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Involvement of Cu^{++}-Catalyzed Peroxidation in Degradation of Collagen and Protective Mechanism of Sodium Salicylate on this Peroxidative Reaction

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ABSTRACT

The present study examines firstly, the inhibition of collagen gelation to explore the possible involvement of Cu^{++}-catalyzed peroxidation in rheumatoid arthritis and secondly, the effect of sodium salicylate on this peroxidative reaction to provide a possible explanation for its mechanism of anti-inflammatory action.

Incubation of collagen obtained from rat skin with Cu^{++} and H_2O_2 resulted in the inhibition of gelation in terms of maximal turbidity and lag phase, but either Cu^{++} or H_2O_2 alone essentially gave no effect in the collagen gelation. In the presence of sodium salicylate the inhibited gelation of collagen induced by Cu^{++} and H_2O_2 was reversed with the dependency of the concentration of sodium salicylate. Moreover, the rate of H_2O_2 decomposition by Cu^{++} was accelerated by sodium salicylate and this decomposition of H_2O_2 was found to be saturable in terms of concentration of this drugs.

Thus it can be expected that Cu^{++}-catalyzed peroxidation attacks collagen resulting in change of structural or functional integrity of collagen, and sodium salicylate may act on this peroxidative process, possibly through the enhancement of catalatic action of Cu^{++}. From these results Cu^{++}-catalyzed peroxidation can be in part responsible for degradation of joint tissue in rheumatoid arthritis and sodium salicylate may exert its anti-inflammatory action by this peroxidative reaction.

Key Words: Collagen gelation, Cu^{++}-catalyzed peroxidation, Sodium salicylate

INTRODUCTION

Copper (Cu^{++}) has been found to be an essential structural element in many enzymes where it is thought to function as a catalyst in oxidation-reduction process (Weser *et al.*, 1979). These include lysyl oxidase, cytochrome oxidase, tyrosinase, dopamine-β-hydroxylase and superoxide dismutase and so forth (Evans, 1973).

On the other hand, in non-enzymatic system, copper itself and copper complexed with various ligands are known to catalyze superoxide dismutation (Brigelius *et al.*, 1974; Rabani *et al.*, 1973) or the decomposition of hydrogen peroxide (H_2O_2) into H_2O and O_2 (Sharma *et al.*, 1970; Sharma and Schubert, 1969).

However, Cu^{++}-catalyzed peroxidative reactions were reported as another catalytic actions of Cu^{++} (Chan and Kesner, 1980; Ishimitsue *et al.*, 1979). These peroxidative reactions are strongly powerful and capable of oxidizing various compounds including fatty acids (Smedly-MacLean and Pearce, 1934), phospholipids (Chan *et al.*, 1982), pyridine nucleotides (Chan and Kesner, 1980) and nucleic acids (Massie *et al.*, 1972).

Although the details of Cu^{++}-catalyzed peroxidative reaction are still well understood, there are many reports that this peroxidative reactions seem to be mediated through the production of reactive oxygen species, especially hydroxyl radical (Chan and Kesner, 1980; Chan *et al.*, 1982; Chung *et al.*, 1984; Lee *et al.*, 1986).

But in view of the facts that reactive oxygen species are involved in the tissue damage in inflam-

matory reaction such as rheumatoid arthritis (Weissmann, 1979), the Cu^{++}-catalyzed peroxidation may also be a potential factor responsible for tissue damage caused by reactive oxygen species.

In this study, this inhibition of collagen gelation was examined to test the involvement of Cu^{++}-catalyzed peroxidation in the degradation of collagen. In addition, the effect of sodium salicylate in this system was evaluated to provide a possible explanation for its mechanism of anti-inflammatory action.

