Eugenol-Mediated Superoxide Generation and Cytotoxicity in Guinea Pig Neutrophils

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Abstract—Eugenol is the medicament used routinely as an anodyne and antiseptic in dentistry and a food flavour and fragrance agent. The drug stimulates the superoxide (O$_2^-$) release of guinea pig neutrophils without a lag time. The production rate increases with the drug concentration and reaches a plateau at 5 mM. However, the induction accompanies the cytotoxicity. The stimulation system of O$_2^-$ production is sensitive to mild heating (45°C, 15 min). The system proceeds without artifacts which may be mediated by a radical chain reaction with H$_2$O$_2$ and hydroxy radical, since neither catalase, mannitol nor azide changes the rate. Ca$^{2+}$ and Ni$^{2+}$ in the medium enhance the activity, but Mg$^{2+}$ and Zn$^{2+}$ have no effect. EDTA inhibits completely, suggesting that intracellular metal ions are involved in this system. Phenolic compounds used as dental medicaments other than eugenol act as potent stimulators of the O$_2^-$ production, with the following order of potency: thymol > eugenol > o-cresol. Resorcinol, guaiacol and hexachlorophene show little activity. This order of potency agrees with the order of hydrophobicity of these chemicals and that of the cytotoxicity to neutrophils. The data suggest that phenolic antiseptic drugs bind to the cell surface hydrophobically, trigger the oxygen burst and make the plasma membrane fragile at a high dose of drugs.

Eugenol (2-methoxy-4-allylphenol) is the active phenolic constituent in oil of cloves and a common ingredient in various dental pastes. Its most useful property is analgesia (1). It inhibits the action potential of nerve fibers in cat dental pulp (2) and acts on the phrenic nerve and phrenic nerve-diaphragm preparation as a membrane-stabilizing (local anaesthetic) drug (3). Recently, it is reported that the analgesic effect may be due to the ability to inhibit prostaglandin $\mathrm{E}_2$ biosynthesis in the dental pulp (4). As another pharmacological action, vasodilation, one component of acute inflammation, was reported (5).

Eugenol acts on soft tissues as an irritant and a toxic agent (6, 7). In acute inflammation of dental pulp, the region of pulp injury under the dentin is heavily infiltrated with neutrophils. Eugenol is often applied to acute pulpitis as an antiseptic and anodyne. We have found that eugenol stimulates the superoxide production in guinea pig neutrophils (8).

In this paper, we report that eugenol induces superoxide production in neutrophils, and phenolic antiseptic medicaments other than eugenol also have this action and discuss the involvement of oxygen metabolites produced by activated neutrophils in the pharmacological function of eugenol.

Materials and Methods

1. Preparation of neutrophils: Neutrophils were obtained from the peritoneal cavity of a male guinea pig (300–400 g) 16–24 hr after an intraperitoneal injection of 20 ml of 0.1% oyster glycogen in isotonic saline. The peritoneum was washed with 50 ml of warm phosphate-buffered saline (PBS). The obtained exudate cell preparation contained 60–70% neutrophils as measured by the May-Grünwald Giemsa staining method. Cytotoxicity of neutrophils was measured by the
dye exclusion method using trypan blue.

2. Superoxide generation: Superoxide was measured continuously at 37°C by the reduction of ferricytochrome c at 550 nm as described previously (8). The standard assay mixture (3 ml) consisted of a HBS medium (130 mM NaCl, 10 mM Hepes, 5.6 mM glucose, 2.5 mM CaCl₂, 1 mM MgCl₂ and 0.035% bovine serum albumin, pH 7.2). In the assay, 3x10⁶ cells were routinely used. The reaction was initiated by addition of 10 μl of eugenol or other antiseptics dissolved in dimethylsulfoxide (DMSO) and then vigorously shaking the vessels. The rates of O₂⁻ production were calculated from the linear portion of the reaction curves. All assays were made in duplicate.

