Korean J. of Pharmacology
Vol. 21, No. 1, pp 62-67, 1985

Role of Ascorbic Acid in the Depolymerization of Hyaluronic Acid by Fe^{++} and H_2O_2 [1]

Jung-Soo Lee, Myung-Hee Chung[2], Jung-Kyoo Lim,
Chan-Woong Park and In-Joon Cha*

Department of Pharmacology, College of Medicine, Seoul National University, Seoul 110, Korea
*Department of Pharmacology, Inje Medical College, Pusan 601, Korea**

ABSTRACT

In the iron-catalyzed Haber-Weiss reaction to produce OH., the requirement for $O_2^-\cdot$ is only to reduce Fe^{+++}. Possibly, the role of $O_2^-\cdot$ can be replaced by other reducing agents. Ascorbate is one of them in biological system. In the present study, the ability of ascorbate to produce OH· in the presence of Fe^{++} and H_2O_2 was investigated by observing the degradation of hyaluronic acid and ethylene production from methional.

Ascorbate stimulated the degradation of hyaluronic by Fe^{++} and H_2O_2. That was confirmed by both viscosity change and gel-permeation chromatographic analysis. The observed degradation was almost completely prevented by catalase and OH· scavengers. In support of the above results, ascorbate enhanced the prouction of ethylene from methional in the presence of Fe^{++} and H_2O_2.

Other reducing agents (cysteine, glutathione, NADH and NADPH) showed similar activities to ascorbate in the degradation of hyaluronic acid and ethylene production. But no stimulatory effects were observed with their oxidized forms such as NAD and NADP. Thus, it appears that reduction of the metal ion was needed for OH· production.

Among the metal ions tested, Fe^{++} showed most potent catalytic action in the production of OH·

The results obtained support that ascorbate can substitute $O_2^-\cdot$ in the metal-catalyzed reactions, particularly with Fe^{++} by which OH· is produced with H_2O_2. The significance of the ascorbate-dependent production of OH· was considered with respect to possible role of ascorbate in the damage of inflamed joints.

Key Words: ascorbic acid, oxygen radical, iron and hyaluronic acid

INTRODUCTION

It is well recognized that superoxide radical ($O_2^-\cdot$) is produced in a wide range of biological reactions involved in the metabolism of O_2· Because of its toxic actions, it appears to be implicated in the tissue damage in various pathological conditions (Demopoulos *et al.*, 1980; Leibovitz and Siegel, 1980; Taylor, 1983). Destruction of the joint components associated with inflammation may be one of the situations where the activated oxygen is involved (Weissmann *et al.*, 1980; Fantone and Ward, 1982). In particular, hyaluronic acid is depolymerized and synovial fluid loses its lubricating properties (McCord,

1. This was supported by 1984 Research Grant of The Ministry of Education, Republic of Korea
2. To whom reprint requests should be addressed

1974; Halliwell, 1978a). In the situation, $O_2^-\cdot$ would be released from the neutrophils infiltrating into the inflamed sites (Weissmann *et al.*, 1980; Fantone and Ward, 1982).

In the degradation of haluronic acid as well as its many other deleterious effects observed, however, it is indicated that $O_2^-\cdot$ is not the directly damaging species. Many reports point to involvement of a highly reactive species such as hydroxyl radical ($OH\cdot$) which is formed from an interaction of $O_2^-\cdot$ and H_2O_2 formed by its dismutation reaction ($O_2^-\cdot + O_2^-\cdot + 2H^+ \rightarrow H_2O_2$) catalyzed iron salt (Fridovich, 1975; Kellogg and Fridovich, 1977; Weiss *et al.* 1978; McCord and Day, 1978; Halliwell, 1979b), so called iron-catalyzed Haber-Weiss reaction (1):

$$O_2^-\cdot + H_2O_2 \rightarrow OH\cdot + OH^- + O_2 \quad (1)$$

In this reaction, Fe^{++} reacts with H_2O_2 by a Fenton type reaction (2) to form $OH\cdot$ and the role of the $O_2^-\cdot$ is to reduce the ferric iron thus formed as in reaction (3), the net result being reaction (1).

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH\cdot + OH^- \quad (2)$$
$$Fe^{+++} + O_2^-\cdot \rightarrow Fe^{++} + O_2 \quad (3)$$

However, if the requirement of $O_2^-\cdot$ is to reduce Fe^{+++}, other reducing agents may be able to perform the role equally well. In fact, Winterbourn (1979; 1981) showed evidence that this was the case with ascorbate. By comparing the ability of various reducing agents in biological system to produce ethylene from methional [as an indication of $OH\cdot$ production (Beauchamp and Fridovich, 1970)] in the presence of Fe^{++} and H_2O_2, she found that only ascorbate stimulated $OH\cdot$ production and further that it was even stronger than $O_2^-\cdot$ in the stimulation. This finding suggests that if Fe^{++} is available in biological fluids, $OH\cdot$ production can be mediated by ascorbate and this pathway would be predominant over the $O_2^-\cdot$-dependent one.

On the other hand, many studies have reported toxic effects of ascorbate to various biological materials. They include inactivation of vaccinia virus (Turner, 1964), peroxidation of membrane lipids (Rehncrona *et al.*, 1980), enzyme inactivation (Schaeffer *et al.*, 1975) and degradation of hyaluronic acid (Matsumura and Pigman, 1965; Wong *et al.*, 1981). In all cases, however, these effects were shown to be markedly enhanced in the presence of metal ions such as iron, in particular and to be inhibited by catalase and very often also by $OH\cdot$ scavengers. Although detailed mechanism of the toxic action of ascorbate has not been settled yet, the findings suggest that ascorbate especially in the presence of iron as a catalyst can be toxic through generating reactive oxygen species.

Biological significance of the toxic action of ascorbate has not been clearly understood. But with the observation that ascorbate in the presence of Fe^{++} can depolymerize hyaluronic acid with involving $OH\cdot$ as a mediator, Wong *et al.*, (1981) have recently suggested that this action of ascorbate can contribute to the degradation of synovial fluid in many inflammatory types of arthritis. In view of the central role of $OH\cdot$ in the toxicities mediated by reactive oxygen species, the substitution of ascorbate for $O_2^-\cdot$ in the iron-catalyzed Haber-Weiss reaction will be one possible mechanism which may explain the ascorbate-mediated toxic action and also this reaction could be extended to another mechanism causing the damage observed in inflamed joints.

In the present study, to test this possibility, ability of ascorbate to produce $OH\cdot$ in the presence of various metal ions including Fe^{++} and H_2O_2 was investigated by observing the degradation of hyaluronic acid and ethylene production from methional and the *in vivo* feasibility of the ascorbate-dependent mechanism as a contributor to the damage in arthritis was discussed.

