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Studies on curcumin and curcuminoids. XIV. Effect of curcumin on hyaluronic acid degradation in vitro

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Summary

The action of curcumin on the depolymerization of hyaluronic acid in vitro was investigated. Curcumin was shown to have a catalytic effect on the depolymerization process. Curcumin did not act as a substrate for xanthine oxydase, and it had no influence on the activity of the enzyme under the given conditions. The catalytic effect of curcumin was inhibited by addition of mannitol, a hydroxyl radical quencher. This indicates that curcumin affects the formation of the reactive hydroxyl radical in the depolymerization process.

Introduction

Hyaluronic acid (HA) is the major macromolecular constituent of synovial fluid, and it accounts for the viscosity of that tissue. It has long been recognized that inflammatory arthritis is accompanied by a loss of viscosity of the synovial fluid (Greenwald, 1986; Greenwald and Moy, 1980). This effect is mainly ascribed to depolymerization of hyaluronic acid. There is, however, no evidence of the existence of the hyaluronidase enzyme in affected joint fluids (Greenwald, 1986). Substantial evidence suggests that oxygen radicals play an important role in the synovial fluid depolymerization (Halliwell, 1982; McCord, 1974; Edwards et al., 1984; Puig-Parellada and Planas, 1978; Greenwald, 1981; Williamson, 1987; Brooks and Day, 1985). During inflammation, a massive invasion of granulocytes and macrophages occurs at the affected site. Phagocytosis is initiated, and a characteristic respiratory burst in which large quantities of oxygen are consumed is started (Williamson, 1987). During phagocytosis the cytotoxic superoxide anion (O_2^-) is produced (Bragt et al., 1980). In vitro experiments have shown that superoxide can react with other compounds to produce the extremely reactive hydroxyl radical ($OH^\cdot$). Formation of hydroxyl radicals is also accounted for in vivo (Halliwell and Gutteridge, 1985; Ingraham and Meyer, 1985).

Hydrogen peroxide is released along with superoxide during phagocytosis. In the presence of trace amounts of free iron salts, superoxide and hydrogen peroxide can react together to produce the hydroxyl radical. The reaction can be expressed as follows:

$$Fe^{2+} + H_2O_2 \rightarrow OH^\cdot + Fe^{3+} + OH^-$$

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This reaction is referred to as the Fenton reaction. The iron II required for the reaction is thought to be derived from the more stable form III of the iron by a reductive process involving the superoxide radical:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

The net result is referred to as the iron catalysed Haber–Weiss reaction:

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^{\cdot} + OH^-$$

Formation of hydrogen peroxide is not only caused by phagocytosis. In aqueous solution, superoxide undergoes the so-called dismutation reaction which may be represented as:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

From this follows that any system generating superoxide must be producing hydrogen peroxide (Halliwell and Gutteridge, 1985). These equations are probably a gross oversimplification of the in vivo mechanism (Halliwell and Gutteridge, 1985), but indicate that the toxicity of superoxide and hydrogen peroxide involve their conversion to the hydroxyl radical, a reaction which in turn will depend on the amount of catalytic iron available. Once generated, hydroxyl radicals will react with the molecules in their immediate surroundings, causing toxic effects.

In vitro results indicate that both superoxide and the hydroxyl radical are capable to reduce the viscosity of hyaluronic acid solutions (Greenwald and Moy, 1980; McCord, 1974). Further studies have shown that anti-inflammatory drugs might act by the prevention of the synovial fluid degradation due to their action on free radicals (Puig-Parellada and Planas, 1978). Hyaluronic acid degradation studies might therefore be useful in the evaluation of the mechanism of action for anti-inflammatory drugs. In this study, the method has been applied in evaluation of the possible anti-inflammatory effect of curcumin. Curcumin is the main colouring principle present in the rhizomes of the perennial plant *Curcuma longa* L., native to South East Asia. Extracts of the plant are reported to have an anti-inflammatory effect including an effect on inflammatory arthritis. The effect is ascribed to the major constituent curcumin (Arora et al., 1971; Deodhar et al., 1980; Ghatak and Basu, 1972; Mukhopadhyay et al., 1982; Rao et al., 1982; Satoskar et al., 1986; Sharma and Chandra, 1987; Srimal and Dhawan, 1973; Yegnanarayan et al., 1976). Previous studies have shown that curcumin inhibits testicular hyaluronidases (Kushwah et al., 1978), but the action on free radicals has not yet been demonstrated.

