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SYNOVIAL FLUID DEGRADATION INDUCED BY FREE RADICALS. *IN VITRO* ACTION OF SEVERAL FREE RADICAL SCAVENGERS AND ANTI-INFLAMMATORY DRUGS

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Abstract—The action of several free radical scavengers and anti-inflammatory drugs to decrease the viscosity of the synovial fluid induced by the reaction between hypoxanthine and xanthine oxidase is evaluated. It confirms the significance of the hydroxyl and superoxide radicals in the synovial fluid degradation and it shows that, with 1,3 diphenilisobenzofuran, singlet oxygen is generated in the xanthine oxidase reaction and suggests that it plays a role in the synovial fluid deterioration. The nonsteroidal anti-inflammatory drugs (Indometacine > Niflumic Acid > Acetaminophen > Imidazole > Mefenamic Acid > Phenylbutazone) as well as the steroidal ones (Prednisone > Dexamethasone > Triamcinolone Acetonide > Hydrocortisone) at 80 μM show the capacity to inhibit the synovial fluid degradation in the indicated order. Acetylsalicylic Acid is inactive. Paradoxically, Acetaminophen and Imidazole are more active for this system than as anti-inflammatory agents. To conclude, the results presented in this paper indicate that the capacity of anti-inflammatory dugs to inhibit the synovial fluid degradation is due to their ability to absorb or destroy free radicals.

McCord has shown the significance of free radicals for inflammatory and arthritic diseases [1, 2]. In the carrageenan food oedema Oyanagui [3] has demonstrated the participation of superoxide radicals at the prostaglandin phase of inflammation and Sharma *et al.* [4] a significant increase in the output of lipid peroxides in the liver. Otomo and Fujihira [5,6] proved the stabilizing effect of anti-inflammatory drugs on hemolysis and lipid peroxidation induced by hydrogen peroxide u.v. irradiation.

This study presents the observations concerning the action of several free radical scavengers and anti-inflammatory drugs on the decrease of viscosity in the synovial fluid induced by the reaction between hypoxanthine and xanthine oxidase [7, 8].

We tested, as free radical scavengers, DL-α-tocopherol, a singlet oxygen scavenger [9] and lipid antioxidant [10]; mannitol [11, 12] and sodium benzoate [13, 14] as hydroxyl radical scavengers; 1,3-diphenilisobenzofuran as a singlet oxygen scavenger [15–17] and magnesium chloride as a superoxide radical scavenger [18]. The anti-inflammatory drugs studied were either the nonsteroidal ones (Indometacine, Niflumic Acid, Acetaminophen, Phenylbutazone, Mefenamic Acid and Acetylsalicylic Acid) or the steroidal ones (Prednisone, Triamcinolone Acetonide, Dexamethasone, and Hydrocortisone) both of which presented a well defined anti-inflammatory action [19]. The anti-inflammatory action of Imidazole was demonstrated by us [20].

Our results suggest that singlet oxygen, hydroxyl and superoxide radicals play a role in the synovial fluid depolymerization and also indicate that the anti-inflammatory drugs protect the synovial fluid degradation due to their action on free radicals.

MATERIALS AND METHODS

Synovial fluid was obtained in the Barcelona slaughter-house by intraarticular extraction from the hind legs of cattle. The synovial fluid extracted was transferred to an isothermic container. In the laboratory it was centrifuged to 12,000 g for 60 min at 4°. The superantant was stored in glass tubes in the cold room at −15°. When needed it was thawed and centrifuged again for 30 min producing a clear synovial fluid.

The technique was the one used by McCord [2]. After 30 min of enzymatic reaction a specimen was obtained and the relative viscosity tested. This was measured by recording the time in seconds required for a given volume (0.7 ml) of the reaction mixture to drain by gravity from the barrel of a plastic syringe through a needle of appropriate size. The relative viscosity assay was repeated five times per reaction mixture and at least four separate reaction mixtures per drug were tested. From this data we calculated the inhibition induced by the drugs in absolute values (sec) and in percentage. The statistical studies were done calculating the mean of the sample differences ±95 per cent confidence limits and their statistical significance between the appropriate samples [21]. The samples incubations and the viscosity assay were performed at 37°.

