ORIGINAL ARTICLE

Interactions of oxidatively modified calf skin collagen with platelets and phagocytes

Milan Ciz [1], Hana Cizova [1], Katerina Pejchalova [1], Viera Jancinova [2], Ivan Goshev [3], Boryana Mihaylova [3], Radomir Nosal [2], Antonin Lojek [1]

[1] Institute of Biophysics of the Academy of Sciences of the Czech Republic, Brno, Czech Republic.
[2] Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences, Bratislava, Slovak Republic.
[3] Institute of Organic Chemistry with Centre of Phytochemistry of the Bulgarian Academy of Sciences, Sofia, Bulgaria.

Correspondence to: Milan Číž PhD, Institute of Biophysics of the Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic.
PHONE: +420-541 517 104, FAX: +420 - 541 211 293, E-MAIL: milanciz@ibp.cz

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Abstract

OBJECTIVES: The effects of non-modified and oxidatively modified calf skin collagen type I on platelet aggregation and the oxidative burst of phagocytes were examined in the framework of a general hypothesis that collagen, platelets and phagocytes cooperate to modulate the oxidative burst of phagocytes and the extent of oxidative stress.

MATERIALS AND METHODS: Calf skin collagen type I was subjected to oxidative modification by hydrogen peroxide or hydroxyl radical. Thermal denaturation of collagen was performed in a spectrophotometer equipped with a temperature gradient device. The aggregation of isolated human platelets obtained after differential centrifugation was measured using a dual-channel aggregometer. The production of reactive oxygen species by human whole blood phagocytes was evaluated by luminol-enhanced chemiluminescence.

RESULTS: Oxidative modification of collagen samples was characterized by a decrease in denaturation transition temperature. Oxidatively modified samples showed a modified SDS-PAGE pattern, evidencing a significant destruction of the collagen. All oxidatively modified collagen samples, independent of the oxidation treatment applied, lost their platelet-aggregating and phagocyte oxidative burst-inducing activity.

CONCLUSION: The results suggest that reactive oxygen species were able to modify collagen. On the other hand, oxidatively modified collagen lost its activating properties towards platelets and phagocytes.

INTRODUCTION

Collagen is one of the main components of the vascular subendothelial environment. After vessel wall disruption, collagen becomes exposed and acts as a potent stimulator of platelet adhesion and aggregation (Varga-Szabo *et al.* 2008). It has been widely accepted that collagen plays an important role in hemostasis and thrombogenesis (Ono *et al.* 2008). This is one of the possible mechanisms for preventing blood leakage in cases of some injuries of the blood vessel wall (physiological case). This may also happen in the case of different diseases, leading to the destruction of the internal surface of

Abbreviations & units

DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
PRP	platelet-rich plasma
ROS	reactive oxygen species
T_d	temperature of denaturation

the blood vessel and the exposure of the collagen fibrils network to the blood stream (pathological case). Thus, thrombi might be formed, on one hand, as a reparation procedure, but, on the other, with possible negative consequences.

This response of the platelets is possible only when they are in a normal state, i.e., unaffected by any other pathological process. As far as it concerns collagen itself, it may induce platelet aggregation only if it is in the form of fibrils consisting of native molecules. The latter means that their structure is not changed as a consequence of other factors – heating, pH (hydrolytic processes), chemical modifications, etc. In this sense, the results of such an experiment characterize both the platelets – respectively, the health status of the blood donor patient, and the nativity of the collagen preparation – respectively, the reliability of the procedure applied for its isolation and purification.

Oxidative modifications of collagen, respectively, of the connective tissue (extracellular matrix), may be provoked (may proceed) at least by two mechanisms: the external impact of different oxidizing substances and *in vivo* by inflammatory processes (Mattana *et al.* 1999; Olszowski *et al.* 2003; Tiku *et al.* 2003). During the latter, reactive oxygen species (ROS) are generated (Lojek *et al.* 2008). If the mechanisms of elimination of their eventual excess are not sufficiently effective, they may attack all protein and non-protein targets, including collagen (VilarRojas *et al.* 1996; Winterbourn, 2008).

