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Effects of Harmaline and Harmalol on the Oxidative Injuries of Hyaluronic Acid, Lipid and Collagen by Fe^{2+} and H_2O_2

In-Sung Cho, Yong-Kyoo Shin and Chung-Soo Lee*

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

ABSTRACT

β-Carboline alkaloids including harmaline have been shown to inhibit enzymatically or nonenzymatically induced-lipid peroxidation of microsomes. This study was done to explore the antioxidant ability of harmaline and harmalol on the oxidative injuries of hyaluronic acid, lipid and collagen by Fe^{2+} and H_2O_2. Their scavenging actions on reactive oxygen species were also examined.

Harmaline, harmalol, superoxide dismutase, catalase and DMSO inhibited both degradation of hyaluronic acid by Fe^{2+} and H_2O_2 and lipid peroxidation of microsomes by Fe^{2+}. In these reactions, DABCO inhibited degradation of hyaluronic acid but did not affect lipid peroxidation. β-Carbolines inhibited degradation of cartilage collagen by Fe^{2+}, H_2O_2 and ascorbic acid. The reduction of ferricytochrome c due to autoxidation of Fe^{2+}, which is inhibited by superoxide dismutase, was not affected by harmaline and harmalol. They also did not have a decomposing action on H_2O_2. Hydroxyl radical production in the presence of Fe^{2+} and H_2O_2 was inhibited by harmaline, harmalol and DMSO.

Harmaline and harmalol may inhibit the oxidative injuries of hyaluronic acid, lipid and cartilage collagen by Fe^{2+} and H_2O_2 through their scavenging actions on reactive oxygen species, OH· and probably iron-oxygen complexes and exert antioxidant abilities.

Key Words: Harmaline, Harmalol, Antioxidant action

INTRODUCTION

Oxygen free radicals and other oxygen-derived species including lipid peroxides have been suggested to play an important role in the pathogenesis of certain human diseases, such as rheumatoid arthritis (Merry *et al.*, 1989), atherosclerosis (Witztum and Steinberg, 1991), postischemic tissue injury (Downey, 1990), and diabetes (Wolff *et al.*, 1991). Reactive oxygen species oxidize protein (Gutteridge and Wilkins, 1983) and unsaturated fatty acids (Gutteridge *et al.*, 1982), damage deoxyribonucleic acid (Jeon *et al.*, 1986) and depolymerize hyaluronic acid (Lee *et al.*, 1985).

Neutrophils are implicated in the injury of tissue components in inflammatory diseases, rheumatoid arthritis and ulcerative colitis (Malech and Gallin, 1987). Many neutrophils are infiltrated at synovial fluid in rheumatoid arthritis. Neutrophils discharge reactive oxygen species and lysosomal enzymes when stimulated by particulate and soluble stimulating substances (Fantone and Ward, 1982). Stimulation of

the respiratory burst produces $O_2^{\cdot-}$, H_2O_2 and probably OH. In addition, contents of metal ions, iron and copper, are enhanced (Sorensen, 1978). Iron is thought to catalyze effectively the oxidative tissue injury, and the oxidoreduction of iron plays a central role in tissue destruction (Halliwell and Gutteridge, 1989b). The autoxidation of iron appears to produce reactive oxygen metabolites, such as OH•, perferryl ion ($Fe^{3+}-O_2^{\cdot-}$, $Fe^{2+}-O_2$) and ferryl ion ($FeOH^{3+}$, FeO^{2+}). Iron-oxygen complex has also been proposed as the causative agent for the oxidative tissue damage (Minotti and Aust, 1987). Thus, in the inflamed sites, iron may promote degradation of joint components caused by the oxidants liberated from activated neutrophils.

