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Collagen degradation by superoxide anion in pulse and gamma radiolysis

Jean-Claude Monboisse [a], Monique Gardès-Albert [b], Alain Randoux [a], Jacques-Paul Borel [a] and Christiane Ferradini [b]

[a] *Laboratory of Biochemistry, CNRS UA 610, UFR Medicine, Reims* and [b] *Laboratory of Physical Chemistry, R. Descartes University, Paris (France)*

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Delipidated collagen fibrils reconstituted from acid-soluble calf skin collagen, suspended in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate, were submitted to pulse radiolysis in Febetron devices or to gamma radiolysis in a ^{60}Co irradiator. A collagen degradation process was found. The kinetics of this degradation was followed by evaluation of the amount of 4-hydroxyproline present in the small peptides liberated during the irradiation period. The yield of 4-hydroxyproline small peptides was low (0.1 mol/100 eV for an initial collagen concentration 3.2 μM). It increased linearly with the dose of irradiation and the concentration of collagen in suspension. The kinetic competition between $O_2^{\cdot-}$ dismutation and $O_2^{\cdot-}$ reaction with collagen was studied by pulse radiolysis at several concentrations of collagen. A value of the kinetic constant of $k(O_2^{\cdot-} + \text{collagen}) = 4.8 \cdot 10^6$ mol$^{-1} \cdot$ l $\cdot$ s^{-1} was determined.

Introduction

Extracellular matrix is the main site where inflammatory phenomena develop. Collagen fibrils are destroyed during the degradative stage of inflammation and several authors have suggested that oxygen-derived free radicals may be involved in these degradation processes. Greenwald and Moy [1] showed the susceptibility of acid-soluble collagen to oxy free radicals generated by the xanthine oxidase-hypoxanthine system. Curran et al. [2] demonstrated that soluble collagen is degraded upon exposure to ozone or hydroxyl radicals generated by Fenton's reaction. This treated collagen becomes more susceptible to enzymatic proteolysis with trypsin. The effect of high-dose gamma irradiation on soluble collagen was reported by Davidson and Cooper [3] and by Shieh and Wierbicki [4].

In previous studies, we showed that superoxide anion, $O_2^{\cdot-}$ generated either by the xanthine oxidase-hypoxanthine system or by gamma radiolysis, attacked the helical parts of collagen fibrils reconstituted in vitro and liberated dialyzable 4-hydroxyproline-containing peptides into the incubation medium [5,6]. Radiolysis methods permit the quantitative study of processes involving free radicals. The aim of this work is to quantitate the effect of $O_2^{\cdot-}$ on collagen fibrils by pulse and gamma radiolysis.

Material and Methods

Reagents

All the common reagents (analytical grade) were obtained from Prolabo (Paris, France) or Merck (Darmstadt, F.R.G.). Ethylenediaminetetraacetic acid (EDTA), ferricytochrome *c* (type VI), hypo-

Correspondence: J.-C. Monboisse, Laboratory of Biochemistry, CNRS UA 610, UFR Medicine, 51 rue Cognacq-Jay, 51095 Reims Cedex, France.

xanthine and xanthine oxidase (X 1875) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

7-Nitrobenz-2-oxa-1,3-diazol-4-yl chloride and diethylenetriaminepentaacetic acid (DTPA) were provided by Aldrich Chimie (Strasbourg, France).

Desferrioxamine (Desferal) was bought from Ciba-Geigy (Basel, Switzerland).

Deionized water was filtered through a MilliQ water system (Millipore Corporation, Bedford, MA, U.S.A.) up to a resistivity close to 18 $M\Omega \cdot cm$. The solution used for all the incubations was a 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate.

N_2O and O_2 (N48 grade) were supplied by Air Liquide (Paris, France) and they were more than 99.99% pure.

Preparation of acid-soluble collagen

Acid-soluble collagen was prepared from calf skin according to Piez et al. [7]. Significant amounts of lipids were removed by extraction in a chloroform/methanol mixture (2 : 1, v/v) at 4°C for three 24 h periods.