3. Hyperthermia treatment: The cells (1.5x10⁷ cells/ml) were suspended in HBS medium. A 1 ml aliquot of cell suspension was transferred into a glass conical tube and capped with aluminium foil. After heating at 45, 50 and 55°C, the tubes were cooled in ice water. Aliquots (0.2 ml) of heated cell suspension (3x10⁶ cells) were stimulated with 5 mM eugenol and 5 ng/ml 12-0-tetradecanoyl 4-phorbol 13-acetate (TPA), and the O₂⁻ production rate was measured.

4. Materials: Superoxide dismutase (SOD, Type I from bovine blood), ferricytochrome c (Type III, from horse heart), catalase (2x crystallized, from bovine liver), and TPA were purchased from Sigma Chemical Co. Eugenol and other antiseptics were obtained from Nakarai Chemical Co. and dissolved in DMSO at 1.5 M.

Results

1. Induction of O₂⁻ production and cytotoxicity by eugenol: Neutrophils produce O₂⁻ immediately after exposure to eugenol (8). After measurement of O₂⁻ production for 5 min, cell viability was checked by the dye exclusion method. Figure 1 shows that the initial O₂⁻ production rate is directly proportional to the concentration up to 2 mM of eugenol, and cell viability decreases slightly. At a concentration higher than 5 mM of eugenol, the activity remains constant, but almost all of cells are damaged. Within 30 sec, there is marked cell injury to less than 50% viability at 5 mM of eugenol (data not shown).

2. Response to hyperthermia: Human neutrophils treated by mild heating (50°C, 15 min) failed to produce any O₂⁻ after exposure to TPA, but retained the ability to exclude trypan blue (9). In guinea pig neutrophils, treatment of heat at 50°C or higher causes decrease of viability as shown in Table 1. The cells treated at 45°C showed the same viability as the control, but became insensitive to the stimuli of 5 mM eugenol and 5 nM TPA. This shows that the induction by high concentration of eugenol also requires the intactness of the O₂⁻ production system, which may be heat-labile.

3. Effect of SOD, catalase, mannitol and azide: Various oxygen metabolites of the respiratory burst in neutrophils: perhydroxy radical, OH⁻ (hydroxy radical) and H₂O₂, modify overall O₂⁻ production by chain radical reactions, and H₂O₂ converted from O₂⁻ may result in O₂⁻ consumption through peroxidation by endogeneous myeloperoxidase (10). So, this possibility was tested using mannitol as a scavenger of OH⁻, catalase for removal of H₂O₂, and high concentration of azide as an inhibitor of myelo-
peroxidase (11, 12). Neither catalase, mannitol nor azide could influence the activity (Table 2). As reported previously (8), SOD eliminated the activity completely and the autoclaved (120°C, 20 min) enzyme partially eliminated it, suggesting that the inhibition is due to the enzyme activity of SOD.

4. Effect of metal ions: Ca²⁺ and Ni²⁺ partially enhanced the activities, while Mg²⁺ and Zn²⁺ had no influence (Table 3). Eugenol is used practically in dentistry in the form of

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell viability (%)</th>
<th>( \text{O}_2^- ) production (nmole/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eugenol</td>
</tr>
<tr>
<td>Control</td>
<td>96</td>
<td>4.6±1.0</td>
</tr>
<tr>
<td>45°C</td>
<td>93</td>
<td>0.5±0</td>
</tr>
<tr>
<td>50°C</td>
<td>67</td>
<td>0±0</td>
</tr>
<tr>
<td>55°C</td>
<td>58</td>
<td>0.5±0.5</td>
</tr>
</tbody>
</table>

Cell suspension in HBS medium was heated for 15 min at 1.5×10⁷ cells/ml. A 0.2 ml aliquot of cell suspension (3×10⁶ cells) were stimulated with 5 mM eugenol and 5 ng/ml TPA. Control was measured with cell suspension kept at room temperature.