In this study, we examined the effects of non-modified and oxidatively modified calf skin collagen type I on platelet aggregation in the framework of a general hypothesis that collagen, platelets and professional phagocytes cooperate to modulate the oxidative burst of phagocytes and the extent of oxidative stress. Our results suggest that reactive oxygen species were able to modify collagen, a major constituent of the extracellular matrix. On the other hand, oxidatively modified collagen lost its activating properties towards platelets.

MATERIALS AND METHODS

Chemicals: Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Zymosan particles were opsonized as follows: 0.24 g of zymosan, which was diluted in 24 ml of phosphate buffered saline (PBS, pH 7.4), was mixed with 12 ml of rat serum and incubated at 37°C for 30 minutes. This suspension was washed twice and resuspended in 48 ml of PBS.

Collagen isolation. Calf skin type I collagen was isolated by a standard procedure involving acetic acid extraction, salting out with NaCl, and using ion exchange chromatography on DEAE-cellulose. In order to prevent spontaneous oxidation of collagen by trace amounts of metal ions, the procedure of collagen isolation was carried out with solutions containing 10 mM EDTA/40 mM citrate/20 mM mannitol. The purity of the preparations was verified by means of interrupted electrophoresis. The collagen concentration was determined according to the modified Biuret method using a SLT Rainbow Spectrophotometer (Tecan, Crailsheim, Germany) using 96-well microtiter plates.

Oxidative modification of collagen. Collagen samples were subjected to the oxidative modification by the incubation of collagen solutions (1 mg/ml in 50 mM acetic acid, pH adjusted to 5.0–5.5) with different oxidants: hydrogen peroxide (100 mM H_2O_2 or 300 mM H_2O_2) or hydroxyl radical (100 μM $FeSO_4$/2 mM H_2O_2 or 50 μM $FeSO_4$/5 mM H_2O_2). After the oxidation treatment, all samples were subjected to an extensive dialysis (nanofiltration membrane, Union Carbide, Chicago, IL, USA) against 10 mM HCl. Collagen samples dissolved in 50 mM acetic acid and dialyzed against 10 mM HCl only were designated as non-modified (control) samples.

Temperature of denaturation of collagen. Thermal denaturation of the non-modified and modified collagen samples was performed on a UV-VIS spectrophotometer Specord M-40 (Carl Zeiss, Jena, Germany), equipped with a thermal gradient device TSE-1. The heating rate used was 0.5°C/min within the temperature range of 22–50°C.

Platelet aggregation. In isolated human platelets obtained after differential centrifugation, platelet aggregation was measured by using a dual channel aggregometer (Chronolog, Havertown, PA, USA). For preparation of platelets, whole blood was collected from healthy donors (who had not taken any drugs in the 14 days before the experiment) by venipuncture and added to 0.1 vol. of 3.8 % sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood at 260 g for 15 min. PRP was removed, mixed with a solution containing 4.5 % citric acid and 6.6 % glucose (50 μl/1 ml PRP), and centrifuged at 1070 g for 10 min. Finally, platelets were resuspended in Tyrode's solution and washed at 1070 g for 6 min. Platelet counts were in the order of 3.0×10^8 platelets/mL. After 1 min stabilization at 37°C, the aggregation of platelets (450 μl) was initiated by addition of 20 μl of tested samples. The development and duration of aggregation was registered on a linear recorder for 5 min. Changes in aggregation were evaluated from the amplitude of aggregation curves (for details see Nosal & Jancinova, 2002).