Delipidated acid-soluble collagen was dissolved in 0.1 M acetic acid to 1.2 g/l and the solution dialyzed against 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate at 4°C for two 24 h periods. The concentration of neutral collagen solution was adjusted to 1 g/l on the basis of its hydroxyproline content. This solution was allowed to settle at 37°C for 30 min in order to make it fibrillate [6] while remaining in suspension. Molar concentrations were calculated on the basis of a molecular weight of 300000 for collagen.

Pulse radiolysis

Pulse radiolysis experiments were performed in a Febetron 708 (R. Descartes University, Paris) delivering electron pulses (800 keV, 4 ns at half-height, optical path 1 cm) and in a Febetron 707 (DPC, CEN Saclay) delivering electron pulses (1.7 MeV, 8 ns at half-height, optical path 2.5 cm).

The content of the cell (0.5 or 1.5 ml depending on the device used) was irradiated by a single pulse. Doses were about 200 Gy in Febetron 700 and varied in the different experiments from 20 to 80 Gy per pulse in Febetron 707. The variations of dose from pulse to pulse were monitored by measuring the total pulse charge with a charge integrating current and the doses were calibrated with KSCN ($\varepsilon_{472,(SCN)_2^-} = 7580\ mol^{-1} \cdot l \cdot cm^{-1}$) [8]. The changes in absorbance were followed at 277 nm ($\varepsilon_{277,O_2^-} = 1050\ mol^{-1} \cdot l \cdot cm^{-1}$ [9].

Under the experimental conditions, the variations of absorbance at 277 nm depended only on the concentration of O_2^- in the cell. The absorbance of the solution before starting the irradiation was taken as the baseline (see Appendix).

Gamma radiolysis of collagen fibril suspension

Gamma radiolysis was performed in a ^{60}Co irradiator with a total activity of 230 Ci and a dose rate equal to $1.6 \cdot 10^{21}\ eV \cdot l^{-1} \cdot h^{-1}$ ($7.1 \cdot 10^{-2}\ Gy \cdot s^{-1}$). Dosimetry was performed by Fricke's method assuming $G_{Fe^{3+}} = 15.6$ mol per 100 eV ($15.6 \cdot 10^{-7}$ mol/J) and $\varepsilon_{Fe^{3+}} = 2160\ M^{-1} \cdot cm^{-1}$ (see Appendix for definition of G). 5 ml volumes of the suspension of collagen were saturated by N_2O or O_2 for 20 min prior to irradiation. Doses varied between 8 and 1200 Gy and irradiation periods from 15 to 270 min. In some experiments, the irradiations were performed in the presence of 0.5 mM DTPA or 0.05 mM desferrioxamine.

Evaluation of collagen fibril degradation

The irradiated suspension of collagen fibrils was centrifuged at $30000 \times g$ for 30 min at 4°C. A 2 ml aliquot of the supernatant was precipitated by ethanol at 80% (v/v) at 4°C, allowed to stand for 24 h and centrifuged at $30000 \times g$ for 30 min at 4°C [6]. The supernatant was evaporated to dryness under nitrogen and hydrolyzed in 6 M HCl at 105°C for 18 h. Proline and hydroxyproline were evaluated by a quantitative fluorometric technique [10]. Controls with non-irradiated collagen were performed simultaneously.

Gamma radiolysis of bovine serum albumin

In a control experiment, bovine serum albumin (1 g/l in the same buffer) was submitted to gamma radiolysis. The supernatant after ethanol precipitation was hydrolyzed by 6 M HCl and its content in amino acids was measured by the ninhydrin reaction [11] and expressed as equivalents of norleucine.