MATERIALS AND METHODS

Hyaluronic acid (Grade III from human umbilical cord), catalase (from bovine liver), NADH, NADH, NADPH, NAD, NADP, cysteine, glutathione (reduced form), Chromosorb 102 (80-100 mesh)

and Sepharose 4B-200 were obtained from Sigma Chemical Corp.. Ascorbate were purchased from Junsei Chemical Co. Ltd.; mannitol from Merck; sodium benzoate, $NiSO_4$, $PbNO_4$ and $ZnSO_4$ from Wako Pure Chemical Industries Ltd. and H_2O_2 from Shinyo Pure Chemicals Co. Ltd.. Ethylene gas was obtained as a gift from Dr. K.I. Chung, Korea Advanced Institute of Science and Technology.

Measurement of hyaluronic acid degradation

Degradation of hyaluronic acid was determined by measuring the change in the viscosity and gel-permeation chromatographic analysis.

Viscometry: Viscometry was performed in a Coulter Harkness viscometer. A typical reaction procedure was as follows: Hyaluronic acid (0.9 mg/ml) was preincubated in the solution containing 150 mM KCl and 50 mM potassium phosphate, pH 7.5 for 10 min. Reaction was started by adding various combinations of Fe^{++}, H_2O_2 and ascorbate to give final concentrations as indicated in the experiments. When effects of the inhibitors were tested, they were added prior to the start of reaction. Total reaction mixture was 10 ml and temperature was 25°C. At various intervals, 1.0 ml aliquots were removed to the viscometer kept in water bath at 25°C. The changes in viscosity were expresesed as flow times (sec) displayed in the viscometer.

Gel-Permeation Chromatography: Hyaluronic acid was treated with Fe^{++}, H_2O_2 and ascorbate as described in the previous section. After treatment for 1 h, the reaction was stopped by addition of 50 μl of 1 mg/ml catalase and 0.1 ml of 1 M sodium formate to 10 ml of reaction mixture. In the control study, the agents were found to prevent the decrease in the viscosity when added in the beginning of the reaction. Ten ml of the reaction mixture was applied on the Sepharose 4B column (85 x 1.6 cm) which was equilibrated with eluting buffer of 0.5 M sodium acetate, pH 7.0. The column was eluted at a flow rate of 50 ml/h which was regulated with LKB 2132 Microperpex Peristaltic Pump. The effluent was fractionated with a LKB 2111 MultiRac and 4.0 ml fractions were collected to assay uronic acid according to the method of Bitter and Muir (1962). The chromatography was operated at room temperature.

Determination of Ethylene Formation

0.5 mM methional in 50 mM potassium phosphate, pH 7.5 was preincubated for 5 min and then mixed with various combinations of Fe^{++}, H_2O_2 and ascorbate at the concentrations as shown in the experiments. Reactions were performed in 12.5 ml glass vials sealed with rubber caps and incubataed at 37°C with vigorous shaking. The total volume of the reaction mixture was 1.0 ml. At 30 min, 1.0 ml aliquots of the gas phase above the reaction mixture were sampled with gas tight syringe and assayed for ethylene. The gas was produced as an end product when methional was oxidized by OH· (Beauchamp and Fridovich, 1970). Ethylene was determined on a gas chromatograph (GC, Varian Aerograph Series 1800) equipped with 1/8 inch x 3 meter stainless steel column of Chromosorb 102 and a flame ionization detector. The temperatures of the column, detector and injector were maintained at 70°C, 200°C and 80°C, respectively. The gas flow was 25 ml/min for carrier (N_2), 35 ml/min for hydrogen and 30 ml/min for air. The amount of ethylene produced was calculated from integrated areas of chromatogram of pure ethylene gas.

RESULTS

Effects of ascorbate on hyaluronic acid degradation with Fe^{++} and H_2O_2

Fig. 1 shows that in the presence of 10 μM Fe^{++} and 0.5 mM H_2O_2, ascorbate (7.5 μM) increased

the rate of reduction in the viscosity of hyaluronic acid. At the concentrations used, Fe^{++}, ascorbate or Fe^{++} + ascorbate showed only slight effect on the viscosity. But when hyaluronic acid was incubated with Fe^{++} and H_2O_2, its viscosity was reduced with time and their effect was markedly enhanced by ascorbate. Some decrease in the viscosity was observed with H_2O_2 alone. That may be due to contamination of trace metal ions in the reaction medium (Wong et al., 1981). The decrease in the viscosity of hyaluronic acid reflects depolymerization or degradation of this polysaccharide. Thus, the observed findings suggest that ascorbate accelerates the degradation of hyaluronic acid by Fe^{++} and H_2O_2.

This was also clearly demonstrated by Sepharose 4B gel-permeation chromatographic analysis of the treated hyaluronic acid (Fig. 2). When untreated hyaluronic acid was chromatographed, the effluent showed two peaks (Fig. 2A), first one representing large sized molecules at fractions from 17 to 23 and second one of smaller sized at fractions from 29 to 40. This indicates that the preparation of hyaluronic acid already contained fragmented products. On analysis of hyaluronic acid treated with Fe^{++} and H_2O_2 for 1 h, slight decrease and increase of the first and second peak, respectively were observed in their size with less distinct separation of the two peaks (Fig. 2B). The observed effect was not marked compared to the considerable changes in the viscosity under the same condition (Fig. 1). It appears that of the two methods to detect the degradation, the chromatography was less sesitive. When 7.5 μM of ascorbate added to this system, the chromatographic pattern was markedly changed with the first peak almost abolished (Fig. 2C). Fig. 3 shows sequential changes of eluting patterns with incubation time. Uronic acids in the first peak were shifted to the right side of lower molecular size and at 5 h, only one large peak remained at the position of the second peak indicating complete degradation of hyaluronic acid.

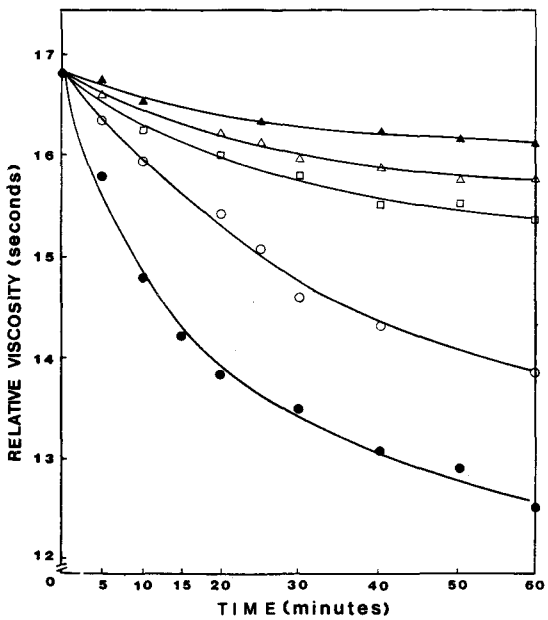

Fig. 1. Effect of Fe^{++}, H_2O_2 and/or ascorbate in the viscosity of hyaluronic acid. Hyaluronic acid (0.9 mg/ml) was incubated with various combinations of Fe^{++}, H_2O_2 and ascorbate in 10 ml of reaction mixture containing 150 mM KCl and 50 mM potassium phosphate, pH 7.5 at 25°C. The concentration of Fe^{++}, H_2O_2 and ascorbate were 10 μM, 0.5 mM and 7.5μM, respectively. At various intervals, one ml aliquots were taken to measure the viscosity change. Other details are described in the Materials and Methods.
Symbols: ▲, ascorbate; △, Fe^{++} and ascorbate; □, H_2O_2; ○, Fe^{++} and H_2O_2; ●, Fe^{++}, H_2O_2 and ascorbate.