β-Carboline alkaloids have been shown to have multiple pharmacological actions, such as monoamine oxidase inhibition (Fuller *et al.*, 1986), convulsive or anticonvulsive action (Loew *et al.*, 1985) and anxiolytic effect (Barbaccia *et al.*, 1986). They are found in mammalian brain, liver, platelet and plasma (Airaksinen and Kari, 1981). Harmane and related β-carbolines are reported to inhibit enzymatically or nonenzymatically induced-lipid peroxidation of liver microsomes (Tse *et al.*, 1991). β-Carbolines are thought to have antioxidant ability. However, the antioxidant action mechanism of them has not been elucidated. In this study, the antioxidant ability of harmaline and harmalol on the oxidative injuries of hyaluronic acid, lipid and collagen by Fe^{2+} and H_2O_2 was investigated. Their scavenging actions on reactive oxygen intermediates were also examined.

MATERIALS AND METHODS

Harmaline, harmalol, hyaluronic acid (Grade III from human umbilical cord), tracheal cartilage collagen, superoxide dismutase (from bovine blood, SOD), catalase (from bovine liver), dimethyl sulfoxide (DMSO), 1,4-diazabicyclo (2, 2,2) octane (DABCO), 2-thiobarbituric acid (TBA), ferricytochrome c and 2-α deoxyribose were purchased from Sigma Chemical Co.. $FeSO_4$ was obtained from Kanto Chemical Co., ; H_2O_2 from Junsei Chemical Co., Ltd.. Other chemicals were of analytical reagent grade.

Viscometry

Viscosity of hyaluronic acid was measured using a modified Cannon capillary viscometer. The reaction mixtures contained 1 mg/ml hyaluronic acid, 150 mM KCl, 50 mM KH_2PO_4 buffer, pH 7.5 and other compounds. The viscosity change was measured at 25℃ and expressed as a flow time (sec).

Preparation of rat liver microsomes

The microsomal fraction was prepared from the rat liver according to the method of Appel *et al.* (1981). Male Sprague-Dawley rats weighing about 150 g were used. Animals were killed by decapitation. After bleeding, livers were quickly removed, placed in ice cold buffer I (0.25 M sucrose, 0.02 M Tris-HCl, 0.5 mM EDTA, pH 7.4), cut into small pieces and homogenized in 4 vol. of buffer I using polytron homogenizer (Brinkman, Model PT-20). After removal of cell debris, nuclei and mitochondria by centrifugation for 10 min at 500, 1,000 and 10,000 g, respectively, the microsomal fraction was pelleted by centrifugation at 100,000 g for 60 min. The resultant pellets were suspended in homogenizing buffer II (0.12 M KCl, 0.05 M Tris-HCl, pH 7.4) and centrifuged at 20,000 g for 20 min. The supernatants were centrifuged at 100,000 g for 60 min, and the pellets were resuspended in buffer II. Protein concentration was determined by the method of Lowry *et al.* (1951) and stored at −70℃

Measurement of lipid peroxidation

Lipid peroxidation of microsomes was estimated from measuring malondialdehyde concentration by thiobarbituric acid method. Liver microsomes (0.2 mg protein/ml) were contained in the reacton mixture consisting of 150 mM KCl, 10 μM $FeSO_4$, harmaline (or harmalol) and 50 mM NaH_2PO_4, pH 7.4. Reaction was started by the addition of iron and the final volume was 1.0 ml. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The chromophore was de-

veloped by boiling in a water bath for 10 min. After cooling to the room temperature, the absorbance was measured at 532 nm (Gutteridge, 1981; Gutteridge *et al.*, 1982). The concentration of malondialdehyde was expressed as n mol/mg protein using the molar extinction coefficient of 1.52×10^5/M/cm (Placer *et al.*, 1966).

Assay of iron-induced superoxide generation

Superoxide anion generated from autoxidation of iron was investigated with reduction of ferricytochrome c (Park *et al.*, 1987). Measurement of superoxide generation was done in 2 ml of reaction mixtures containing 75 μM ferricytochrome c, 100 μM $FeSO_4$, 1 mM harmaline (or harmalol), 150 mM KCl and 50 mM NaH_2PO_4, pH 7.4. Reaction was initiated by adding $FeSO_4$, and reduction of ferricytocrome c was read spectrophotometrically at 550 nm.