Studies with $O_2^{\cdot-}$ produced by a chemical reaction

The $O_2^{\cdot-}$-scavenging activity of collagen was measured using the reaction of xanthine oxidase on hypoxanthine as an $O_2^{\cdot-}$-generating system. The reaction mixture (2 ml) contained the following reagents: (a) 1 ml of 0.5 mM hypoxanthine solution; (b) 0.1 ml of 1 mM ferricytochrome *c* solution; (c) 0.05 ml of xanthine oxidase solution (final concentration 75 units/l); (d) 0.2 ml of collagen fibril suspension (final concentration 3.2 μM). All reagents were initially prepared in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate and 0.8 mM EDTA. The final volume was adjusted to 2 ml with the same phosphate buffer.

The formation of $O_2^{\cdot-}$ for 2 min was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* monitored at 550 nm in a Beckman DU-40 spectrophotometer at 20 °C, and compared to that measured in the additional presence of 1.5 mM KCN or 5 mM EDTA. $O_2^{\cdot-}$ production was calculated using a ferricytochrome *c* absorption coefficient of $15.5 \cdot 10^{-3}$ $M^{-1} \cdot cm^{-1}$ [12].

Results

Pulse radiolysis

$O_2^{\cdot-}$ decay in the absence of collagen, shown in Fig. 1, permitted the calculation of an apparent kinetic constant of $O_2^{\cdot-}$ dismutation of $9 \cdot 10^5$ $mol^{-1} \cdot l \cdot s^{-1}$.

The decay of $O_2^{\cdot-}$ concentration, when measured in the presence of different collagen concentrations, were plotted versus time as shown in Fig. 2a–c.

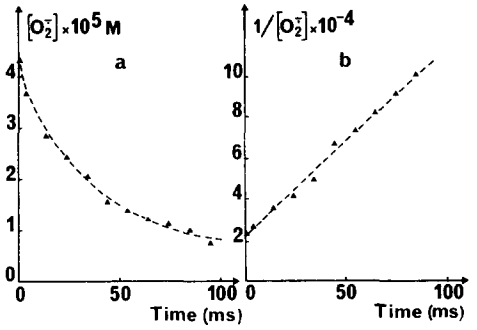

Fig. 1. $O_2^{\cdot-}$ radical decay (a) and second-order kinetic law (b) observed in the absence of collagen. Irradiations by pulse radiolysis in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate, under air. The curve in (b) was calculated from data given in (a).

Kinetic constants were derived from the plots as described in the Discussion and the competition ratio was found to depend on the initial concentrations of collagen and $O_2^{\cdot-}$.

Gamma radiolysis

Fig. 3 shows again that the amount of liberated hydroxyproline increased linearly with the radiation dose as well as, for a given dose, with the collagen concentration. The yield of hydroxyproline (G_{4Hyp}) was 0.1 mol per 100 eV ($0.1 \cdot 10^{-7}$ mol/J) for a collagen concentration of 3.2 μM. At concentrations of collagen of 1.1 and 3.2 μM,

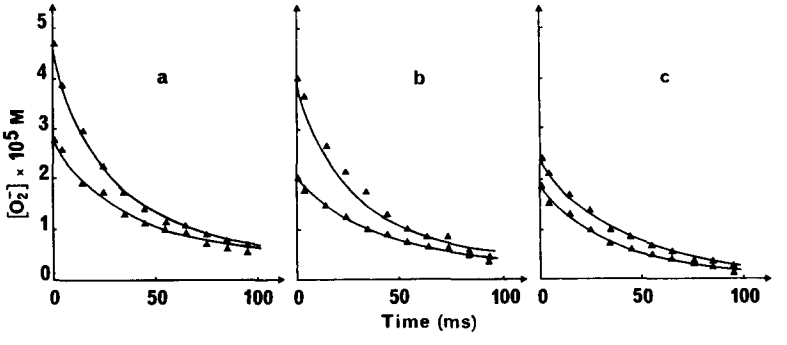

Fig. 2. $O_2^{\cdot-}$ radical decay in the presence of collagen at different concentrations: (a) $0.8 \cdot 10^{-6}$ M, (b) $1.6 \cdot 10^{-6}$ M, (c) $3.2 \cdot 10^{-6}$ M, and with each collagen concentration at two different initial $O_2^{\cdot-}$ concentrations. Irradiations by pulse radiolysis in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate, under air. Dose per pulse ranged between 20 and 80 Gy. ▲ represent experimental data. Curves were traced from calculations according to the theoretical equations (see text).