MATERIALS AND METHODS

Preparation of skin collagen

Collagen was prepared from the skin obtained from male rat weighing about 100 g according to procedure of Oegema *et al.* (1975). Collagen solution in 0.1 M acetic acid was lyophilized and stored at −20°C. Collagen when used, was dissolved in 0.5 M acetic acid to approximately 2 mg/ml, dialyzed extensively against 0.005 M acetic acid at 4°C and the resulting collagen in 0.005 M acetic acid was used in the experiments. Protein concentration of collagen was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Effect of Cu^{++}-catalyzed peroxidation on collagen gelation

Collagen (200 μg/ml) was mixed in 4 ml of reaction medium containing 0.14 M NaCl, 10 mM sodium phosphate buffer, pH 7.4 with or without varying concentration of $CuSO_4$ and H_2O_2 and incubated for 24 h at 4°C. the reaction was started by adding H_2O_2 and terminated by adding 0.1 mM EDTA and 10 μg/ml catalase. After incubation, dissolved air was removed by vaccum at 4°C and then 3 ml of aliquot was taken to a cuvette. Gelation was initiated by heating up the cuvette to 37°C in the temperature-controlled chamber of Pye-Unicam SP 1750 Spectrophotometer. Gelation was monitored by recording the increase in turbidity at 400 nm. When the effect of sodium salicylate in this system was studied, experimental procedure was the same as described above except that collagen was incubated with 20 μM Cu^{++} and 0.5 mM H_2O_2 in the presence or absence of sodium salicylate.

Effect of sodium salicylate on the decomposition of H_2O_2 by Cu^{++}

0.5 mM H_2O_2 in 4ml of reaction medium containing 0.14 M NaCl and 10 mM sodium phosphate buffer, pH 7.4 was incubated with 20 μM Cu^{++} in the presence or absence of varying concentrations of sodium salicylate at 4°C for 24 h. During the incubation 0.5 ml of aliquot was taken at time intervals as indicated and assayed for H_2O_2 remaining in this aliquot by iodometric method (Allen *et al.*, 1952).

RESULTS

1) Inhibition of collagen gelation by Cu^{++} and H_2O_2

The skin collagen from rat incubated with 20 μM Cu^{++} and 0.5mM H_2O_2 at 4°C for 24 h was markedly inhibited in its gelation that occured when heated up to 37°C. The inhibition was observed only in the presence of both Cu^{++} and H_2O_2. Either Cu^{++} or H_2O_2 alone essentially gave no effect (Fig. 1). Furthermore, when collagen was incubated with either 10 μg/ml catalase or 0.1 mM EDTA, almost no detectable change in gelation was observed (data not shown). These results suggest that both Cu^{++} and H_2O_2 were needed for peroxidative attack to collagen resulting in inhibition of its gelation.

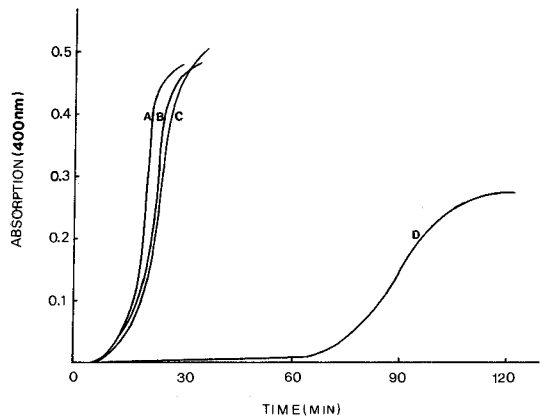

Fig. 1. Effect of Cu^{++} and H_2O_2 on collagen gelation. A reaction mixture (4ml) containing 200 μ/ml collagen, 0.5 mM H_2O_2, 20 μM $CuSO_4$ and 0.14 M NaCl, 10 mM KH_2PO_4 buffer, pH 7.4 was incubated for 24 h at 4°C. The reaction was started by adding H_2O_2 and stopped by EDTA and catalase (final concentrations of them were 100 μM and 10 μg/ml, respectively). After treatment of collagen with Cu^{++} and H_2O_2, gelation was initiated by heating up the reaction mixture to 37°C and measured spectrophotometrically by observing the increase in turbidity at 400 nm A; control (no Cu^{++} and H_2O_2), B; 20 μM Cu^{++} only, C; 0.5 mM H_2O_2 only, D; 20 μM Cu^{++} and 0.5 mM H_2O_2.