Assay of H_2O_2 decomposition

The concentration of H_2O_2 was measured by the method of Allen *et al.* (1952). The reaction mixtures contained, in a final volume of 1.0 ml, 120 mM KCl, 0.1 mM H_2O_2, 1 mM harmaline (or harmalol), 10 μM sodium azide, 50 mM Tris-HCl, pH 7.4 and other compounds. After reaction stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml ammonium molybdate) was added to the above mixture, absorbance change was read spectrophotometri-cally at 350 nm.

Assay of the thiobarbituric acid reactivity of 2-α deoxyribose

Amount of hydroxyl radical generated was estimated from the thiobarbituric acid (TBA) reactivity of 2-α deoxyribose (Gutteridge, 1981; Halliwell and Gutteridge, 1981). The reaction mixtures contained 1 mM 2-α deoxyribose, 10 μM iron (II), 0.1 mM H_2O_2, 150 mM KCl, 50 mM NaH_2PO_4 buffer, pH 7.4 and other compounds in a final volume of 1.0 ml. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% TBA in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The reaction mixtures were heated in a boiling water bath for 10 min. After cooling to the room temperature, the reaction mixtures were centrifuged at 3000 rpm for 10 min. The fluorescence was read at the wavelengths of excitation, 532 nm and emission, 553 nm.

Electrophoretic analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Lammeli (1970) on 5% acrylamide slab gels using Mini Protein II (Bio-Rad). SDS-PAGE was carried out at 40 mA for 80 min. The gels were stained with 0.125% and 0.025% Coomasie Brilliant Blue R-250 and destained in 7% and 10% acetic acid. The stained gels were photographed.

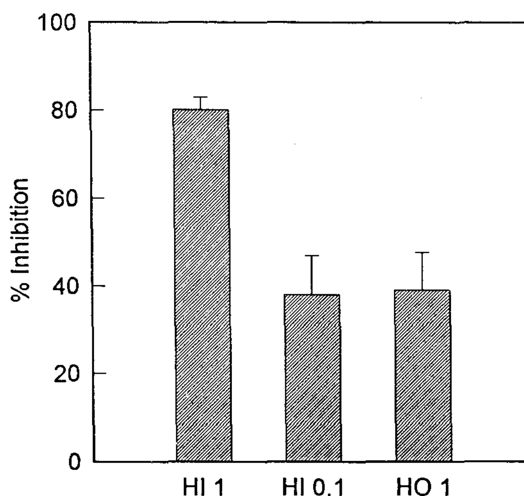

Fig. 1. Inhibitory effects of harmaline and harmalol on degradation of hyaluronic acid by Fe^{2+} and H_2O_2. Hyaluroic acid (1 mg/ml) was incubated with 10 μM $FeSO_4$ and 0.1 mM H_2O_2 in the presence of β-carbolines or not for 1 h. Viscosity of intact hyaluronic acid was 27.8±0.6 sec (S.D.), n = 5. Designated concentrations of Fe^{2+} and H_2O_2 had a decreased viscosity of 10.2±0.6 sec (SD), n=6. Values are expressed as % inhibition and are means ±S.D., n=4~6. HI 0.1, 0.1 mM harmaline; HI 1, 1 mM harmaline; HO 1, 1 mM harmalol.

RESULTS

Inhibitory effects of harmaline and harmalol on degradation of hyaluronic acid by Fe^{2+} and H_2O_2.

Reactive oxygen species appear to be implicated in the tissue damage in various pathologic conditions. Iron is considered to stimulate the oxidative tissue injury. As shown in Fig. 1, viscosity of hyaluronic acid was significantly decreased by 10μM Fe^{2+} and 0.1 mM H_2O_2, and at 60 min of incubation at 25℃, the viscosity change of 10.2±0.8 sec (S.D., n=6) occured. Viscosity of intact hyaluronic acid was 27.8±0.6 sec (S.D.), n=5. The effect of Fe^{2+} plus H_2O_2 was apparently greater than that of Fe^{2+} or H_2O_2 alone (data not shown).