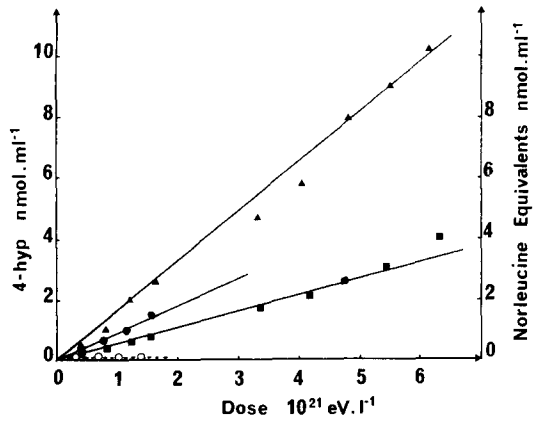

Fig. 3. Dose-dependent effect of gamma radiolysis on collagen breakdown followed by determination of 4-hydroxyproline-containing peptides. Gamma radiolysis incubations were performed in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate, under air. Collagen concentration: ■, 1.1 μM; ●, 2.2 μM; ▲, 3.2 μM. Dose rate: $1.6 \cdot 10^{21}$ eV·l^{-1}·h ($7 \cdot 10^{-2}$ Gy/s). Degradation of bovine serum albumin is expressed as norleucine equivalents (o------o). Each value is the mean of four determinations.

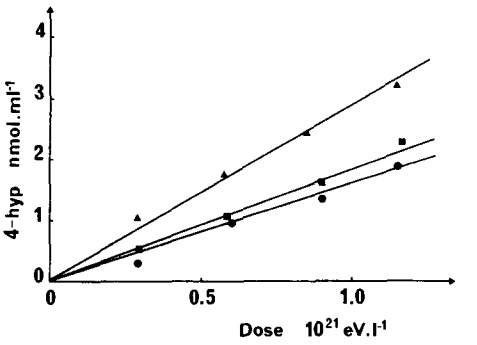

Fig. 4. Effect of DTPA and desferrioxamine on collagen breakdown followed by determination of 4-hydroxyproline-containing peptides. Gamma radiolysis incubations were performed in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate, under air, at a collagen concentration of $3.2 \cdot 10^{-6}$ M. ●, without addition; ▲, with 0.05 mM desferrioxamine; ■, with 0.5 mM DTPA. Dose rate = $1.6 \cdot 10^{21}$ eV·l^{-1}·h ($7 \cdot 10^{-2}$ Gy/s). Each value is the mean of four determinations.

when the irradiation times were extended up to 270 min (dose, $6 \cdot 10^{21}$ eV/l), the liberation of hydroxyproline remained linear with the doses. The rate of collagen breakdown was not different whether the incubations were performed under air or oxygen. Under N_2O (when only formate radicals, $COO^{\cdot-}$, were formed), no 4-hydroxyproline-containing peptides were liberated (data not shown). When the collagen suspensions were incubated for 24 h with 0.2 mM H_2O_2, no 4-hydroxyproline-containing peptides were found in the medium.

When gamma irradiations of 3.2 μM collagen suspension were performed in the presence of superoxide dismutase (50 U/ml), a strong inhibition of the collagen breakdown was observed (0.68 $\pm$ 0.32 vs. 4.28 $\pm$ 0.34 nmol 4-hydroxyproline liberated in the incubation medium, for an irradiation dose of $3.2 \cdot 10^{21}$ eV/l).

In a control experiment, bovine serum albumin was submitted to gamma radiolysis. No degradation products, expressed as norleucine equivalents, were found in the medium (Fig. 3).

Ruling out contamination by metallic cations or superoxide dismutase

Gamma radiolysis was performed in the presence of 0.5 mM DTPA or 0.05 mM desferrioxamine (Fig. 4). In the latter case, an increase of collagen degradation was noted.