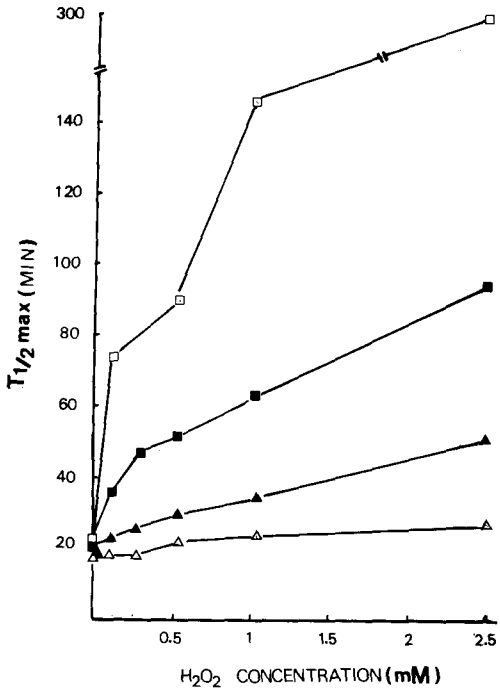

Fig. 2. Effect of varying concentrations of Cu^{++} and H_2O_2 on collagen gelation. The conditions were the same as in Fig. 1. Extent of inhibition of gelation was expressed as $T_{1/2}max$, the time required to attain half-maximum turbidity of gelated collagen. △; no Cu^{++} and ▲, ■ and □; 5, 10 and 20 μM Cu^{++}, respectively.

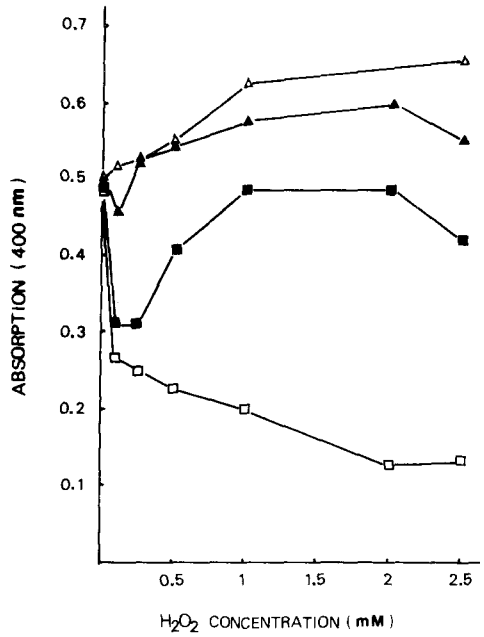

Fig. 3. Effect of varying concentrations of Cu^{++} and H_2O_2 on maxiumum absorption of gelated collagen. The conditions were the same as in Fig. 1. △: no Cu^{++} and ▲, ■ and □; 5, 10 and 20 uM Cu^{++}, respectively.

Fig. 2 showed the effect of concentration of Cu^{++} and H_2O_2 on lag phase of gelation. When the extent of prolongation of gelation was represented as $T_{1/2max}$, the time required to attain half-maximum turbidity, no significant change in $T_{1/2max}$ was observed in the presence of Cu^{++} or H_2O_2 alone. However, with constant Cu^{++} concentration $T_{1/2max}$ was prolonged with increasing concentration of H_2O_2. The same result was observed when fixed H_2O_2 concentration with increasing concentration of Cu^{++} was used.

Maximal inhibition of collagen gelation was also changed by Cu^{++} and H_2O_2. In all concentrations of H_2O_2 tested, maximum turbidity at 400 nm was decreased with increasing concentration of Cu^{++}. But in the cases with 5 and 10 μM of Cu^{++}, turbidity change was biphasic, i.e., turbidity decreased in the lower concentration of H_2O_2 followed by increasing in the higher concentration of H_2O_2 (Fig. 3). Probably these results seem to be due to different degrees of aggregation of gelated collagen.

