The degradative action of peroxynitrite on high-molecular-weight hyaluronan

Monika Stankovská[1], Eva Hrabárová[2], Katarína Valachová[1], Marianna Molnárová[1], Peter Gemeiner[2] & Ladislav Šoltés[1]

1. Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia
2. Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia

Correspondence to: Dipl. Engineer Ladislav Šoltés, DSc.
Institute of Experimental Pharmacology, Slovak Academy of Sciences,
Dúbravská cesta 9, 841 04 Bratislava, Slovakia
TEL.: +421-2-59410670, FAX: +421-2-54775928
EMAIL: ladislav.soltes@savba.sk

Submitted: September 2, 2006 *Accepted: October 28, 2006*

Key words: **hyaluronan degradation; viscosity; sodium peroxynitrite; manganese dioxide; rotational viscometry**

Neuroendocrinol Lett 2006; **27**(Suppl.2):31–34 **PMID:** 17159774 NEL270806A06 © Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJECTIVES: This contribution presents the results of the kinetics of HA degradation by peroxynitrite, which represents one of the main reactive oxygen species degrading various biomacromolecules under inflammatory conditions.
METHODS: Two simple procedures have been adapted to prepare sodium peroxynitrite: the first containing an excess of H_2O_2, and the second in which the H_2O_2 excess had been decomposed by MnO_2 treatment. The kinetics of hyaluronan degradation by action of peroxynitrite was monitored by rotational viscometry.
RESULTS: High-molecular-weight hyaluronan was degraded by peroxynitrite. The degradation was increased in the presence of $ONOO^-$ previously treated by MnO_2 in order to remove residual hydrogen peroxide. One of the reasons of this finding could be that by the action of the residual metal the pathway of $ONOO^-$ decomposition starts to be manifested immediately on mixing traces of metals originally present in the HA sample with the ions of manganese.
CONCLUSIONS: Trace amounts of transition metal(s) should be taken into consideration on evaluating the experimental results. Purchase of the marketed peroxynitrite product appears to be the appropriate approach to simplify and standardize the quality of ONOONa.

Abbreviations

MDa	- megagram/mol
Mw	- molecular weight
EPR	- electron paramagnetic resonance
NMR	- nuclear magnetic resonance
HA	- hyaluronan
rpm	- rotational speed per minutes
η	- dynamic viscosity
ε	- extinction coefficient

Introduction

Peroxynitrite anion ($ONOO^-$) is a reactive species generated *in vivo* by fast recombining reactions between superoxide anion radical ($O_2^{\bullet -}$) and a free radical of nitric oxide ($NO^\bullet$): the second order rate constant equals $6.7 \times 10^9\ M^{-1}.s^{-1}$ [10]. Large quantities of $O_2^{\bullet -}$ and $NO^\bullet$ are produced by neutrophils and monocytes upon inflammatory stimulation [5]. It may be relevant to point out, however, that

ORIGINAL ARTICLE

the hydrogenated form of peroxynitrite anion, ONOOH, is a weak acid with a pKa value of 6.8 [14]. Thus under slight acidosis accompanying inflammation processes, i.e. at e.g. pH 6.8, the ratio of [$ONOO^-$] to [ONOOH] is 50:50.

The chemistry of peroxynitrite anion as well as that of peroxynitrous acid may be of high importance, taking into account that the molecules can undergo several different decomposition ways by which further reactive/oxidative species are generated (cf. Scheme 1). The protonation of $ONOO^-$ to peroxynitrous acid, with a half-life of 1 s, is proceeding to $NO^\bullet$ and $^\bullet OH$ [2,3,7,20]. The fraction of the generated hydroxyl radicals however was reported to range from 0.1–5% [1], through 10 [16] up to 40% [7].

In the presence of CO_2, formation of $NO_2^\bullet$ and $CO_3^{\bullet-}$ was observed [4,8,13]. In the presence of metal ions, peroxynitrite decomposition with the formation of NO_2^+ was reported [11]. Undoubtedly, the formed radicals ($^\bullet OH$, $NO_2^\bullet$) and the cation (NO_2^+) may play an important role in physiological/pathophysiological processes.

Biomacromolecules, such as nucleic acids, proteins, polysaccharides, have often been employed to study *in vitro* degradative action(s) of various oxidants. Both the given biopolymers and the applied oxidative conditions are adjusted usually to mimic "pathological" events – such as atherosclerosis, rheumatoid diseases, meningitis, etc. The key task is to detect the chemical and/or physical changes occurring in the target biomacromolecule.