Oxidative burst of whole blood phagocytes. Heparinized (50 IU/ml) blood samples were obtained from healthy male volunteers with their informed consent. The sampling procedure was in accordance with the

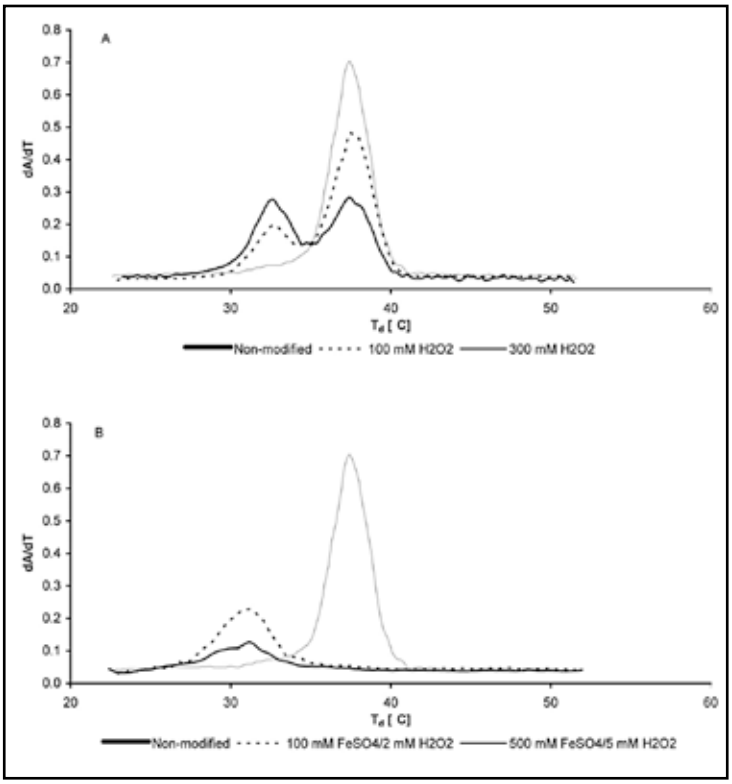

Figure 1. Temperature of denaturation in representative samples of collagen oxidatively modified by hydrogen peroxide (panel A) or hydroxyl radical generated in a system of ferrous sulphate/hydrogen peroxide (panel B) and compared to that of non-modified collagen.

ethical standards of the responsible committee of the Institute of Biophysics on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The number of leukocytes in the blood and their relative differentiation counts were determined using a Coulter counter STKS (Coulter Corporation, Miami, FL, USA). Luminol-enhanced chemiluminescence of human phagocytes in the whole blood was measured using an LM-01 microplate luminometer (Immunotech, Prague, Czech Republic). The principle of the method is based on luminol interaction with the phagocyte-derived oxidizing species, which results in large measurable amounts of light at a peak wavelength of 425 nm. Briefly, the reaction mixture consisted of 10 µl whole blood, 1mM luminol (stock solution of 10mM luminol in 0.2M borate buffer, pH 9.0) and one of the activators: 62.5 µg/mL opsonized zymosan particles (OZP), 0.81 µM phorbol-12-myristate-13-acetate (PMA) or 1.14 µM N-formyl-Met-Leu-Phe (fMLP). The total reaction volume of 200 µl was adjusted with Hanks balanced salt solution (pH 7.4). The assays were run in duplicates. The CL emission expressed as relative light units (RLU) was recorded continuously for 60 min at 37°C.

Statistical analysis. All data are expressed as the mean ± standard error of the mean (SEM), n = 8. The data were analyzed by one-way analysis of variance (ANOVA), followed by a Newman-Keuls test with a level of significance of $p < 0.05$.

Table 1. Temperature of denaturation (T_d) in non-modified and oxidatively modified collagen samples.

Collagen sample modification	T_d1 (°C)	T_d2 (°C)
Non-modified	-	37.60 ± 0.20
100 mM H_2O_2	32.80 ± 0.20	37.44 ± 0.20
300 mM H_2O_2	32.64 ± 0.20	37.36 ± 0.20
100 µM $FeSO_4$ + 2 mM H_2O_2	30.80 ± 0.20	-
50 µM $FeSO_4$ + 5 mM H_2O_2	31.52 ± 0.20	-

RESULTS

It is obvious from the data that the procedures used led to the modification of calf skin collagen samples characterized by the decrease in denaturation temperature (see ***Table 1***). The results showed a single transition at 37.60°C for the non-modified collagen. This value is low – it should be close to 40°C if the preparation tested is considered to be native. The value observed might be a consequence of a partial denaturation – the cumulative effect of the quality of the starting material, the isolation procedure (esp. enzyme treatment), the duration and conditions of storage, etc.