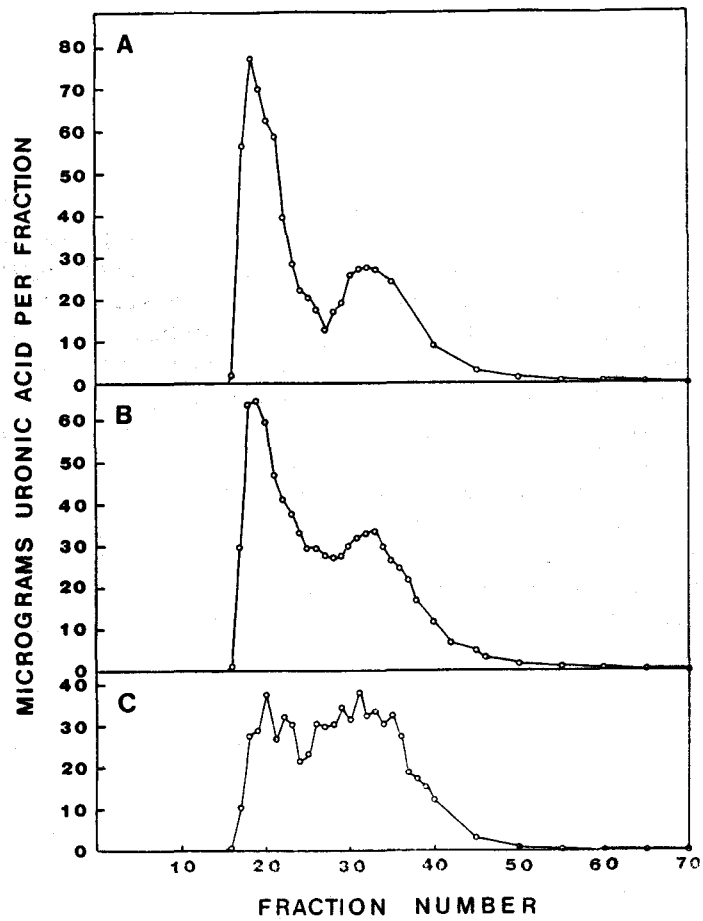

Fig. 2. Sepharose 4B elution patterns of hyaluronic acid treated with Fe^{++} and H_2O_2 in the presence or absence of ascorbate. Hyaluronic acid (0.9 mg/ml) was incubated with 10 μM Fe^{++} and 0.5 mM H_2O_2 in the absence or presence of 7.5 μM ascorbate for 1 h under the same conditions as in Fig. 1 and the reaction was terminated as described in the Materials and Methods. Ten ml of the reaction mixtures were applied on the column and fractions of 4.0 ml were collected and assayed for uronic acid. The chromatographic conditions and assay for uronic acid were described in the Meterials and Methods. A, control; B, treated with Fe^{++} and H_2O_2; C, treated with Fe^{++} and H_2O_2 in the presence of ascorbate.

Effects of catalase and various scavengers on hyaluronic acid degradation by Fe^{++}, H_2O_2 and ascorbate

In the previous experiments, Fe^{++} and H_2O_2 were able to degrade hyaluronic acid and ascorbate showed its effect on the degradation only in the presence of Fe^{++} and H_2O_2 (Fig. 1 and 2). Fe^{++} has been reported to produce OH· through reaction (2) (Repine *et al.*, 1981) and this radical as most reactive oxygen species was shown to cause degradation of hyaluronic acid (McCord, 1974; Halliwell, 1978a). Thus, it appears that the degradation was due to OH· and ascorbate attributed its action to the promotion of OH· production from Fe^{++} and H_2O_2.

As expected, the decrease in the viscosity of hyaluronic acid by the complete system was prevented by catalase and also by OH· scavengers, bezoate, formate and mannitol (Table 1). Their effects were increased with increasing their concentrations. In the presence of 20 μg/ml of catalase and 100 mM of each OH· scavenger, the viscosity of hyaluronic acid was almost the same as that in the presence of

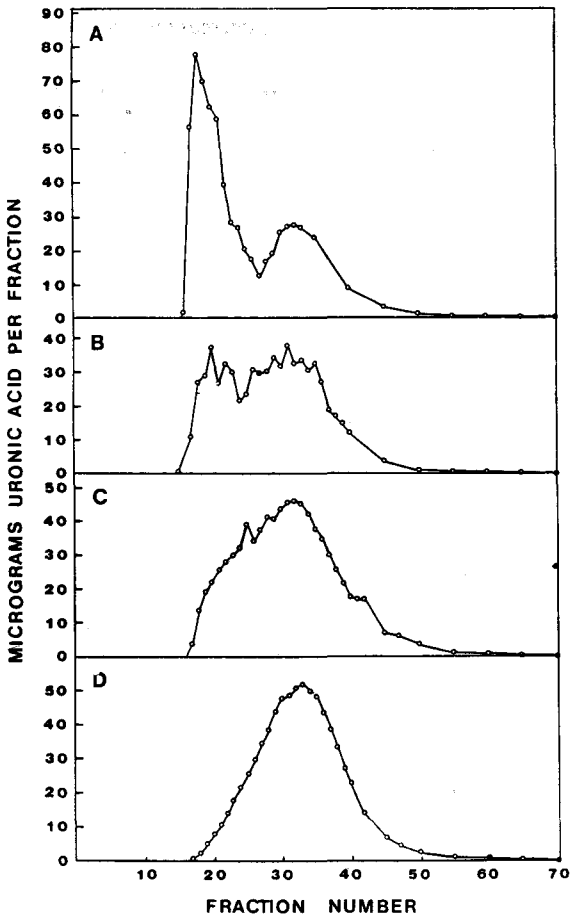

Fig. 3. Changes of Sepharose 4B elution patterns of hyauronic acid treated with Fe^{++}, H_2O_2 and ascorbate for different incubation time. Hyaluronic acid (0.9 mg/ml) was incubated with 20 μM Fe^{++}, 0.5 mM H_2O_2 and 7.5 μM ascorbate for varying period of incubation. All other experimental conditions were the same as in Fig. 2. A, zero time; B, 1 h; C, 2h and D, 5 h incubation.