One mM of harmaline and harmalol effectively inhibited degradation of hyaluronic acid by Fe^{2+} and H_2O_2, and the inhibitions of 80.1% and 39.0% were observed, respectively.

To explore which reactive oxygen species are involved in degradation of hyaluronic acid by Fe^{2+} and H_2O_2, effects of scavengers of reactive oxygen species on the degradative effect of Fe^{2+} and H_2O_2 were examined. Ten μM Fe^{2+} and 0.1 mM H_2O_2 induced degradation of hyaluronic acid was inhibited by 10 μg/ml SOD, a scavenger of $O_2^{\cdot-}$, 10 μg/ml catalase, a scavenger of H_2O_2, 1 mM DMSO, a scavenger of OH• and 1 mM DABCO, a quencher of $^1O_2$ (Fig. 2).

Inhibition of Fe^{2+}-induced lipid peroxidation and collagen degradation by harmaline and harmalol

Antioxidant abilities of harmaline and harmalol on lipid peroxidation of microsomes by Fe^{2+} alone were investigated. A 10 μM Fe^{2+}

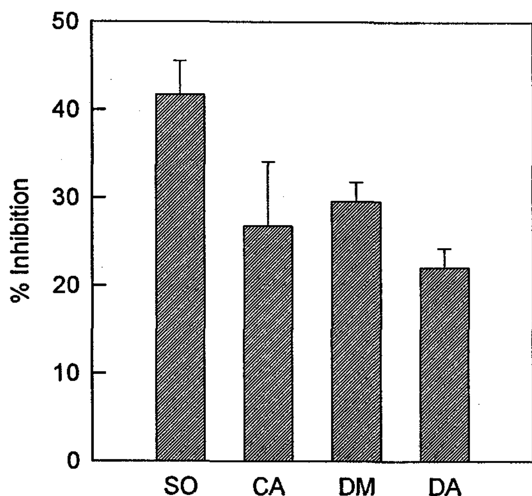

Fig. 2. Inhibition of Fe^{2+} and H_2O_2-induced degradation of hyaluronic acid by scavengers of reactive oxygen species. Hyaluronic acid (1 mg/ml) was incubated with 10 μM $FeSO_4$ and 0.1 mM H_2O_2 *in the presence of* scavengers or not for 1 h. Values are expressed as % inhibition and are mean±S.D., n=3. SO, 10 μg/ml SOD; CA, 10 μg/ml catalase; DM, 1 mM DMSO; DA, 1 mM DABCO

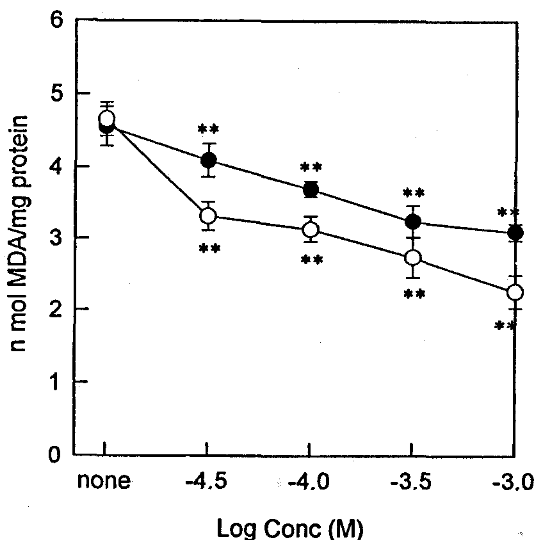

Fig. 3. Inhibitory effects of harmaline and harmalol on Fe^{2+}-induced lipid peroxidation. Liver microsomal fraction(0.2 mg protein/ml) was incubated with 10 μM $FeSO_4$ in the presence of β-carbolines or not for 30 min. Values are means±S.D., n=5. ●, harmaline; ○, harmalol. **p<0.01 by Student's *t*-test.