Materials and Methods

Materials

Pure, crystalline curcumin was synthesized after the method of Pabon (Pabon, 1964). Hyaluronic acid from human umbilical cord, xanthine oxidase grade I from buttermilk, hypoxanthine and mannitol were provided by Sigma. Acetyl salicylic acid (ASA) was provided by Norsk Medisinaldepot, Norway.

In all the experiments a 0.05 M phosphoric acid buffer, pH = 7.3 containing 0.2 mM EDTA was used as solvent. Addition of iron salt was required under the given conditions. A stock solution of 4 mM ferric chloride in water (not in phosphate buffer where a precipitate will form) was used, from which 10 μl is added to the final 3.0 ml reaction mixture. This gives a final concentration of 20 μM ferric ion.

As superoxide source was used the hypoxanthine/xanthine oxidase system (Greenwald, 1986). Xanthine oxidase (XO) (25 UN/ml) was diluted with buffer to a final concentration of 1 UN/ml. Hypoxanthine was dissolved in buffer to give a final concentration of 5 mM.

Solutions of hyaluronic acid were obtained by adding 18 mg hyaluronic acid to a final volume of 15 ml buffer followed by gentle tumbling of the solution for 30 min at ambient temperature. 1.6 ml stock solution were added to the final 3.0 ml reaction mixture. A specific viscosity of 1.5–2 will then be obtained.

Curcumin is slightly soluble in water at pH = 7.3. A saturated solution of curcumin in buffer

was used in these experiments. 0.5 mg curcumin was suspended in 10 ml buffer followed by gentle tumbling for 1 h in a dark room. After centrifugation of the mixture, 1.2 ml of the supernatant were added to the final 3.0 ml reaction mixture. Organic solvents like acetone and ethanol which are good solvents for curcumin were also tried out. They did, however, interfere with the reaction and could not be used. Mannitol and acetyl salicylic acid were dissolved in buffer (100 μM).

Methods

The effect of oxygen radicals on HA can be followed directly by monitoring the viscosity of the solution as a function of time. 3.0 ml plain buffer are added to the viscosimeter, and the flow time is measured. The instrument is rinsed with 50 ml of distilled water, 50 ml of ethanol and 20 ml of acetone and then dried by vacuum followed by a hot air stream. Traces of organic solvent will strongly influence the results, but by following the mentioned procedure a clean and dry instrument can be obtained within a few minutes.

In a test tube 1.6 ml hyaluronic acid solution were mixed with 1.2 ml buffer containing the additives (test substances or inhibitors) of interest. The final volume was made 3.0 ml by addition of buffer. 10 μl of iron chloride solution and 40 μl of xanthine oxidase were added. No reaction will occur as no hypoxanthine is present. The solution was transferred to the viscosimeter and the flow time was measured. The measured flow time is the zero time value used for the calculation of specific viscosity reduction. The test tube preparation was then repeated, but now the 0.2 ml plain buffer were replaced with the same volume of the hypoxanthine solution. The reaction was initiated by addition of 40 μl XO. The solution was transferred to the viscosimeter, and the initial flow time was measured after 1.5 min (elapsed time from the addition of XO) and then at $t = 15$, 30, 45 and 60 min.

A Cannon-Ubbelodhe semimicro viscosimeter was used; as viscosity is sensitive to temperature, the viscosimeter was placed in a water bath at constant temperature (25 °C) and protected from light.

Specific viscosity is a measure of the fractional change in solution viscosity attributable to the solute. Specific viscosity is given as the relative viscosity (η_{rel}) − 1, where the relative viscosity is the ratio of the flow time of the solution to the flow time of the solvent. To evaluate the results, per cent reduction in specific viscosity was calculated.

The influence of curcumin on the activity of xanthine oxidase was measured by monitoring the conversion of hypoxanthine to uric acid by UV spectroscopy (Halliwell and Gutteridge, 1985). The molar absorbance of uric acid is 1.22×10^4 cm^{-1} at 290 nm.