Fe^{++} and ascorbate which were shown to cause slight decrease of viscosity (about 10%). This indicates that they showed complete inhibition at the concedntrations used (the % inhibition shown in the Table 1 was calculated from the ratios of the reductions in flow times of the treated hyaluronic acid with Fe^{++}, H_2O_2 and ascorbate in the presence to the absence of the scavengers without correction of the viscosity decrease caused by Fe^{+}£ and ascorbate). Superoxide dismutase did not show any effectgs on the viscosity change of hyaluronic acid (data not shown). The preventive effect was also observed on the gel-permeation chromatography. As shown in Fig. 4, with increasing concentration of formate, the eluting pattern of degraded hyaluronic acid treated with the complete system was gradually restored to the pattern of untrated hyaluronic acid and the the degradation was completely inhibited at 100 mM (Fig. 4E).

Stimulation of ethylene production from methional

OH· involvement was further tested by assaying ethylene production from methional. Methional when attacked by OH· was reported to produce ethylene as an end-product (Beauchamp and Fridovich,

Table 1. Effect of catalase and OH· scavengers on the decrease in viscosity of hyaluronic acid by Fe^{++}, H_2O_2 and ascorbate

Compounds	Concentration	* % inhibition of the reduction of viscosity by Fe^{++}, H_2O_2 and ascorbate
Catalase	5 μg/ml	39.2
	10 μg/ml	69.6
	20 μg/ml	86.7
Sodium benzoate	1 mM	16.9
	10 mM	62.0
	100 mM	88.7
Mannitol	1 mM	15.0
	10 mM	58.0
	100 mM	90.6
Sodium formate	1 mM	13.1
	10 mM	57.0
	100 mM	90.4

Data expressed are mean of 3-5 experiments. Hyaluronic acid was treated with Fe^{++}, H_2O_2 and ascorbate for 1h as in Fig. 1 in the absence and presence of the scavengers. * % inhibition was calculated from the ratio of flow time reducations observed with hyaluronic acid treated with Fe^{++}, H_2O_2 and ascorbate in the presence to absence of the added components,

Talbe 2. Effect of Fe^{++}, H_2O_2 and/or ascorbate on the production of ethylene from methional

Additions	Ethylene formed in 30 min (nmoles)
None	2.6
10 μM ascorbate	2.9
0.5 mM H_2O_2	2.5
10 μM ascorbate + 0.5 mM H_2O_2	3.9
10 μM ascorbate + 10 μM Fe^{++}	7.3
10 μM Fe^{++} + 0.5 mM H_2O_2	35.6
10 μM Fe^{++} + 0.5 mM H_2O_2 + 10 μM ascorbate	46.6

Data are expressed as mean of 3-5 experiments. Various combinations of Fe^{++}, H_2O_2 and ascorbate were added to 0.5 mM methional and mixed in 50 mM potassium phosphate, pH 7.4. After 30 min incubation, ethylene was assayed by gas chromatography. The experimental details are described in the Materials and Methods.

1970) and the phenomenon has been employed to detect OH· production in biological systems (Klebanoff and Rosen, 1978; Weiss *et al.*, 1977 and 1978; Cohen and Cederbaum, 1979). As shown in Table 2, each agent alone or ascorbate + H_2O_2 did not produce ethylene from methional. Little amount (7.3 nmoles) was also observed with ascorbate and Fe^{++}. But significant amount (35.6 nmoles) was produced with Fe^{++} and H_2O_2, and it was again stimualed by ascorbate (46.6 nmoles). The results may support again

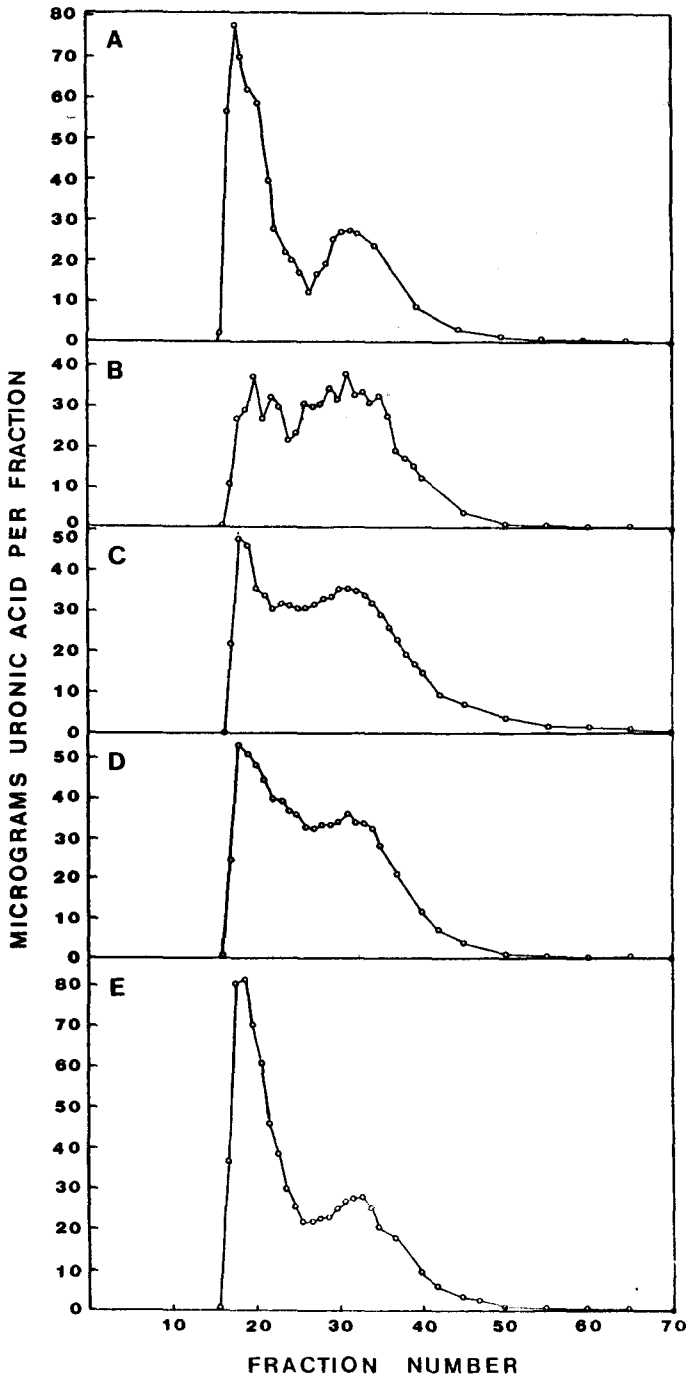

Fig. 4. Sepharose 4B elution patterns of hyauronic acid treated with Fe^{++}, H_2O_2 and ascorbate in the presence of formate. Hyaluronic acid was treated for 1 h in the presence of different concentrations of formate. All other experimental conditions were the same as in Fig. 2. A, no treatment; B, C, D and E, treated in the presence of 0, 1, 10 and 100 mM formate, respectively.

the role of ascorbate to stimulate the production of OH· from the reaction of Fe^{++} and H_2O_2 which is a primary source of this radical and seem to be in good agreement with the observations obtained in the degradation and scavenger studies.