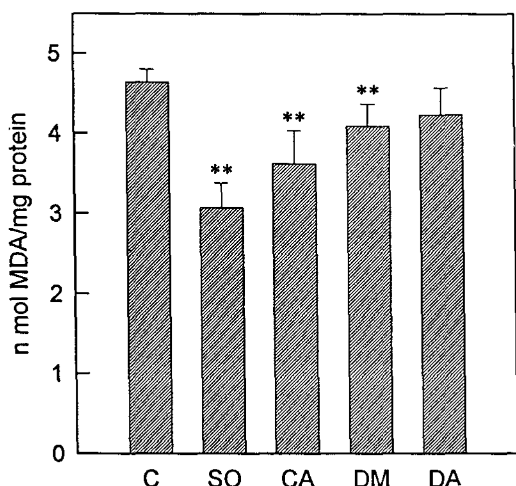

Fig. 4. Effects of scavengers of reacitive oxygen species on Fe^{2+}-induced lipid peroxidation. Liver microsomes(0.2 mg protein/ml) were incubated with 10 μM $FeSO_4$ in the presence of scavengers or not for 30 min. Values are means±S.D., n=5. C, no addition; SO, 30 μg/ml SOD; CA, 30 μg/ml catalase; DM, 10 mM DMSO; DA, 10 mM DABCO. **$p<0.001$ by Student's *t*-test.

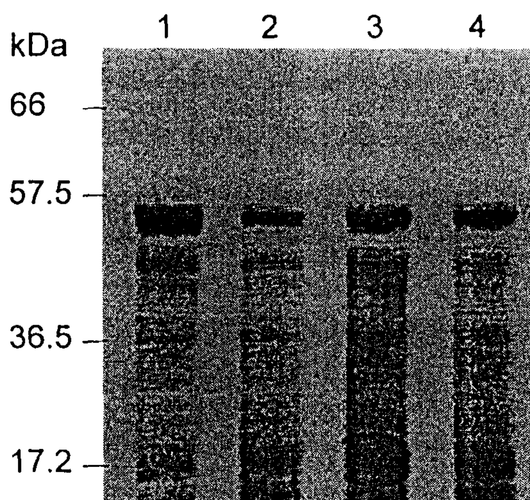

Fig. 5. Inhibition of oxidative degradation of cartilage collagen by carbolines. Tracheal cartilage collagen was incubated with 10 μM $FeSO_4$, 500 μM H_2O_2 and 100 μM ascorbic acid in the presence of carbolines for 6 h at 37℃. lane 1, intact collagen and lane 2, collagen without carbolines; lane 3, collagen+1 mM harmaline; lane 4, collagen+1 mM harmalol with $FeSO_4$, H_2O_2 and ascorbic acid. Reference proteins were bovine serum albumin (Mr, 66,000), catalase (Mr, 57,500), lactic dehydrogenase (Mr, 36.500) and myoglobin (Mr, 17,200).

produced 4.56±0.27 n mol MDA/mg protein (S. D., n=5) at 30 min of incubation. Fig. 3 shows that 10 μM Fe^{2+}-induced lipid peroxidation was inhibited by harmaline and harmalol in a dose dependent fashion. In the presence of 1 mM of harmaline and harmalol, the peroxidative action of Fe^{2+} was inhibited by 32% and 50.4%, respectively.

Involvement of reactive oxygen species in lipid peroxidation induced by Fe^{2+} was examined. As shown in Fig. 4, the peroxidative action of Fe^{2+} was inhibited by 30 μg/ml SOD and 30 μg/ml catalase, while 10 mM DMSO showed a slight inhibitory effect. The effect of 10 mM DABCO was not detected.

Tracheal cartilage collagen was degraded by 10 μM $FeSO_4$, 500 μM H_2O_2 and 100 μM ascorbic acid, and cleavage of a major band (Mr. 53,000) was detected (Fig. 5). The oxidative degradation of cartilage collagen was inhibited by 1 mM harmaline and 1 mM harmalol.