The $O_2^{\cdot-}$-scavenging activity of the collagen preparation was evaluated by mixing it with the hypoxanthine-xanthine oxidase system and measuring the Cu,Zn-superoxide dismutase-inhibitable reduction of ferricytochrome *c*. The addition of 3.2 μM collagen decreased the reduction of ferro-

TABLE I

SCAVENGING ACTIVITY OF COLLAGEN ON $O_2^{\cdot-}$ PRODUCED BY THE HYPOXANTHINE-XANTHINE OXIDASE SYSTEM EVALUATED BY THE SUPEROXIDE DISMUTASE-INHIBITABLE REDUCTION OF FERRICYTOCHROME *c*

Incubations were performed in 50 mM phosphate buffer, pH 7.4, containing 100 mM sodium formate and 0.8 mM EDTA, under air and at 20 °C. The superoxide dismutase inhibitable reduction of ferricytochrome *c* was recorded spectrophotometrically at 550 nm. Each result corresponds to the mean $\pm$ S.D. of four determinations.

Incubations	$O_2^{\cdot-}$ production (nmol/2 min)	
	without collagen	with 3.2 μM collagen
Control	16.77 $\pm$ 0.50	13.45 $\pm$ 0.43
+ 1.5 mM KCN	17.71 $\pm$ 0.56	13.16 $\pm$ 0.38 (n.s.) [a]
+ 5 mM EDTA	17.03 $\pm$ 0.44	14.19 $\pm$ 0.35 (n.s.) [a]

[a] n.s. = not significant compared to the control value.

cytochrome *c* by 15% (Table I). The effect was not suppressed by addition of 5 mM EDTA or 1.5 mM KCN.

Discussion

The above experiments permitted the quantitation of interaction between a suspension of collagen fibrils and the superoxide ion, $O_2^{\cdot-}$, formed by irradiation of water by an electron accelerator (pulse radiolysis) or by a gamma source (gamma radiolysis). In both cases, the presence of sodium formate in the solution prevented the formation of $OH^{\cdot}$ radicals (see Appendix). The amount of $O_2^{\cdot-}$ formed in the pulse radiolysis cell was quantitated by recording the absorbance of the mixture at 245 nm or at 277 nm depending on the optical path of the cell. The occurrence of proteolysis was demonstrated by the liberation of small peptides containing hydroxyproline from the fibrils of collagen under the influence of $O_2^{\cdot-}$. The effect of $O_2^{\cdot-}$ was quantitated by specific determination of hydroxyproline content in these small peptides.

It was demonstrated that the amount of liberated hydroxyproline increased linearly with the radiation dose and with the collagen concentration. The evaluation of the absorbance of $O_2^{\cdot-}$ in the absence and in the presence of collagen in pulse radiolysis experiments suggested that $O_2^{\cdot-}$ radicals competed between spontaneous dismutation and reaction with collagen fibrils.

We combined the kinetic data of the reactions observed with and without collagen:

$$O_2^{\cdot-} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad k_0 = 9 \cdot 10^5 \text{ mol}^{-1} \cdot \text{l} \cdot \text{s}^{-1}$$

and

$$O_2^{\cdot-} + \text{collagen} \rightarrow \text{products} \quad k_c$$

into a differential equation giving the rate of decay of $O_2^{\cdot-}$ radicals in the presence of collagen:

$$\frac{-\mathrm{d}[O_2^{\cdot-}]}{\mathrm{d}t} = 2k_0 \cdot 10^{pK_A - pH}[O_2^{\cdot-}]^2 + k_c[O_2^{\cdot-}][\text{collagen}]$$

In every experiment, the initial collagen concentrations were considered to remain constant with time.