Effects of other reducing agents on the action of Fe^{++} and H_2O_2

If the stimulatory effect of ascorbate observed in the previous experiments can be assumed to be ascribed to the reduction of Fe^{+++} to Fe^{++}, other reducing agents may also be expected to perform the role as well. With several reducing compounds occurring in the biological system, their substitution for ascorbate was tested. Indeed, the degradation of hyaluronic acid by Fe^{++} and H_2O_2 was stimulated by these agents. As shown in Fig. 5 and 6, their effects were demonstrated on both the viscosity change and chromatographic eluting pattern. Moreover, their stimulatory effects were almost equal to that of ascorbate. But no stimulation was observed with oxidized form, for example, NAD and NADP (Fig. 7). This may further support that reduction of Fe^{+++} to Fe^{++} is a required process in the stimulation observed with the reducing agents. These compounds were also examined on their ability to stimulate the production of ethylene from methional in the presence of Fe^{++} and H_2O_2. As expected, ethylene production was enhanced by these agents. As shown in Table 3, the extent of stimulation was almost the same as that with ascorbate. Here again, no stimulation was observed with NAD and NADP. The results accord well with those observed in the degradation experiments.

Comparison of the catalytic actions of various metal inos

Effects of varions metal ions on hyaluronic acid degradation and ethylene formation were observed in the presence of ascorbic aric and H_2O_2. All the metal ions tested were shown to stimulated the

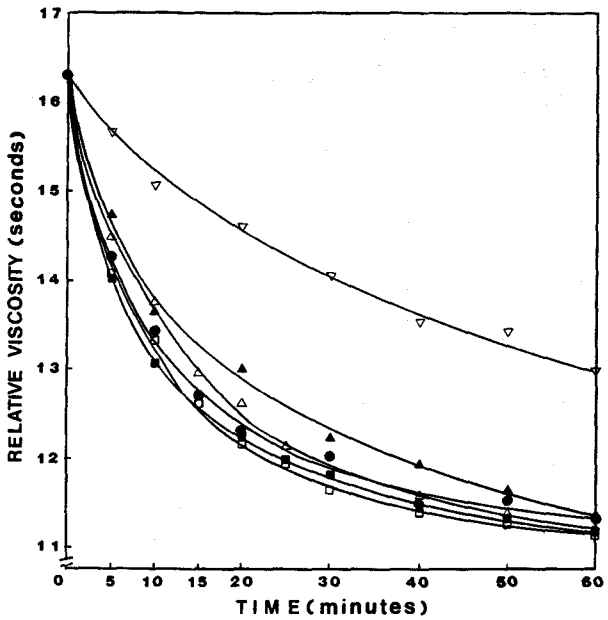

Fig. 5. Effects of ascorbate and other reducing agents on the decrease in the viscosity of hyaluronic acid by Fe^{++} and H_2O_2. Hyaluronic acid (0.9 mg/ml) was incubated with 10 μM Fe^{++} and 0.5 mM H_2O_2 in the presence of 7.5 μM of ascorbate and other reducing agents. At various intervals, 1.0 ml aliquots were taken to measure the viscosity. Other experimental conditions were the same as in Fig. 1. Symbols; △, with no reducing agents; ▲, glutathione; ●, ascorbate; ▲, cysteine; □, NADH and ■, NADPH.

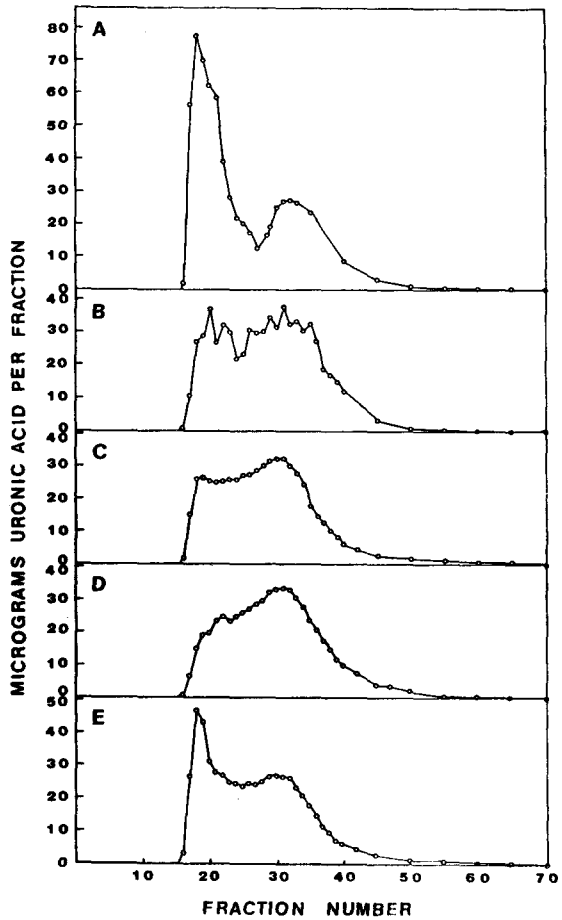

Fig. 6. Sepharose 4B elution patterns of hyaluronic acid treated with Fe^{++} and H_2O_2 in the presence of various reducing agents. Hyaluronic acid was treated for 1 h with 10 μM Fe^{++} and 0.5 mM H_2O_2 in the absence or presence of 7.5 μM of the reducing agents and then reaction mixtures were analyzed on the column. All other experimental conditions were the same as in Fig. 2. A, no treatnent; B,C,D and E, treated in the presence of asocrbate, NADH, cysteine and glutathione, respectively.

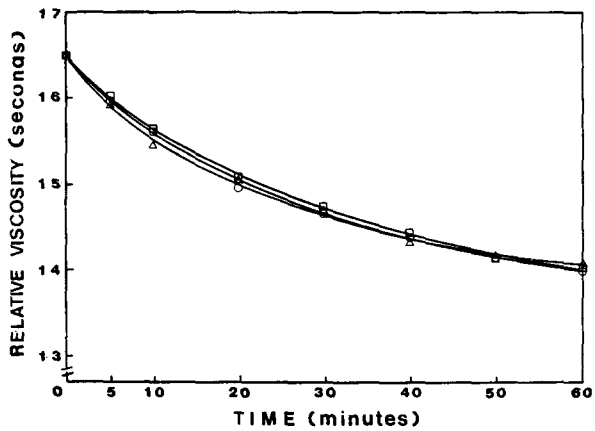

Fig. 7. Effect of NAD and NADP on the decrease in the viscosity of hyaluronic acid by Fe^{++} and H_2O_2. Hyaluronic acid (0.9 ml/ml) was treated with 10 μM Fe^{++} and 0.5 mM H_2O_2 in the absence (□) or presence of 7.5 μM of NAD (△) and NADP (○). Other experimental conditions were the same as in Fig. 1.

