-induced histamine release "short-circuits" the cAMP-associated stage but has a similar mechanism to the second stage of antigen-induced histamine release.\(^7\) The data in Fig. 4A and 4B indicate that \(\text{H}_2\text{O}_2\)-induced histamine release resembles that induced by A-23187. It remains to be elucidated whether \(\text{H}_2\text{O}_2\) directly functions as a releasing stimulus. It is possible to postulate that reaction of \(\text{H}_2\text{O}_2\) with some cellular components (e.g. peroxidation) may produce some changes leading to histamine release. The results described above showed the direct evidence that active species of oxygen evolved from urate synthesizing system could be stimuli of inflammation. We postulate that the inflammatory activities of active species of oxygen would have some significance in the symptoms of gout. In fact, further studies would be necessary to elucidate that these phenomena occur really in vivo.

**SUMMARY**

Xanthine oxidase-hypoxanthine system (XOD-HPX) was found to cause an acute foot edema when injected into the foot paw of rats. The observations that superoxide dismutase, catalase and oxygen radical scavengers like p-benzoquinone, benzoic acid and D-mannitol were effective in depressing the foot edema suggested that active species of oxygen (\(\text{O}_2^-, \text{H}_2\text{O}_2\) and \(\text{OH}'\)) were responsible for the induction of the paw swelling. Histamine might play a major role as a mediator in this inflammation because diphenhydramine and cromoglycate suppressed the edema markedly. Further studies to elucidate the mechanism of XOD-HPX-induced foot edema revealed that \(\text{H}_2\text{O}_2\) evolved from XOD-HPX efficiently released histamine from peritoneal mast cells of rats. \(\text{H}_2\text{O}_2\)-induced histamine
release may be one of, although not a sole, major factors in XOD-HPX-induced foot edema. The significance of these observations in relation to gout is discussed.

REFERENCES

1) Babior BM, Kipnes RS, Curnutte JT: J Clin Invest 52;741 (1973)
5) Packer L, Walton J: CHEMTECH 276(1977)
質 疑 応 答

質 問    坂内  慶彦（東京大学総合医研究科）
肥満細胞の脱顆粒にはⅡ相があるが、先生の場合は第Ⅱ相と考えてよいか。

応 答    大森  >('Dibutyryl cAMP によって、全く抑制されなかったことから考えて、H\textsubscript{2}O\textsubscript{2} による release には第Ⅱ相の機構が関与している可能性があると思われるが、H\textsubscript{2}O\textsubscript{2} の肥満細胞に対する作用点については今後詳細に検討したい。