Integration of the equation gave the expression of the variation of $O_2^{\cdot-}$ radical concentration, $[O_2^{\cdot-}]_t$, at time t (concentration at time $0 = [O_2^{\cdot-}]_0$).

$$\ln\left(\frac{[O_2^{\cdot-}]_t}{[O_2^{\cdot-}]_0}\right)\left(\frac{[O_2^{\cdot-}]_0 + \dfrac{k_c[\text{collagen}]}{2k_0 10^{pK_A - pH}}}{[O_2^{\cdot-}]_t + \dfrac{k_c[\text{collagen}]}{2k_0 10^{pK_A - pH}}}\right) = -k_c[\text{collagen}] \cdot t$$

Using the data obtained with three different concentrations of collagen (Fig. 2), we verified that this equation was fitting with all concentrations of collagen tested and calculated the kinetic constant:

$$2k_0 10^{pK_A - pH} = 9 \cdot 10^5 \text{ mol}^{-1} \cdot \text{l} \cdot \text{s}^{-1}$$

$$k_c = (4.8 \pm 0.8) \cdot 10^6 \text{ mol}^{-1} \cdot \text{l} \cdot \text{s}^{-1}$$

From the kinetic constant, k_c, we calculated the initial competition ratio (R):

$$(R) = \frac{k_c[\text{collagen}][O_2^{\cdot-}]_0}{k_c[\text{collagen}][O_2^{\cdot-}]_0 + 2k_0 10^{pK_A - pH}}$$

(R) was 0.18, 0.30 and 0.46, respectively, with collagen concentrations of $0.8 \cdot 10^{-6}$ M, $1.6 \cdot 10^{-6}$ M and $3.2 \cdot 10^{-6}$ M, with an initial $O_2^{\cdot-}$ concentration of $2 \cdot 10^{-5}$ M. The percentage of initial $O_2^{\cdot-}$ which reacted with collagen fibrils increased with the concentration of collagen.

In gamma radiolysis experiments all the $O_2^{\cdot-}$ produced (calculated according to $G_{O_2^{\cdot-}} = 6$ mol/100 eV) reacted with collagen fibrils (concentration of $O_2^{\cdot-}$ in the stationary state is $3 \cdot 10^{-9}$ M for a collagen concentration of 3.2 μM). On the other hand, the initial yield of hydroxyproline, $G_{4\text{Hyp}}$, was low. For instance, in gamma radiolysis $G_{4\text{Hyp}}$ was 0.1 mol/100 eV for an initial collagen concentration of 3.2 μM, despite complete $O_2^{\cdot-}$ trapping by collagen fibrils. It appeared that in gamma radiolysis experiments at this concentration of collagen, only 1.5% of $O_2^{\cdot-}$ radicals were efficient for the breaking of collagen fibrils into small peptides.

To explain this discrepancy, it might be speculated that $O_2^{\cdot-}$ reacted on collagen molecules by more than one mechanism. For instance, if the targets were peptide bonds, only a limited number of them might be sensitive enough for hydrolysis to occur, and this sensitivity might well depend on

the nature of the amino acid residues present in the vicinity or on the secondary structure of the molecule. In addition, some of the free radicals might react with the amino acid residue side chains without leading to peptide linkage hydrolysis.

A kinetic interpretation of the phenomena may be suggested as follows:

(1) $O_2^{\cdot-}$ + collagen → $Coll_1^{\cdot}$ / $Coll_2^{\cdot-}$ + H_2O_2 or O_2

(2) $Coll_1^{\cdot} + Coll_2^{\cdot}$ → collagen

(3) $Coll_1^{\cdot}$ (or $Coll_2^{\cdot}$) + collagen → products + 4-hydroxyproline-containing peptides

Reaction 1 corresponds to the trapping of $O_2^{\cdot-}$ by collagen fibrils, giving two (or more) radicals, $Coll_1^{\cdot}$ and $Coll_2^{\cdot}$, that can recombinate to form collagen according to reaction 2 (recovering process) in competition with reaction 3 which symbolizes the step(s) towards hydroxyproline-containing peptide liberation.