Table 3. Effects of various reducing agents and their oxidized forms on ethylene production from methional by Fe^{++} and H_2O_2

Additions	Ethylene formed in 30 min(nmoles)
Fe^{++} + H_2O_2	36.1
+ 10 uM ascorbate	46.6
+ 10 uM NADH	46.5
+ 10 uM NADPH	43.3
+ 10 uM NAD	36.6
+ 10 uM NADP	36.4
+ 10 uM cysteine	44.1
+ 10 uM GSH	44.7

Data are expressed as mean 3-5 of experiments. Methional 0.5 mM was incubated with 10 μM Fe^{++} and 0.5 mM H_2O_2 in presence of various reducing agents or their oxidized forms, NAD and NADP. All the experimental conditions were the same as in Table 2.

Table 4. Effects of various metal ions on the decrease in viscosity of hyaluronic acid in the presence of H_2O_2 and ascorbate

Additions	the decrease in Viscosity(%)
+ 10 μM Fe^{++}	100
+ 10 μM Ni^{++}	56.6
+ 10 μM Zn^{++}	44.5
+ 10 μM Cu^{++}	61.6
+ 10 μM Sn^{++}	61.6
+ 10 μM Pb^{++}	53.8
+ 10 μM Hg^{++}	51.0

Data are expressed as mean of 3-5 experiments. Hyaluronic acid was incubated with 0.5 mM H_2O_2 and 7.5 μM ascorbate for 1 h in the presence of various metal ions under the same conditions as in Fig. 1. The reduction in the flow time with Fe^{++} was 5.6 sec which was taken as 100%.

Table 5. Ethylene production with H_2O_2 and ascorbate in the presence of various metal ions.

Additions	Ethylene formed in 30 min (nmoles)
0.5 mM H_2O_2 and 10 μM ascorbate	3.9
+ 10 μM Fe^{++}	46.6
+ 10 μM Cu^{++}	19.8
+ 10 μM Zn^{++}	14.5
+ 10 μM Ni^{++}	15.4

Data are expressed as mean of 3 experiments. 0.5 mM methional was incubated for 30 min with ascorbate and H_2O_2 in the presence of various metal ions under the same experimental conditions as in Table 2.

degradation (Table 4). But the highest stimulation was found with Fe^{++}. Effect of other metal ions were about half that of Fe^{++}. In the control study, these metal ions either alone or with ascorbate showed no detectable change on the viscosity of hyaluronic acid. Similar results were also observed in the ethylene production (Table 5). With Fe^{++}, the amount of ethylene produced for 30 min was 46.6 nmoles which was much higher than that with Cu^{++}, Zn^{++} or Ni^{++}.

Discussion

The results of the present study suggest that ascorbate and H_2O_2 in the presence of catalytic amount of Fe^{++}, react to form OH·. The degradation of hyaluronic acid by the Fenton reagents, Fe^{++} and H_2O_2 was augmented by ascorbate (Fig. 1 and 2). This degradation was almost completely inhibited by catalase as well as OH· seavengers, benzoate, mannitol and formate (Table 1 and Fig. 4). Ethylene production was also enhanced by ascorbate in the reaction of methional with Fe^{++} and H_2O_2 (Table 2). Although it has been shown that ethylene production is not necessarily specific for OH· (Pryor and Tang, 1978), in the present system, it is highly likely that OH·was responsible; ethylene production was only observed by Fe^{++} and H_2O_2 which is a well established producer of OH· (Repine *et al.*, 1981), and while ascorbate did not show significant production of ethylene with either Fe^{++} or H_2O_2 and at concentration used, the production by Fe^{++} and H_2O_2 was enhanced by ascorbate, indicating that with ascorbate present, OH· is also produced with increased rate. Under these circumstances, this radical is more likely to have been the source of ethylene than is any other reaction product. Therefore, ascorbate in the present system can be proposed to act for OH· production with a combination of reaction (2) and reaction (4):

$$2F^{+++} + \text{ascorbate} \rightarrow 2Fe^{++} + \text{dehydroascorbate} \qquad (4)$$

which has already been demonstrated by Halliwell and Foyer (1976).

One thing to be mentioned here is that production of OH· was observed with ascorbate and iron in the absence of added H_2O_2, but involving H_2O_2 as an intermediate. This reaction is likely to be initiated by the autoxidation of ascorbate by iron (Halliwell and Foyer, 1976). The resulting H_2O_2 can react with this metal ion to form OH·. Accordingly, the effect of ascorbate in the present system may be due to a simple addition of this mechanism to reaction (2) rather than through reactions (2) and (4). In fact, degradation hyaluronic acid and ethylene production were observed with Fe^{++} and ascorbate (Fig. 1, 2 and Table 2). But this mechanism is not likely since these effects were much smaller than those stimulated by ascorbate in the presence of Fe^{++} and H_2O_2. In other words, the OH· production in the presence of the three components was higher than the sum of productions expected from individual reactions of either ascorbate-Fe^{++} or Fe^{++}-H_2O_2. Therefore, the above conclusion is favored which suggests that ascorbate substitutes O_2^-· as a reducing agent in reaction (3) and increases the concentration of Fe^{++} to catalyze reaction (2) to form OH·.

It has been reported that among the several reducing agents in the biological system, only ascorbate was capable of enhancing OH· production in the reaction of Fe^{++} and H_2O_2 (Winterbourn, 1979), implying that the substitution of O_2^-· in the reaction (3) seems to be unique to ascorbate. But in the present study, all the reducing agents (Fig. 5 and 6) tested but not their oxidized forms (Fig. 7) showed stimulatory effects on both degradation of hyaluronic acid and ethylene production, and further they were similar to ascorbate in the degree of stimulation (Fig. 5,6 and Table 3). The results suggest a possibility that substances in the body with reducing ability can replace the action of O_2^-· to enhance OH· production through reactions (2) and (4).

The reaction of O_2^-· with Fe^{++} and H_2O_2 may be an important source of OH· in biological system, and the destruction of tissue components by OH· generated though the reaction has already been accepted as one of the mechanisms to explain the oxygen-induced tissue damage (Fridovich, 1978). *In vitro* evidence for this mechanism includes degradation of collagen and hyaluronic acid by xanthine/xan-

thine oxidase system and activated neutrophils (McCord, 1974; Greenwald and Moy, 1979; 1980), alloxan-induced damage of islet cells of pancreas (Fischer and Hamburger, 1980), microsomal lipid peroxidation due to drug metabolism by cytochrome p-450 (Paick *et al.*, 1985) and membrane damage and enzyme incactivation by $O_2^-\cdot$ generated from mitochondria (Roh *et al.*, 1985). Free iron concentration of body fluid was found to be in micromolor range sufficient to promote the OH· fromation (Gutteridge *et al.*, 1981) and the role of Fe^{++} as a catalyst was also proved in the peroxidative process *in vivo* (Rowley *et al.*, 1984), possibly through this mechanism. Thus, the *in vivo* feasibility of the superoxide-dependent formation of OH· in the presence of Fe^{++} has been supported (Halliwell, 1982).