Results and Discussion

In contradiction to what is given in the literature for other non-steriodal anti-inflammatory drugs, curcumin shows a catalytic effect on the

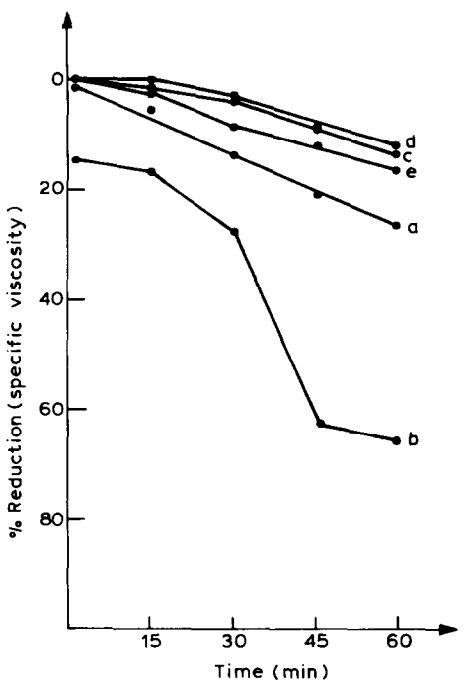

Fig. 1. Depolymerization of hyaluronic acid (HA) in the hypoxanthine/xanthine oxidase system demonstrated as a reduction of specific viscosity as a function of time. A: HA solution. B: HA + curcumin. C: HA + mannitol. D: HA + mannitol + curcumin. E: HA + acetyl salicylic acid.

depolymerization of hyaluronic acid under the given conditions. The results are given in Fig. 1. The formation of reactive oxygen radicals causing depolymerization of hyaluronic acid possibly consists of two steps; the enzymatic generation of superoxide by the hypoxanthine/xanthine oxidase system followed by dismutation of superoxide and generation of hydrogen peroxide, and generation of the hydroxyl radical by the iron catalysed Haber–Weiss reaction. Curcumin can theoretically act in one or both of these steps. To investigate the direct influence of curcumin on the enzyme activity, conversion of hypoxanthine to uric acid was measured spectrophotometrically at 290 nm without and in the presence of curcumin. An increase in absorbance of 0.001 units/10 s were observed over a period of 1 h independent of the addition of curcumin. This indicates that curcumin has no influence on the activity of xanthine oxidase. The absorption spectrum of the solution in the wavelength range of 300–500 nm remained constant throughout the reaction. The curcumin concentration was apparently reduced with less than 10% according to these spectra.

By replacing hypoxanthine with curcumin in the hyaluronic acid degradation test it was further demonstrated that curcumin is not a substrate for XO. With curcumin as substrate, no decrease in viscosity was observed over a period of 1 h.

These results indicate that the catalytic effect of curcumin on hyaluronic acid depolymerization can not be ascribed to a direct effect on the first step of oxygen radical formation in this process. This was further underlined by addition of mannitol to the reaction mixture. Mannitol is a quencher of hydroxyl radicals (McCord, 1974). In the presence of mannitol, only a minor change in viscosity was observed independent of the addition of curcumin (Fig. 1, lines C, D). This change is probably caused by the presence of remaining superoxide in the solution.

The catalytic effect of curcumin on hyaluronic acid depolymerization was compared to the effect of ASA in the same system. ASA is a drug commonly used in the treatment of inflammation. ASA is shown to have no inhibitory effect on synovial fluid degradation, and a different mechanism of action is postulated (Puig-Parellada and Planas, 1978). It is further shown that the chemically related compound salicylic acid can generate oxygen radicals in aqueous media (Williamson, 1987). However, in this test system, ASA showed a marked inhibitory effect on hyaluronic acid depolymerization. The mechanism of action of curcumin is therefore probably not related to the postulated alternative mechanism of ASA.

To explain the results obtained in these experiments it can be postulated that curcumin has an influence on the second step of oxygen radical formation in the depolymerization process; by influencing the Haber–Weiss reaction or by acting as a source of hydroxyl radicals. This is in agreement with previous studies which show that curcumin also has a catalytic effect on the peroxidation of linoleic acid by lipoxygenase (Tønnesen, 1989). Further investigations on curcumin as a potential source of oxygen radicals will be made. The observed anti-inflammatory effect of curcumin suggests that this compound has a different mechanism of action than the other NSAIDs tested.

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