Table 1. Response to hyperthermia

<table>
<thead>
<tr>
<th>Addition</th>
<th>( \text{O}_2^- ) production (nmol/5 min)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.5±0.3</td>
<td>100</td>
</tr>
<tr>
<td>Superoxide dismutase (50 μg/ml)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autoclaved superoxide dismutase (50 μg/ml)</td>
<td>6.2±0.3</td>
<td>73</td>
</tr>
<tr>
<td>Catalase (100 μg/ml)</td>
<td>8.5±0.6</td>
<td>99</td>
</tr>
<tr>
<td>Mannitol (10 mM)</td>
<td>7.6±0.1</td>
<td>90</td>
</tr>
<tr>
<td>NaN₃ (50 mM)</td>
<td>7.9±0.6</td>
<td>93</td>
</tr>
</tbody>
</table>

The materials tested in this experiment were added to the cell suspension (3×10⁶ cells) for 2 min prior to contact with 5 mM eugenol. Relative activity was expressed as a percentage of that obtained without effectors.

Table 2. Effect of superoxide dismutase, catalase, mannitol and azide on \( \text{O}_2^- \) production of neutrophils

<table>
<thead>
<tr>
<th>Addition</th>
<th>( \text{O}_2^- ) production (nmol/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>( \text{CaCl}_2 ) (2 mM)</td>
<td>8.1±1.7</td>
</tr>
<tr>
<td>( \text{MgCl}_2 ) (2 mM)</td>
<td>5.4±0.4</td>
</tr>
<tr>
<td>( \text{CaCl}_2 ) (2 mM) + ( \text{MgCl}_2 ) (2 mM)</td>
<td>7.0±2.0</td>
</tr>
<tr>
<td>( \text{ZnCl}_2 ) (2 mM)</td>
<td>5.7±2.1</td>
</tr>
<tr>
<td>( \text{NiSO}_4 ) (2 mM)</td>
<td>7.5±0.1</td>
</tr>
<tr>
<td>EDTA (2 mM)</td>
<td>0±0</td>
</tr>
</tbody>
</table>

HRS medium without \( \text{CaCl}_2 \) and \( \text{MgCl}_2 \) was used as a basal medium. Cells (3×10⁶) were preincubated in the medium only (control) or the medium containing various ions and EDTA for 2 min and then exposed to 5 mM eugenol.
the zinc oxide mixture. ZnCl₂ shows no effect on the activity (Table 3) and cytotoxicity induced by eugenol (data not shown). EDTA inhibits the activity completely. This suggests that the system requires fluxes of metal ion, probably Ca²⁺ into the cell.

5. The action of phenolic compounds used as antiseptics: The effects of phenolic compounds known as antiseptic medicaments on O₂⁻ production and cytotoxicity were investigated (Table 4). Eugenol converts the quinone form of 3,4-dihydroxy allylbenzene and methanol by oxidation (13). This suggests that the drug may act on the O₂⁻ generation system as an electron donor, but not as a stimulator of the plasma membrane. The activity of guaiacol which has the same structure except for the allyl portion is compared with those of o-cresol and resorcinol. Guiacol considered structurally as an electron donor shows no activity, while o-cresol which has a methyl moiety instead of a methoxyl moiety and high phenolic coefficient shows the activity. This indicates that the hydrophobicity rather than the reducing ability of the drug seems to be important in induction. In another experiment, a more hydrophobic antiseptic, thymol, and a more bactericidal drug, hexachlorophene, are tested. Phenol coefficients of thymol and hexachlorophene are 30 and 125 respectively (14). Thymol shows about four times higher O₂⁻ production and acts more cytotoxically than eugenol. Hexachlorophene shows neither O₂⁻ production nor cytotoxicity.