Scavenging effects of harmaline and harmalol on reactive oxygen species

Autoxidation of iron liberates reactive oxygen species, and iron causes formation of OH• and iron-oxygen complexes. Ferricytochrome c was reduced by the addition of 100 μM Fe^{2+}, and the reduction was effectively inhibited by 10 μg/ml SOD (Fig. 6). A 1 mM of harmaline and harmalol had no effect on the reduction of ferricytochrome c by Fe^{2+}.

In biological systems, H_2O_2 has been implicated as a precusor for more reactive oxygen species and can also form complexes with iron. Direct scavenging effects of harmaline and harmalol on H_2O_2 were observed. H_2O_2 was

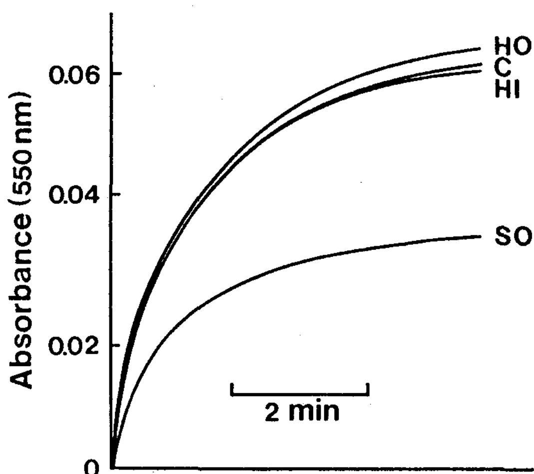

Fig. 6. Effects of harmaline and harmalol on reduction of ferricytochrome c by Fe^{2+}. Reduction of ferricytochrome c was initiated by the addition of 100 μM $FeSO_4$ in the presence of β-carbolines, and absorbance was read at 550 nm. C, no addition; HI, 1 mM harmaline; HO, 1 mM harmalol; SO, 10 μg/ml SOD.

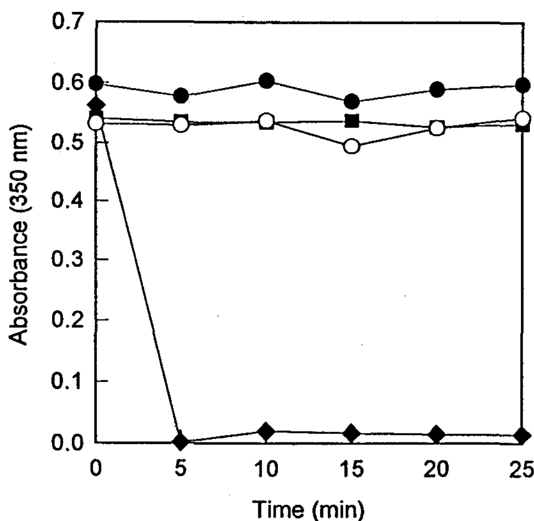

Fig. 7. Effects of harmaline and harmalol on H_2O_2 decomposition. A 100 μM H_2O_2 was incubated with 1 mM harmaline (●), 1 mM harmalol (○) and 10 μg/ml catalase(◆) or not (■) for the designated time, and remaining H_2O_2 was measured as described in Materials and Methods. Values are means, n=3∼4.

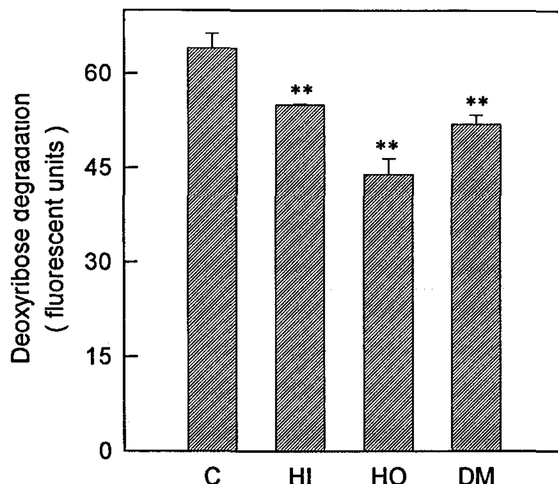

Fig. 8. Effects of harmaline and harmalol on the deoxyribose degradation by Fe^{2+} and H_2O_2. 2-α deoxyribose (1 mM) was incubated with 10 μM $FeSO_4$ and 0.1 mM H_2O_2 in the presence of β-carbolines. C, no addition; HI, 1 mM harmaline; HO, 1 mM harmalol; DM, 10 mM DMSO. Values are relative fluorescent units and are means S.D., n = 5. ** $p < 0.01$ by Student's *t*-test.