2) Effect of sodium salicylate on collagen gelation inhibited by Cu^{++} and H_2O_2

As shown before, gelation was markedly inhibited by 20 μM Cu^{++} and 0.5 mM H_2O_2 in the absence of sodium salicylate. However, when the reaction medium was incubated in the presence of sodium salicylate, lag phase of gelation was shortened and the inhibition of gelation was reversed toward the condition of control. The degree of reversal increased with increasing the concentration of this drug from 16 μg/ml to 64 μg/ml (Fig. 4), which is much lower than that required for maximal suppression of rheumatic inflammation, 250-350 μg/ml of plasma.

3) Effect of sodium salicylate on the decomposition of H_2O_2 by Cu^{++}

Among the various mechanisms which may explain the action of the drug observed in Fig. 5, it might be possible to enhance the catalase-like action of Cu^{++} in this experiment. To prove it in a separate experiment, the rate of H_2O_2 decomposition by Cu^{++} was determined in the presence and absence of sodium salicylate.

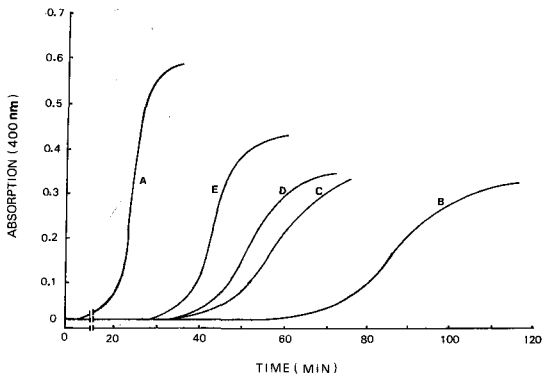

Fig. 4. Effect of sodium salicylate on the collagen gelation inhibited by Cu^{++} and H_2O_2. Collagen was treated with the same conditions as in Fig. 1. except the presence of varying amount of sodium salicylate. A; no Cu^{++} and H_2O_2, B; 20 μM Cu^{++} and 0.5 mM H_2O_2, C; 20 μM Cu^{++}, 0.5 mM H_2O_2 and 16 μg/ml Na salicylate, D; 20 μM Cu^{++}, 0.5 mM H_2O_2 and 32 μg/ml Na salicylate, E; 20 μM Cu^{++}, 0.5 mM H_2O_2 and 64 μg/ml Na salicylate.

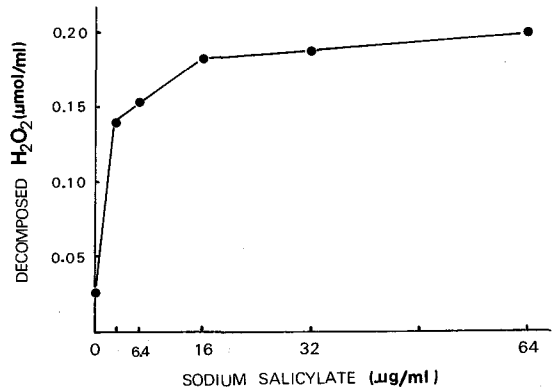

Fig. 6. The relationship between the decomposition rate of H_2O_2 by Cu^{++} and varying concentration of sodium salicylate. The conditions were the same as in Fig. 5. but the amount of H_2O_2 decomposed by Cu^{++} for 24 h was measured in the presence of varying amount of sodium salicylate.

When 0.5 mM H_2O_2 was incubated with 20 μM Cu^{++} at 4°C for 24 h H_2O_2 was decomposed at a slower rate. But the rate of H_2O_2 decomposition was accelerated with increasing the concentration of sodium salicylate. When concentration of sodium salicylate in the reaction medium was 3.2 μg/ml, about six-fold stimulation was observed (Fig. 5). However, this decomposition of H_2O_2 was found to be saturable in terms of concentrations of drug. As