The reagents employed were: Hypoxanthine and Xanthine Oxidase grade 1 from Sigma, Imidazole, Magnesium Chloride, Sodium Benzoate and Hydrocortisone from Merck, Acetylsalicylic Acid, *N*-acetyl-*p*-aminophenol (Acetaminophen), EDTA, from Scharlau, DL-α-tocopherol from Calbiochem were dissolved in acetone, 1,3 diphenilisobenzofuran from Fluka

Table 1. Effect of some free radical scavengers on the degradation of bovine synovial fluid

Free radical scavengers	Concentration (μM)	Δ in Relative viscosity (seg)	Inhibition (%)
Control	—	8.28 ± 0.65*†	100
α-Tocopherol	80	6.21 ± 1.36*‡	75.00
Mannitol	80	6.18 ± 1.19*	74.63
Sodium benzoate	80	5.27 ± 1.55*	63.64
1,3 Diphenilisobenzofuran	800	4.71 ± 0.98*	56.88
Magnesium chloride	800	4.35 ± 0.89*	52.53

The synovial fluid degradation was induced by the following reaction mixture: 5 ml of bovine synovial fluid; 0.05 ml EDTA 0.1 M; 0.5 ml hypoxanthine 10 mM; after 30 min preincubation at 37° enzymatic reaction was activated by the addition of xanthine oxidase (0.10 U in 0.05 ml). The free radical scavengers (Table 1) and anti-inflammatory drugs (Table 2) were added at time zero to the amount of 0.05 ml in the concentration indicated in the Tables. The final pH was 7.5.

* $P < 0.001$ of the sample differences.

† Mean of the sample differences ±95 per cent confidence limits between samples of a complete reaction mixture without xanthine oxidase and drug and samples of a complete reaction mixture without drug.

‡ Mean of the sample differences ±95 per cent confidence limits between samples of a complete reaction mixture without drug and samples of a complete reaction mixture.

were prepared as a homogenous suspension in 3% (v/v) *N,N'*-dimethylformamide, Triamcinolone Acetonide from Pierrel, Dexamethasone from Roussel-Uclaf, Mefenamic Acid from Parke-Davis, Mannitol from Carlo Erba, and Indometacine, Niflumic Acid, Phenylbutazone and Prednisone U.S.P. grade.

RESULTS

The results obtained with the different free radical scavengers, are presented in Table 1, which show the concentration necessary to induce an inhibition of more than 50 per cent and the value of this inhibition in absolute values (sec), and in percentages. The two hydroxyl radical scavengers tested (Benzoate and Mannitol) arrested significantly the synovial fluid deterioration at concentrations of 80 μM. The two singlet oxygen scavengers tested showed different responses: DL-α-tocopherol is active at 80 μM and 1,3-diphenilisobenzofuran is active at 800 μM. Magnesium chloride, a superoxide radical scavenger, is active at 800 μM. All the samples tested showed statistically significant differences ($P < 0.001$).

Table 2 shows, the action of different anti-inflammatory drugs in a concentration of 80 μM. At this concentration all the drugs inhibited higher than 50 per cent, except Acetylsalicylic Acid, which is inactive and Hydrocortisone which only inhibited a 38.4 per cent. The decrease in relative viscosity is always significant ($P < 0.001$) for all the anti-inflammatory drugs tested except for acetylsalicylic acid.

There is a strong negative linear correlation between the anti-inflammatory activities (ED_{50} in the rat paw edema) according to Vane [22] and their capacity to inhibit and decrease in viscosity shown in the present paper. The coefficient of correlation for the nonsteroidal anti-inflammatory drugs (except Acetaminophen and Imidazole) is −0.93 ($P < 0.01$) and for the steroidal ones is −0.97 ($P < 0.05$).

It is also shown in Table 2 that at the same concentration the steroidal drugs are not so active as the nonsteroidal ones. The maximum inhibition attainable is 74 per cent for Prednisone, and 66 per cent for Dexamethasone. Neither *N,N'*-dimethylformamide nor acetone used as a solvent showed any significant action on synovial fluid degradation.