completely decomposed by adding 10 μg/ml catalase within 5 min of incubation. Fig. 7 shows that 1 mM of harmaline and harmalol did not have a decomposing action on H_2O_2. In addition, they did not affect degradation of hyaluronic acid by H_2O_2 alone at the same experimental conditions (data not shown). Hydroxyl radical produced was measured with the TBA reactivity of 2-α deoxyribose. As can be seen in Fig. 8, the increased TBA reactivity of 2-α deoxyribose in the presence of 10 μM Fe^{2+} and 0.1 mM H_2O_2 was decreased by 1 mM harmaline, 1 mM harmalol and 10 mM DMSO.

DISCUSSION

Damage of the joint components associated with inflammation is thought to be ascribed partially to reactive oxygen species (Merry *et al.*, 1989; Weiss, 1989). In the inflammatory situa-

tion, reactive oxygen species could be released from neutrophils infiltrating into the inflamed sites (Fantone and Ward, 1982). Besides this phenomenon, the iron content of synovial fluid is elevated in rheumatoid arthritis, and there is actual deposition of iron complexes in the synovial membranes (Sorensen, 1978; Ogilvie-Harris and Fornaiser, 1980). Thus, iron-catalyzed formation of OH• is expected at the inflamed sites. However, since synovial fluid has little antioxidant system, including superoxide dismutase, reactive oxygen species are not detoxified and cause a damage of joint components (McCord, 1974). In particular, hyaluronic acid is depolymerized, and synovial fluid losses its lubricating properties, causing friction in the joint (Kefoed and Barcelo, 1978).

Viscosity of hyaluroic acid was significantly decreased by Fe^{2+} and H_2O_2. Inhibition of the peroxidative action of Fe^{2+} and H_2O_2 by scavengers of reactive oxygen species indicates that $O_2^{\cdot-}$, H_2O_2, OH• and $^1O_2$ may be involved in degradation of hyaluronic acid by Fe^{2+} and H_2O_2. β-Carboline alkaloids including harmane appear to inhibit lipid peroxidation of microsomes induced by either Fe^{3+}ADP and NADPH or Fe^{3+}ADP and dihydroxyfumarate through inhibition of oxidative enzymes in the microsomal system (Tse *et al.*, 1991). However, their effects on the production system of reactive oxygen species have not been elucidated. Harmaline and harmalol significantly inhibited degradation of hyaluronic acid by Fe^{2+} and H_2O_2. The inhibitory effect of harmaline was greater than that of harmalol.

Iron has been shown to initiate lipid peroxidation by forming OH•, perferryl species, ferryl species or $Fe^{2+}/Fe^{3+}/O_2$ complex (Halliwell and Gutteridge, 1989b). Lipid peroxidation of microsomes was induced by Fe^{2+} alone. The peroxidative action of Fe^{2+} on lipid was inhibited by SOD and catalase but was not affected by DABCO, while DMSO had a slight inhibitory effect. OH• and $^1O_2$ are highly reactive species that can damage most types of biomolecules (McCord and Day, 1978). However, the peroxidative action of Fe^{2+} in the presence or absence of reducing agents was slightly inhibited or not affected by scavengers of OH• and $^1O_2$. Thus, involvement of iron-oxygen complex in iron-induced lipid peroxidation is suggested (Minotti and Aust, 1987). On the other hand, OH• may not play an important role in iron-induced lipid peroxidation. Fe^{2+}-induced lipid peroxidation was significantly inhibited by harmaline and harmalol. In contrast to degradation of hyaluronic acid, lipid peroxidation was more effectively inhibited by harmalol than by harmaline. The inhibitory effects of harmalol and harmaline on lipid peroxidation was apparently greater than that of DMSO at the same concentration. These findings indicate that harmaline and harmalol might have scavenging action on iron-oxygen complex. The antioxidant ability of β-carbolines was also detected in the oxidative degradation of structural protein.