The effect of peroxynitrite on hyaluronan (HA) degradation was studied for the first time by Li et al. [12]. The electrophoretic and viscometric analyses showed a reduction of HA molecular weight. In the year 2003, Al-Assaf et al. [1] presented the efficiency of the peroxynitrite anion/peroxynitrous acid system to induce chain scission of a high-molecular-weight hyaluronan. The authors observed that while the hydroxyl radicals were extremely efficient, namely nine of ten $^\bullet OH$ caused HA chain scission, peroxynitrous acid (ONOOH), and/or the related peroxynitrite anion ($ONOO^-$), was found to be significantly less efficient in breaking HA macromolecules. Corsaro et al. used the EPR and NMR method to study the chemistry of HA degradation by peroxynitrite. The observed data were very similar to those reported for HA degradation by hydroxyl radicals [6].

This contribution presents results on the kinetics of HA degradation by peroxynitrite. We compared the action of two peroxynitrite preparations, one containing an excess of H_2O_2 and another one in which H_2O_2 had been decomposed by MnO_2 treatment.

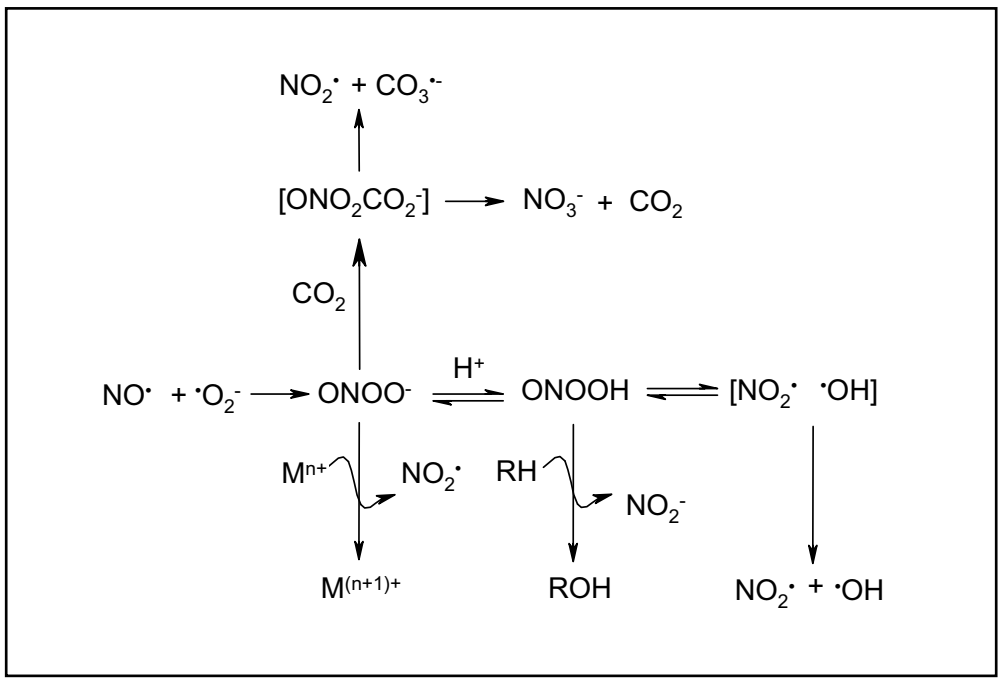
Materials and methods

Biopolymer

A high-molecular-weight hyaluronan sample (coded F1750762) with the average-molecular-weight Mw=1.378 MDa [17].

Chemicals

Analytical purity grade NaCl and MnO_2 were from Slavus Ltd., Bratislava, Slovakia. Aqueous solution of H_2O_2 (≈30%) and $NaNO_2$ p.a. were purchased from Chemapol, Prague, Czech Republic, HCl 35% and NaOH extra pure were purchased from mikroCHEM, Pezinok, Slovakia. Water used was of redistilled deionized quality grade.



Scheme 1. Adapted from Radi R. et al., 2001 [15].

Preparation of peroxynitrite

The substance – ONOONa – can be prepared simply from $NaNO_2$ [4,5]. Briefly: Into 2 ml of cold intensively stirred aqueous solution of $NaNO_2$ (0.5 M) and H_2O_2 (0.5 M) 1 ml of cold HCl (1.0 M) was added. The solution of HCl should be injected into the reaction vessel as one single load, and – immediately (within ≤ 0.5 second) – the reaction system should be alkalized with the addition of a cold NaOH solution (1.0 ml; 1.5 M). The formation of ONOONa is indicated by yellow color of the product. The procedure was performed on ice. The actual concentration of ONOONa solution was determined by spectrophotometric measurement at 302 nm (ε=1 670 $M^{-1}.cm^{-1}$) directly before the HA degradation study.

The peroxynitrite solution was divided into two parts: Part 1: The unreacted H_2O_2 was decomposed by addition of 4 mg of MnO_2 powder per each 1 ml of the peroxynitrite solution during one-hour storage in a cold and dark place (solution A). Part 2: ONOONa solution without addition of MnO_2 (solution B).