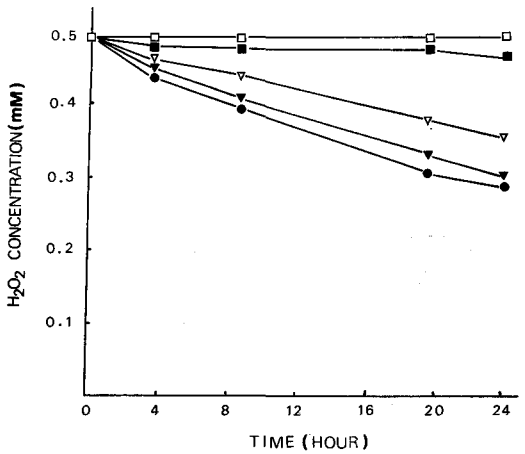

Fig. 5. Effect of sodium salicylate on the decomposition of H_2O_2 by Cu^{++}. H_2O_2 (0.5 mM) was incubated with 20 μM Cu^{++} in the presence or absence of sodium salicylate at 4°C and 0.5 ml of aliquot was taken at time intervals as indicated and assayed for H_2O_2. □; no Cu^{++} and Na^+ salicylate, ■; 20 uM Cu^{++} only, ▽; 20 μM Cu^{++} and 3.2 μg/ml Na^+ salicylate, ▼; 20 μM Cu^{++} and 16 μg/ml Na salicylate, ●; 20 μM Cu^{++} and 64 μg/ml Na salicylate.

shown in Fig. 6, almost maximum stimulation was observed when concentration ratio of Cu^{++} and sodium salicylate was 1:1, suggesting rather specific stoichiometry in coordination between two factors is necessary.

DISCUSSION

Reactive oxygen species are thought to play a major role in tissue damage in chronic inflammatory diseases (Weissman, 1979) and several reports showed that some physicochemical properties of hyaluronic acid and collagen are altered by these species in rheumatoid arthritis (Greenwald and Moy, 1979; McCord, 1983).

Among the reactive oxygen species, H_2O_2 by itself is not very reactive as an oxidant compared to the other reactive oxygen species such as hydroxyl radical and singlet oxygen. Rather, H_2O_2 can serve as a precursor for the more reactive oxygen species which may attack various tissue components (Kameta *et al.*, 1979; Kellog and Fridovich, 1977). Several reactions in which H_2O_2 produces more reactive species have

been proposed. These are enzymatic production of singlet oxygen by myeloperoxidase in the presence of halide ions (Klebanoff *et al.*, 1976), Haber-Weiss reaction to form hydroxyl radical and singlet oxygen by the interaction with superoxide radical (Fridovich, 1978).

However, another possible reaction related to H_2O_2 is Cu^{++}-catalyzed peroxidative reaction (Chan and Kesner, 1980; Lee *et al.*, 1986; Ishimitsue *et al.*, 1979). Although the details of this peroxidative mechanism are not still well understood, high reactivity inherent to this metal ion and H_2O_2 may provide a potential factor responsible for some of tissue toxicity caused by the reactive oxygen species. From this aspects, to test the possibility that Cu^{++}-catalyzed peroxidation may attack on collagen, skin collagen was employed as a test material and the effects of Cu^{++} and H_2O_2 on the gelation were observed of collagen representing fibril formation in vitro of this structural protein (Wood and Keech, 1960). These results showed both Cu^{++} and H_2O_2 were necessary for this peroxidative attack to collagen and collagen gelation was markedly inhibited by Cu^{++} and H_2O_2 (Wood and Keech, 1960).

These findings suggest that Cu^{++}-catalyzed peroxidation attacks collagen resulting in change of structural or functional integrity since inhibition of collagen gelation reflects alteration of collagen molecule or collagen degradation. And these results were also supported by some reports that this peroxidative reaction can cause destruction of articular cartilage (Chung *et al.*, 1983, 1984; Venkatasubramanian and Joseph, 1977).

In another aspect, copper is a commonly found metal in most biological fluids and tissues (Underwood, 1962). But Gerber (1974) observed that the level of plasma histidine frequently decreased in patients with rheumatoid arthritis. Because histidine is a potent physiological chelator of Cu^{++}, this decreased histidine level may make copper more available for peroxidative catalysis of biological substances. Moreover, in severe rheumatoid arthritis the copper concentration increases more than two-fold in the serum and synovial fluid (Lober *et al.*, 1968; Sorenson, 1978) and the elevated copper concentration may play as a destructive agent on joint tissue in this pathologic condition. At the same time, in acute stage of this disorder, massive infiltration of neutrophils into inflamed joint has been observed, which may result in localized high concentration of H_2O_2 (Nathan *et al.*, 1979; Root *et al.*, 1975).