Many antioxidants are suggested to protect tissue components against the oxidants by scavenging action on reactive oxygen intermediates and by chelating action on reactive metal ions (Halliwell and Gutteridge, 1989a). In this of view, the mechanism of antioxidant actions of harmaline and harmalol was investigated. Superoxide production due to autoxidation of Fe^{2+} was not affected by harmaline and harmalol. In addition, harmalol did not affect superoxide production by xanthine and xanthine oxidase system (data not shown). Thus, neither of them may have scavenging action on O_2^- and chelating action on iron. H_2O_2 is considered to act as a precusor for highly reactive oxygen species (Kellogg and Fridovich, 1977) and to cause macromolecular damages directly (Lee *et al.*, 1985) or through the formation of complex with metal ions (Halliwell and Gutteridge, 1989b). Harmaline and harmalol did not have a decomposing action on H_2O_2. Highly reactive OH• can be detected sensitively by measuring TBA reactivity of 2-α deoxyribose. Increased TBA reactivity of 2-α deoxyribose in the presence of Fe^{2+} and H_2O_2, which is inhibited by DMSO, was decreased by harmaline and harmalol. They appear to exert antioxidant ability by scavenging action on OH•. Compounds, which have scavenging action on reactive oxygen species, such as OH• and HOCl rather than inhibition of prostaglandin synthesis, are suggested as an effective antiarthritic agents (Halliwell *et al.*, 1988). Harmaline and

harmalol may exert antioxidant ability against the oxidative tissue injury catalyzed by iron in the inflamed sites by scavenging action on reactive oxygen species, OH • and probably iron-oxygen complexes.

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=국문초록=

Fe^{2+}와 H_2O_2에 의한 Hyaluronic Acid, Lipid와 Collagen의 산화성 손상에 나타내는 Harmaline과 Harmalol의 영향

중앙대학교 의과대학 약리학교실

조 인 성·신 용 규·이 정 수*

Harmaline을 포함한 β-Carboline 알카로이드들은 마이크로조움의 효소성 또는 비효소성 지질 과산화를 억제한다고 제시되고 있으나, 이들의 항산화 작용기전은 분명하지 않다. 본 연구에서는 Fe^{2+}와 H_2O_2에 의한 hyaluronic acid, 지질과 콜라겐의 산화성 손상에 있어 harmaline과 harmalol의 항산화 능력을 관찰하였다. 또한 반응성 산소대사물에 대한 이들의 제거작용을 조사하였다.

Harmaline, harmalol, superoxide dismutase, catalase와 DMSO는 Fe^{2+}와 H_2O_2에 의한 hyaluronic acid의 변성과 Fe^{2+}에 의한 지질 과산화를 억제하였다. 이들 반응에서 DABCO는 hyaluronic acid의 변성을 억제하였으나 지질 과산화에 영향을 나타내지 않았다. β-Carboline은 Fe^{2+}, H_2O_2와 ascorbic acid에 의한 cartilage collagen의 변성을 억제하였다. Superoxide dismutase에 의하여 억제되는 Fe^{2+}의 자가산화에 따른 ferricytochrome c의 환원은 harmaline과 harmalol의 영향을 받지 않았다. 또한 이들은 H_2O_2에 대하여 분해작용을 나타내지 않았다. Fe^{2+}와 H_2O_2의 존재하에서 OH· 생성은 harmaline, harmalol과 DMSO에 의하여 억제되었다.

Harmaline과 harmalol은 반응성 산소대사물인 OH·과 아마도 철이온-산소 복합체에 대한 제거작용으로써 Fe^{2+}와 H_2O_2에 의한 hyaluronic acid, 지질과 콜라겐의 산화성 손상을 억제하고, 항산화 능력을 나타낼 것으로 추정된다.