Inactive ONOONa solution, used as a control, was produced by the same procedure, except that addition of NaOH followed 10 s after the addition of HCl (solution C).

Hyaluronan degradation study by rotational viscometry

For the degradation studies, 20.0 mg of HA sample was dissolved in 0.15 M aqueous NaCl in two steps: first, 4.0 ml solvent was added in the morning, next 3.36 ml (A, C), or 3.54 (B) ml portion of the solvent was added after 6 h. The solution was kept overnight in the dark, at room temperature. The following morning, two different volumes of the working ONOONa solution were mixed: 640 µl (12.5 mM solution A and unknown concentration of solution C), or 460 µl (17.3 mM solution B) to yield the final concentration 1 mM at 30 s moderate stirring.

The resulting solution (8.0 ml) was immediately transferred into the Teflon® cup reservoir of the rotational viscometer. The record of the viscometer output parameters started 2 min after onset of the experiment. The changes of dynamic viscosity (η) and torque were monitored at 25±0.1 °C by using a digital rotational viscometer Brookfield LVDV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.) equipped with a cup-spindle pair built of Teflon® at our laboratory [19]. At the spindle rotational speed of 180 rpm, the shear rate was 237.6 s^{-1}. The HA sample degradation was monitored in 3 min intervals for up to 120 min or up to the viscosity value 5.80 mPa•s.

Results and discussion

Figure 1 represents the kinetics of hyaluronan degradation by different oxidative systems. Curve "HA" indicates that in the case of a simple hyaluronan solution, without any oxidants, the dynamic viscosity increases slightly due to a well-known phenomenon of rheopexy [18]. However, as evident from curve C, addition of even a small amount of solution C resulted in a slight but significant decrease of the η value within the first 11 minutes. Afterwards, the HA solution was again characterized by a slight rheopexy. Curve B is direct evidence of the degrading action of peroxynitrite. As seen, the value of η decreases from the initial value of 9.76 mPa•s at 2 minutes to the value η = 5.80 mPa•s within the further time period of 109 min. A relatively unexpected result is represented in curve A, documenting only the action of pure peroxynitrite – free of any traces of H_2O_2. As evident from the curve A, in the whole time interval investigated the η values were significantly lower than those values represented in curve B.

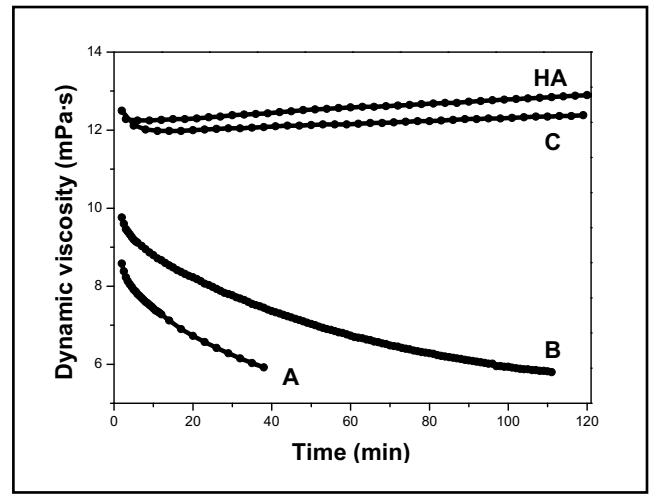


Figure 1. Kinetics of HA degradation. Biopolymer solution incubated in absence of ONOONa (HA); in presence of inactive ONOONa (C); with addition of 1 mM ONOONa not treated (B) or treated with MnO_2 (A).

This observation indicates, although indirectly, the well-known effect of residual trace metals in HA on the dynamic viscosity of its solutions [9]. Degradation of hyaluronan by a direct action of peroxynitrite appears to be unlikely. As reported in the section Introduction, peroxynitrite anion can undergo several different decompositions (cf. Scheme 1). The presence of transition metal ions manganese ions with traces of metals originally present in the HA sample – can induce decomposition of peroxynitrite, resulting in generation of a mixture of further reactive/oxidative species [2]. Manganese ions dissolved presumably in the peroxynitrite solution during MnO_2 treatment under alkaline conditions. The degradation of high-molecular-weight hyaluronan occurs probably by the action of the above mentioned reactive/oxidative species.

It is well-known that attack of •OH on hyaluronan triggers a cascade of chain reactions with generation of hyaluronan-radicals [17]. Decomposition of these radicals, namely hydroperoxides, was induced by transition metal ions. This process yields alkoxyl radicals,

which are presumed intermediates of the chain splitting of the hyaluronan macromolecule. In the action of peroxynitrite/peroxynitrite-decomposition species on hyaluronan we assumed a similar cascade of chain reactions as well as the effect of metal ions to be involved in the decay of HA-radicals.