In the present study, Fe^{++} was also most potent in catalyzing the reaction of ascorbate and H_2O_2 to produce OH·. Most of the transition metal ions tested showed about half of the catalytic activity as Fe^{++} (Table 4 and 5). However, in the presence of iron which is an important biological catalyst, the results of this study support that other reducing agents such as ascorbate rather than $O_2^-\cdot$ can participate in the production of OH·. In other words, $O_2^-\cdot$-dependent iron-catalyzed reaction may not be necessarily the only biologically significant mechanism of OH^x production or its toxicity.

A characteristic symptom in many types of arthritis is deterioraion of synovial fluid attributable to depolymerization of hyaluronic acid. It has been suggested that the depolymerization of hyaluronic acid is due to attack of OH· generated by $O_2^-\cdot$-dependent reaction of iron and H_2O_2 (Halliwell, 1978a; Greenwald and Moy, 1980). Release of lysosomal hyaluronidase by invading neutrophils has not been a satisfactory explanation for the depolymerzation of synovial fluid hyaluronidate, since lysosomal hyaluronidase is totally inactive above pH 4.5 (Aronson and Davidson, 1967). The most likely physiological source of $O_2^-\cdot$ in the joint fluid appears to be metabolically activated neutrophils infiltrating inflamed sites (Fantone and Ward, 1982). At the same time, H_2O_2 can also be provided since its production always accompanies $O_2^-\cdot$ production as a product of the dismutation of $O_2^-\cdot$, spontaneously or catalytically by superoxide dismutase (Fantone and Ward, 1982; Klebanoff, 1980).

However, the present results and other reports suggest that the degradation of hyaluronic acid will be mediated by OH· largely generated through the ascorbate-dependent mechanism. When a source of $O_2^-\cdot$ and physiological concentration of ascorbate are available in the medium provided with Fe^{++} and H_2O_2, the reaction involving ascorbate predominates, and there is even inhibition of the $O_2^-\cdot$-dependent pathway (Winterbourn, 1979). This may be supported by the finding that ascorbate can not only do reaction (4) but also react with $O_2^-\cdot$ (Halliwell and Foyer, 1976). In biological fluids such as human plasama to which Fe^{++} is added, OH· production is also primarily ascorbate-dependent(Winterborun, 1981). With the widespread distribution of ascorbate and iron, this may well be the case in most biological fluids, and particularly in the synovial fluid of inflamed joints where high concentration of H_2O_2 is expected.

The concentration of ascorbate in synovial fluid is comparable to those of serum ranging 40 to 140 μM (Koch *et al.*, 1980). During inflammatory diseases including rheumatoid arthritis, the iron content of synovial fluid rises sharply (Sorenson, 1978) and there is actual deposition of iron complexes in the synovial membranes (Ogilvie-Harris and Fornaiser, 1980). At the same time, the infiltration of neutrophils at inflamed joint can cause a localized high concentration of H_2O_2. Takeshige *et al.* (1979) have estimated that when neutrophils were activated, about 22 nmoles of H_2O_2 could be generated per minute per 10^7 cells. Further, synovial fluid contained a barely detectable amount of endogenous catalase (50 ng/ml or less) but contain enough endogenous superoxide dismutase (1 μg/ml) (McCord, 1974). This may provide more favorable condition that most of $O_2^-\cdot$ formed from neutrophils can exist as H_2O_2.

Wong *et al.* (1981) observed that even without H_2O_2 added, hyaluronic acid was depolymerized by ascorbate-dependent mechanism but at higher concentrutions of Fe^{++} and ascorbate. In this reaction, H_2O_2 was involved as a product of dismutation of $O_2^-\cdot$ formed from the reaction, $Fe^{++} + O_2 \rightarrow Fe^{+++} + O_2^-\cdot$. With this finding, they suggested a possibility that only the condition of accumulation of iron in joints during rheumatoid arthritis, together with the presence of ascorbate can be a source of OH· for degradation of synovial fluid *in vivo*. But in inflamed sites where H_2O_2 can be supplemented

from neutrophils, $OH\cdot$ production is expected to increase further.

In the present study, hyaluronic acid in the presence of H_2O_2 was really degraded at much lower concentrations of ascorbate and Fe^{++} than what they used. Thus, it is suggested that the ascorbate-dependent mechanism can serve as a major source of $OH\cdot$ for the degradation of synovial fluid *in vivo*. Furthermore, in view of the finding that in addition to ascorbate, other reducing agents can also replace the action of $O_2^-\cdot$ (Fig. 5 and 6), the relative significance of $O_2^-\cdot$-dependent mechanism will be even more decreased.

In this respect, $O_2^-\cdot$ under the condition that Fe^{++} is available may serve only as a sounce of H_2O_2 in the $OH\cdot$ production. What appears more relevant to oxygen radical-induced tissue damage is the involvement of H_2O_2 and thus, efficient removal of H_2O_2 should be more important preventive measure. In fact, the observation of Brage *et al.* (1980) that catalase was most anti-inflammatory agent in the rat of all reactive scavengers tested appears to be the case.

REFERENCES

Aronson NN and Davidson EA: Lysosmal hyaluronidase from rat liver. II. properties. J Biol Chem 242: 441-447, 1967

Beauchamp C and Fridovich I: A mechanism for the production of ethylene from methional: the generation of the hydroxyl radical by xanthine oxidase. J Biol Chem 245: 4641-4646, 1970

Bragt PC, Bansberg JI and Bonta IL: Anti-inflammatory effects of free radical scavengers and antioxidants: further support for pro-inflammatory roles of endogenous hydrogen peroxide and lipid peroxides. Inflammation 4: 289-299, 1980

Cohen G and Cederbaum AI: Chemical evidence for production of hydroxyl radicals druing microsomal electron tranfer. Science 204: 66-68, 1979

Demopoulos HB, Flamm ES, Pietronigro DP and Seligman ML: The free radical pathology and the microcirculation in the major central nervous system disorders. Acta Physiol Scand Suppl 492: 91-119, 1980

Fantone JC and Ward PA: Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am J Path 107:397-418, 1982

Fischer LJ and Hamburger SA: Inhibition of alloxan action in isolated pancreatic islets by superoxide dismutase, catalase and a metal chelator. Diabetes 29: 213-216, 1980

Fridovich I: Superoxide dismutases. Ann Rev Biochem 44: 147-159, 1975

Fridovich I: The biology of oxygen radicals. Science 201: 875-880, 1978

Greenward RA and Moy WW: Inhibition of collagen gelation by the superoxide radical. Arthritis Rheumat 22: 251-259, 1979

Greenward RA and Moy WW: Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheumat 23: 455-463, 1980

Gutteridge JMC, Rowley DA and Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: detection of free iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J 199: 263-265, 1981

Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: its role in degradation of hyaluronic acid by a superoxide-generation system. FEBS Lett 96: 238-242, 1978a

Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: is it a mechanism for hydroxyl radical production in biochemical systems? FEBS Lett 92: 321-326, 1978b

Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts is a feasible source of hydroxyl radicals *in vivo*. Biochem J 205: 461-462, 1982

Halliwell B and Foyer CH: Ascorbic acid, metal ions and the superoxide radical. Biochem J 155: 697-700, 1976