As our data could also have been explained by an interference by traces of superoxide dismutase or of metallic cations adsorbed onto the collagen molecules, we performed additional experiments. In gamma radiolysis, the additional presence of DTPA, a chelating agent of transition metals, did not modify the degradation rate of collagen. The addition of desferrioxamine, another Fe^{3+}-chelating agent, increased the degradation rate of collagen. It is known that $O_2^{\cdot-}$ is capable of reacting with desferrioxamine to form a desferrioxamine radical [13]. It is hypothesized here that this radical reacts secondarily with collagen. Another experiment of $O_2^{\cdot-}$ production by the hypoxanthine-xanthine oxidase system was performed for the evaluation of the scavenging activity of collagen by ferricytochrome *c* reduction in the presence of EDTA for chelating metals or of KCN for superoxide dismutase. As the effect of the collagen fibrils was not abolished by these inhibitors, we concluded that the hypothesis of such interferences could be ruled out under our experimental conditions, except in the case of an especially strong binding of transition metals to collagen, a situation which has never been demonstrated up to now.

These experiments demonstrate the possibility that collagen is cleaved by $O_2^{\cdot-}$. Cleavage of many proteins by $OH^{\cdot}$ radicals has been already demonstrated [14,15] but examples of degradation by $O_2^{\cdot-}$ are scanty, if at all demonstrated. The sensitivity of collagen to $O_2^{\cdot-}$ may be due to its special triple-helical structure. It does not preclude its sensitivity to $OH^{\cdot}$ (Monboisse, J.C., unpublished observations).

These experiments have been performed in vitro. The in vivo situation may be different because the two types of oxygen radicals might operate simultaneously on the molecules and because the fibrils of collagen are composed of several collagen types and contain proteoglycans which may modify the reactivity of the structures. These questions will be addressed in further experiments but the assessment of the reactivity of every element of the system constitutes a preliminary milestone.

Appendix

Principle of radiolysis method

In irradiated water, free radicals $OH^{\cdot}$, $H^{\cdot}$ and e_{aq}^- are produced at neutral pH with a known *G* value (*G* is the number of molecules per 100 eV of energy absorbed for each species).

$G_{OH^{\cdot}} = G_{e_{aq}^-} = 2.75$ mol per 100 eV ($2.75 \cdot 10^{-7}$ mol/J) [16]

$G_{H^{\cdot}} = 0.55$ mol per 100 eV ($0.55 \cdot 10^{-7}$ mol/J) [16]

In the presence of 100 mM sodium formate and under air ($[O_2]_0 = 2.10$ μM) or under oxygen ($[O_2]_0 = 1$ mM), there is formation of $O_2^{\cdot-}$ radicals according to the following reactions:

(1) $OH^{\cdot} + HCOO^- \rightarrow COO^{\cdot-} + H_2O$,

$k_1 = 3.5 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [17]

(2) $H^{\cdot} + HCOO^- \rightarrow COO^{\cdot-} + H_2$, $k_2 = 5 \cdot 10^8\ M^{-1} \cdot s^{-1}$ [18]

(3) $COO^{\cdot-} + O_2 \rightarrow O_2^{\cdot-} + CO_2$, $k_3 = 4.2 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [19]

In the presence of N_2O, reaction 3 does not take place.

(4) $e_{aq}^- + O_2 \rightarrow O_2^{\cdot-}$, $k_4 = 1.9 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ [20]

In pulse or gamma radiolysis, the yield of O_2^-

radicals, according to the above equations, corresponds to:

$G_{O_2^{\cdot-}} = G_{OH^{\cdot}} + G_{e^-_{aq}} + G_{H^{\cdot}}$, about 6 mol per 100 eV

$(6 \cdot 10^{-7}$ mol/J).

During gamma radiolysis, the $O_2^{\cdot-}$ concentration is low and quasi-constant (about 10^{-9} M), because it corresponds to the equilibrium between the formation and the disparition of $O_2^{\cdot-}$. In pulse radiolysis, a high $O_2^{\cdot-}$ concentration (10^{-5}–$4 \cdot 10^{-5}$ M) is initially produced in a single pulse for a short time (about 50 ns).

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