Thus the presence of transition metal ions in the peroxynitrite solution can accelerate HA degradation in two ways: by decomposition of peroxynitrite as well as by decomposition of HA-radicals. Purchase of the very recently marketed product peroxynitrite (e.g. Alexis Corporation, Lausen, Switzerland) appears to be an appropriate approach to simplify and standardize the quality of ONOONa for studies on the degradation of targeted biomacromolecules.

Acknowledgements

The VEGA grants 2/5002/05, 2/4133/04, 2/7028/07 and the grant APVV-51-017905 are gratefully acknowledged. This work was also supported by the COST B 35 action.

REFERENCES

1 Al-Assaf SA, Navaratnam S, Parsons BJ, Phillips GO. Chain scission of hyaluronan by peroxynitrite. Arch Biochem Biophys. 2003; **441**:73–82.
2 Beckman JS, Beckman TW, Marshall PA. Apparent hydroxyl radical production by peroxynitrite: complications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA. 1990; **87**:1620–4.
3 Beckman JS, Chien J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. Meth Enzymol. 1994; **233**: 229–40.
4 Coddington JW, Hurst JK, Lymar SV. Hydroxyl radical formation during peroxynitrous acid decomposition. J Am Chem Soc. 1999; **121**:2438–43.
5 Conner EM, Grisham MB. Inflammation, free radicals and antioxidants. Nutrition. 1996; **12**:274–7.
6 Corsaro MM, Pietraforte D, Lorenzo AS, Minetti M, Marino G. Reaction of peroxynitrite with hyaluronan and related saccharides. Free Rad Res. 2004; **38**(4):343–53.
7 Edwards JO, Plumb RC. The chemistry of peroxynitrites. Progr Inorg Chem. 1994; **41**:599–635.
8 Goldstein S, Czapski G. Formation of peroxynitrite from the reaction of peroxynitrite with CO_2: Evidence for carbonate radical production. J Am Chem Soc. 1998; **120**:3458–63.
9 Gura E, Hückel M, Müller PJ. Specific degradation of hyaluronic acid and its rheological properties. Polym Degrad Stabil. 1998; **59**:297–302.
10 Huie RE, Padmaja S. The reaction of NO with superoxide. Free Rad Res. 1993; **18**:195–9.
11 Ischiropoulos H, Beckman JS, Crow JP, Ye YZ, Royall JA, Kooy NW. Detection of peroxynitrite. METHODS: A Companion to Meth Enzymol. 1995; **7**:109–15.
12 Li. M, Rosenfeld L, Vilar RE, Cowman MK. Degradation of hyaluronan by peroxynitrite. Arch Biochem Biophys. 1997; 34(2):24–5.
13 Lymar SV, Jiang Q, Hurst JK. Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. Biochemistry. 1999; **35**:7853–61.
14 Parsons BJ, Al-Assaf S, Navaratnam S, Phillips GO. Comparison of the reactivity of different oxidative species (ROS) towards hyaluronan. In: Kennedy JF, Phillips GO, Williams PA, editors, Hascall VC, guest editor. Hyaluronan: Chemical, Biochemical and Biological Aspects. Cambridge: Woodhead Publishing Ltd; 2002, Vol. 1, p. 141–50.
15 Radi R, Peluffo G, Alvarez MN, Navilliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. Free Rad Biol Med. 2001; **30**(5):463–88.
16 Richeson CE, Mulder P, Bowry VW, Ingold KU. The complex chemistry of peroxynitrite decomposition: New insights. J Am Chem Soc. 1998; **120**: 7211–9.
17 Rychlý J, Šoltés L, Stankovská M, Janigová I, Csomorová K, Kogan G, Gemeiner P. Unexplored capabilities of chemiluminescence and thermoanalytical methods in characterization of intact and degraded hyaluronans. Polym Degrad Stab. – in press.
18 Stankovská M, Šoltés L, Vikartovská A, Mendichi R, Lath D, Molnárová M, Gemeiner P. Study of hyaluronan degradation by means of rotational viscometry: contribution of the material of viscometer. Chem Pap-Chem Zvesti. 2004; **58**(5):348–52.
19 Stankovská M, Šoltés L, Vikartovská A, Gemeiner P, Kogan G, Bakoš D. Degradation of high-molecular-weight hyaluronan: a rotational viscometry study. Biologia. 2005; **60**(Suppl.17):149–52.
20 Van der Vliet A, Smith A, Ońeill CA, Halliwell B, Cross CE, Kaur H. Aromatic hydroxylation and nitration of phenylalanine and thyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. FEBS Lett. 1994; **339**:89–92.