Discussion
Characterization of O₂⁻ production in eugenol-stimulated neutrophils was investigated. Representative analgesics, morphine and naloxone, were reported to reduce ferricytochrome c by transferring electrons from NADPH (15), while eugenol acts on neutrophils to induce SOD-inhibitable reduction, but causes no reduction in the absence of cells (Table 2). Induction of O₂⁻ production in our system is dependent on Ca²⁺ (Table 3), inhibited by the SH inhibitor N-ethylmaleimide, and the production proceeds without a lag time (8) as observed in other stimuli of the respiratory burst. The induction system is destroyed irreversibly by mild heating (45°C, 15 min) which causes no staining with trypan blue (a conventional indicator of cell viability) and the same destruction is observed in the most potent chemical inducer of the respiratory burst in neutrophils, TPA (Table 1). It is reported that human neutrophils retain viability after heating at 55°C for 15 min (9), but our data using guinea pig neutrophils (Table 1) and the report using rabbit neutrophils (16) show moderate toxicity. This indicates species difference in response to hyperthermia. It is known that in the O₂⁻
producing system a small amount of $O_2^-$ acts as an electron donor to produce a large amount of $O_2^-$ non-enzymatically (10) and myeloperoxidase, an endogeneous peroxidase in neutrophils, converts phenol derivatives in the presence of H$_2$O$_2$ (17). Catalase, mannitol and NaN$_3$ cause no change in the induction (Table 2), suggesting that almost all $O_2^-$ is trapped by ferricytochrome c, and the reduction is not modified by $O_2^-$ or eugenol-derived metabolites.

The $O_2^-$ production rate is directly proportional to eugenol concentration up to 2 mM, which causes less toxicity to cells, as shown in Fig. 1. At higher than 5 mM, it is toxic, but $O_2^-$ production rate is maintained at the maximum value. The high concentration of the stimuli with detergent activity, digitonin and deoxycholate are also toxic, but cause decrease of $O_2^-$ production (18), different from that in the eugenol-mediated system. Although it is unknown whether the action mechanisms of eugenol at low and high concentration is the same, one can speculate that a high concentration of eugenol activates cells promptly and induces in the later stage an irreversible damage of cell structure, but cell integrity is not required after the onset of oxygen metabolism, because the activation mechanism in guinea pig neutrophils requires energy, but energy is not needed to sustain the activity of the system (19).

Phenolic antiseptics other than eugenol are also able to simulate $O_2^-$ production (Table 4). Microbicidal activity represented with the phenolic coefficient is enhanced by alkyl substitutions in the phenol molecule (20). Induction ability in $O_2^-$ production also correlates well with the degree of alkyl substitutions with the following order of potency: Thymol $>$ eugenol $<$ o-cresol. This result suggests that phenol derivatives with strong hydrophobicity can easily penetrate into the plasma membrane and perturb the membrane to trigger the oxygen burst. Kakinuma reported the same idea using saturated and unsaturated fatty acids of various chain lengths (21). Hirafuji showed that phenolic compounds caused inhibition in PGI$_2$ biosynthesis in rat dental pulp (4), but eugenol was more active than thymol, different from that in our system. A remarkably wide variety of agents from F$^-$ through detergents to inflammatory mediators trigger an oxygen burst (22). To our knowledge, this is the first report to describe that phenolic compounds also stimulate such a burst.

Eugenol has weak antiseptic action and acts on bacteria cytostatically rather than cytotoxically (23). When it is applied topically to sepsis accompanying acute inflammation, bacteriocidal action by eugenol itself and the ability of host defense, e.g., $O_2^-$ metabolites produced by eugenol-stimulated neutrophils may show a synergistic action. This type of drug is ideal as an antiseptic.

In contrast, however, $O_2^-$ is one of phlogistic mediators and is harmful to biological materials. Our data warn that eugenol may cause unexpected neutrophil-mediated tissue damage when it is applied to the inflammed site as an anodyne or antiseptic.

The other pharmacological action of eugenol-induced $O_2^-$ production, vasodilation, is considered apart from the direct action of the drug, because exogeneous $O_2^-$ dilates cat mesentric blood vessels (24). We found that thymol has stronger activity in induction of $O_2^-$ production. With thymol, studies are now in progress to trigger the respiratory burst in neutrophils in order to clarify the pharmacological action mechanism of phenolic antiseptic drugs.

References


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