Table 2. Effect of some anti-inflammatory drugs on the degradation of bovine synovial fluid

Nonsteroidal anti-inflammatory drugs (80 μM)	Δ in Viscosity (seg)	Inhibition (%)
Control	8.28 ± 0.65*†	100
Indometacine	7.97 ± 1.36*‡	96.25
Niflumic acid	7.81 ± 1.49*	94.32
Acetaminophen	6.09 ± 1.55*	73.55
Imidazole	5.77 ± 1.38*	69.68
Mefenamic acid	5.25 ± 1.00*	63.40
Phenylbutazone	5.11 ± 1.38*	61.71
Acetylsalicylic acid	0.97 ± 0.72	11.71
Steroidal anti-inflammatory drugs (80 μM)		
Prednisone	6.13 ± 1.67*	74.03
Dexamethasone	5.52 ± 1.52*	66.66
Triamcinolone acetonide	4.92 ± 1.48*	59.42
Hydrocortisone	3.18 ± 1.59*	38.40

(See Table 1 for legend).

DISCUSSION

The synovial fluid degradation induced by free radicals generated by the reaction between the hypoxanthine and xanthine oxidase was indicated by a marked reduction in viscosity [2].

The superoxide ion, a reduced and unstable form of oxygen [7, 8], is generated in the reaction mixture containing xanthine oxidase plus hypoxanthine. Hydrogen peroxide is produced in the dismutation reaction in which two molecules of superoxide radical give rise to hydrogen peroxide either spontaneously or enzymatically [23]. The fact that the degradative process was inhibited by either superoxide dismutase or catalase [2] indicates that the actual depolymerizing species is the hydroxyl radical generated secondarily by a reaction between the superoxide radical and hydrogen peroxide [14].

The results presented here are in agreement with the above data showing that the two hydroxyl radical scavengers, Mannitol and Benzoate, arrested the synovial fluid degradation. The capacity to inhibit the synovial fluid deterioration by the singlet oxygen scavengers assayed in this paper confirms that singlet oxygen is generated in the xanthine oxidase reaction [17, 24, 25] and suggests that it plays a role in the synovial fluid degradation. The significance of superoxide radicals was obtained with Magnesium Chloride a known superoxide radical scavenger.

The inactivity of Acetylsalicylic Acid suggests that perhaps this drug has a different mechanism from the other anti-inflammatory drugs tested. On the other hand, Acetylsalicylic Acid was not effective in various other experiments on peroxidative mechanism [26, 5]. Acetaminophen and Imidazole both show an action superior to their own as anti-inflammatory drugs [19, 20].

Our results indicate that the nonsteroidal anti-inflammatory drugs inhibited the synovial fluid degradation due to their action on hydroxyl radicals and singlet oxygen produced in the xanthine oxidase reaction. It has been suggested [6] that the inhibition of the erythrocytes hemolysis induced by anti-inflammatory drugs is due to their capacity to absorb and destroy free radicals or peroxides. Recently Kuehl *et al.* [27] suggested that in the enzymatic oxidation of arachidonic acid a hydroxyl radical is generated which is the major inflammatory agent. The formation of an intermediate product, possibly a hydroperoxide, in the inflammatory and peroxydative processes could explain the action of anti-inflammatory drugs in both systems.

The fact that drugs such as Prednisone or Dexamethasone with high activity as anti-inflammatory drugs show low activity as inhibitors of the synovial fluid degradation suggests that the therapeutic efficiency of the steroidal anti-inflammatory drugs is not related to its action on free radicals.

The clinical value of our results must be based on the fact that deterioration of synovial fluid is a symptom which characterizes inflammatory types of arthritis. On the other hand, the phagocytosing polymorphonuclear leucocytes present in the synovial fluid produce superoxide radicals [28, 29] with attendant generation of hydrogen peroxide and hydroxyl radicals. The above reaction was suggested as the *in vivo* mechanism of synovial fluid degradation in inflamed joints. In addition, the same reagents that prevented the synovial fluid degradation were shown [1] to protect phagocytosing leucocytes from premature death and release of hydrolytic enzymes and chemotactic factors which play a role in perpetuating the inflammatory cycle.

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