Kellogg III EW and Fridovich I: Liposome oxidation and erythrocyte lysis by enzymatically generated superox-

ide and hydrogen peroxide. J Biol Chem 252: 6721-6728, 1977
Klebanoff SJ: Oxygen metabolism and the toxic properties of phagocytes. Ann Int Med 93: 480-489, 1980
Klebanoff SJ and Rosen H: Ethylene formation by polymorphonuclear leukocytes: role of myeloperoxidese. J Exp Med 148: 490-506, 1978
Koch P, Sidloi M and Tonks DB: Estimation of serum ascorbic acid in patients and the effect of ascorbic acid and its oxidation products on SMA 12/60 parameters. Clin Biochem 13: 73-77, 1980
Leibovitz BE and Siegel BV: Aspects of free radical reactions in biololgical systems. J Gerontol 35: 45-56, 1980
Matsumura G and Pigman W: Catalytic role of copper and iron ions in the depolymerization of hyaluronic acid by ascorbate. Arch Biochem Biophys 110: 526-533, 1965
McCord JM: Free radical and inflammation: protection of synovial fluid by superoxide dismutase. Science 185: 529-531, 1974
McCord JM and Day ED: Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett 86: 139-142, 1978
Ogilvie-Harris DJ and Fornaiser VL: Synovial iron deposition in osteoarthritis and rheumatoid arthritis. J Rheumatol 7: 30-36, 1980
Paick JS, Kim SW, Chung MH and Kim MS: Effects of nitrofurantoin on lipid peroxidation and reactive oxygen radical generatrion in porcine lung microsome. Korean J Pharmacol 21: 34-47, 1985
Pryor WA and Tang RH: Ethylene formation from methional. Biochem Biophys Res Comm 81: 498-503, 1978
Rehncrona S, Smith DS, Akesson B, Westerberg E and Siesjo BK: Peroxidative changes in brain cortical fatty acids and phospholipids, as characterized during Fe^{++} and ascorbic acid-stimulated lipid peroxidation in vitro. J Neurochem 34: 1630-1638, 1980
Repine JE, Fox RB and Berger EM: Hydrogen peroxide kills strphylococcus aureus by reacting with staphylococcal iron to form hydorxyl radical. J Biol Chem 256: 7904-7906, 1981
Roh JK, Pyo JG, Chung MH, Lim JK and Myung HJ: Generation of superoxide radical from rat brain mitochondria and mechanism of its toxic action to mitochondrial and extramitochondrial components. Korean J Pharmacol 21: 12-26, 1985
Rowely D, Gutteridge JMC, Blake D, Farr M and Halliwell B: Lipid peroxidation in theumatoid arthritis: thiobarbituric acid reactive material and catalytic iron salts in synovial fluid from rheumatoid patients. Clinical Science 66: 691-695, 1984
Schaeffer A, Komlos M and Seregi A: Lipid peroxidation as the cause of the ascorbic acid-induced decrease of ATPase activities of rat brain microsomes and its inhibition by biogenic amines and psychotropic drugs. Biochem Pharmacol 24: 1781-1786, 1975
Sorensen JRJ: An evaluation of altered copper, iron, magnesium, manganese and zinc concentrations in rheumatoid arthirtis. Inorg Perspect Biol Med 2: 1-26, 1978
Takeshige K, Matsumoto T, Shibata R and Minakami S: Simple and rapid method for the diagnosis of chronic granulomatous disease, measuring hydrogen peroxide and superoxide anions released from leukocytes in whole blood. Clinica Chim Acta 92: 329-335, 1979
Taylor AE: Oxygen radical and microcirculation: The Physiologist 26: 151-181, 1983
Turner GS: Inactivation of vaccinia virus by ascorbic acid. J Gen Microbiol 35: 75-80, 1964
Weiss SJ, King GW and LoBuglio AF: Evidence for hydroxyl racdical generatron by human monocytes. J Clin Invest 60: 370-373, 1977
Weissmann G, Smolen JE and Korchak HM: Release of inflammatory mediators from stimulated neutrophils. New Engl J Med 303: 27-34, 1980
Winterbourn CC: Comparison of superoxide with other reducing agents in the biological production of hydroxyl radicals. Biochem J 182: 625-628, 1979
Winterbourn CC: Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate and superoxide. Biochem J 198: 125-131, 1981
Wong SF, Halliwell B, Richmond R and Skowroneck WR: The role of superoxide and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and ascorbic acid. J Inorganic Biochemistry 14: 127-134, 1981

국문초록=

Fe^{++} 및 H_2O_2에 의한 hyaluronic acid 분해에 있어서 ascorbic acid의 역할

서울대학교 의과대학 약리학교실
인제의과대학 약리학 교실*
이정수, 정명희, 임정규, 박찬웅, 차인준*

Hydroxyl raical(OH·)을 생성하는 것으로 알려진 iron-catalyzed Haber-Weiss recation에서 superoxide anion(O_2^-·)은 주로 Fe^{+++}을 Fe^{++}로 환원시키는 데에 작용하는 것으로 추정하고 있다. 이러한 O_2^-·의 역할은 다른 환원제들에 의하여 대체가 가능할 것으로 추측되며 생물계의 환원제의 하나로써 ascorbate가 관심의 대상이 되고 있다. 이에 따라 본 연구에서는 Fe^{++}와 H_2O_2존재하에서 OH· 을 생성하는 ascorbate의 역할을 hyaluronic acid의 변성과 methional로 부터 ethylene 생성에 대한 효과로써 관찰하였다.

Ascorbate는 Fe^{++}와 H_2O_2에 의한 hyaluronic acid의 변성을 촉진하였으며, 이런 현상은 점성도 변화와 Sepharose 4B를 이용한 코로마토그라피에 의하여 확인할 수 있었다. 이때 관찰되는 변성은 catalase와 OH· scavenger에 의하여 거의 완전히 억제되었다. 또한 ascorbate는 Fe^{++}와 H_2O_2에 의한 methional로 부터 ethylene생성을 항진시킴으로써 상기의 결과를 뒷받침 하였다.

다른 환원제들(cysteine, glutathione, NADH와 NADH와 NAKPH)도 ascorbate와 같이 hyaluronic acid의 변성과 methional로 부터 ethylene생성을 촉진하였으나, 그들의 산화형인 NAD와 NADP의 효과는 관찰 할 수 없었다. 그러므로 OH· 생성에 있어 철이온의 환원이 관여함을 시사하였다.

또한 metal ion가운데 Fe^{++}는 OH· 생성에 가장 강력한 촉매작용을 나타내었다.

이상의 결과는 ascorbate가 OH· 을 생성하는 metal-catalyzed reaction에서 Fe^{+++}을 Fe^{++}로 환원하는 O_2^-·의 작용을 대신할 수 있음을 증명하며 이와같은 ascorbate 의존적인 OH· 의 생성은 ascorbate가 조직손상에 관여할 가능성을 시사하였다.