From the result obtained as well as other reports it may suggest that Cu^{++}-catalyzed peroxidation may play a part in the degradation of cartilage and collagen observed in rheumatoid arthritis.

It is well known that the formation of reactive oxygen species in vivo occurs through phagocytosis (Babior *et al.*, 1973; Johnston *et al.*, 1975) and biosynthesis of prostaglandin via cyclooxygenase pathway (Bragt *et al.*, 1980) during the inflammatory reaction. And aspirin-like drugs are thought to exert their anti-inflammatory action through inhibiting of prostaglandin synthesis (Flower, 1974). Recently another additional mechanisms of their anti-inflammatory actions have been suggested (Atkinson and Collier, 1980). For example, some of these drugs could complex with copper. These complexes show superoxide dismutase-like action and greater anti-inflammatory effect in vivo than their parent compounds through interfering with the toxic effects of reactive oxygen species (Brigtius, 1974; Younes and Weser, 1977). However, in the present study the inhibition of gelation induced by Cu^{++} and H_2O_2 was markedly protected by sodium salicylate and the rate of decomposition of H_2O_2 was also accelerated. These findings suggest that sodium salicylate may act on the Cu^{++}-catalyzed peroxidative process, possibly through the enhancement of catalatic action of Cu^{++}.

But it should be considered that sodium salicylate could chelate Cu^{++} and then make this metal ion in less available for peroxidation as a catalyst. In this respect much more studies will be needed to support the mechanism of anti-inflammatory action of sodium salicylate.

In conclusion, Cu^{++}-catalyzed peroxidation can be in part responsible for degradation of joint tissue in rheumatoid arthritis and sodium salicylate may exert its anti-inflammatory action by acting upon this peroxidative process.

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=국문초록=

Cu^{++} 촉매작용에 의한 과산화 현상이 Collagen 손상에 관여함과 Sodium Salicylate에 의한 보호 작용

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김용식

Cu^{++} 촉매작용에 의한 과산화현상이 관절조직손상의 한 형태인 collagen 손상에 관여할 수 있음을 알아보고, sodium salicylate의 항 염증기전의 일부를 설명해 보고자 sodium salicylate가 이 과산화반응에 미치는 효과를 검토하였다. 쥐피부로 부터 얻은 collagen을 이용하여 collagen gelation에 대한 Cu^{++}와 H_2O_2의 효과를 관찰한 결과 Cu^{++} 또는 H_2O_2 단독으로는 gelation에 영향을 미치지 못하였으나, Cu^{++}와 H_2O_2가 동시에 첨가된 경우 gelation이 억제되어 maximal turbidity가 감소되고, lag phase가 연장됨을 보였다. 그리고 같은 반응 조건에서 sodium salicylate 첨가에 의해 Cu^{++}와 H_2O_2에 의해 억제된 gelation이 회복됨을 볼 수 있었으며 회복정도는 salicylate 농도 증가에 의존적이었다. 한편 Cu^{++}에 의한 H_2O_2의 decomposition rate가 sodium salicylate에 의해 증가됨을 보였고, salicylate 농도 증가에 의해 점차 saturation되는 양상을 보였다.

이상의 결과로 부터 Cu^{++} 촉매작용에 의한 과산화 현상은 collagen에 작용하여 구조적 또는 기능적인 변화를 초래함을 알 수 있었고, salicylate에 의해 이러한 과산화 현상이 억제되는 것은 Cu^{++}에 의한 H_2O_2의 decomposition rate를 증가시킨 결과임을 알 수 있었다.

그러므로 Cu^{++} 촉매작용에 의한 과산화현상은 만성염증 반응 특히 rheumatoid arthritis에서 나타나는 관절조직 손상에 관여할 수 있으며, sodium salicylate는 이 과산화반응에 작용하여 항 염증효과를 나타낼 수 있으리라 믿어졌다.