Effective concentrations of 100 and 300 mM of hydrogen peroxide were applied to oxidatively modify the collagen samples (see ***Figure 1a***). The modification of collagen with hydrogen peroxide resulted in two denaturation transitions (see ***Table 1***). This observation might mean that the oxidative treatment affected only a certain part of the protein, and that there is still a certain amount of protein remaining intact in the sample. The treatment with hydroxyl radical resulted in an extensive destruction of the collagen structure and a corresponding significant decrease in the thermal stability of collagen samples (see ***Figure 1b***). These samples showed only one denaturation transition (see Table 1).

All oxidatively modified collagen samples, independent of the oxidation treatment applied, totally lost their platelet-aggregating activity *in vitro* (see ***Figure 2***). It is evident that all oxidation procedures affected the stability of the collagen molecule in solution in the same manner and caused a loss of platelet aggregation activity.

Non-modified collagen itself had a capacity to induce a spontaneous oxidative burst of phagocytes. The oxidatively modified collagen samples lost their capacity to induce the oxidative burst of phagocytes, the changes being independent of the type of oxidative treatment. The effect of collagens on the oxidative burst of activated phagocytes was similar (see ***Figure 3***).

DISCUSSION

The non-modified collagen sample showed an aggregation behavior comparable to that of thrombin. This means that its structure is basically unaffected by the procedures of isolation, purification and storage. On the other hand, the determination of the denaturation temperature showed lower values than expected. Speculatively, this observation might mean that there are certain structural changes, but they do not impair significantly the assemblage of individual collagen molecules into fibrils and the consecutive platelet aggregation activity of collagen. The interesting fact is that, despite the evident structural changes, the platelet aggregation activity of the non-modified samples was preserved.

The oxidation of acid soluble calf skin collagen by different reactive oxygen species (hydrogen peroxide, hydroxyl radical generated according to the Fenton's reaction) caused moderate to significant changes of the denaturation behavior of the protein. The main transition peak was either splitted and redistributed, accompanied by the appearance of a fore-peak, or totally shifted to a lower temperature. Komsa-Penkova *et al.* (2000), have shown that the redistribution of the main transition peak shows a dose-response effect and a dependence on the treatment duration. The most aggressive free radical is the hydroxyl radical causing a decrease in the denaturation temperature by 6–7°C. There are no data in the literature on the problem of proving measurable protein fragmentation or cross-linking. We checked the samples for fluorescent compounds eventually formed by oxidation and subsequent cross-linking, but there was no fluorescence emission recorded.

All oxidized collagen samples were tested for their platelet aggregation activity. A non-oxidized collagen sample and thrombin were applied as standards. Both thrombin and a non-modified collagen sample behaved similarly. The oxidized collagen samples did not induce platelet aggregation independently on the free radical applied. Komsa-Penkova *et al.* (2000) showed that the hydroxyl radical, generated by the system Fe(II)–H_2O_2, caused a significant decrease (approximately 2x) of the available free amino groups. It is known that positively charged amino acid side chains (-NH_2) are critical for platelet aggregation activity induced by collagen samples (Jaffe & Deykin, 1974). The authors have suggested that the modification, evidently caused by oxidative stress, may lead to a random orientation of the collagen chains and diminish their fibril-forming ability. The platelet aggregation is induced *in vitro* by the collagen fibrils, building the framework of the blood vessel walls. Therefore, any treatment affecting fibril formation, e.g. an enzymatic digestion (Wilner *et al.* 1968) applied for increasing the yield of soluble collagen fraction, changes the time course of the aggregation process – in the mentioned case, a retardation and prolongation of the lag phase has been observed.

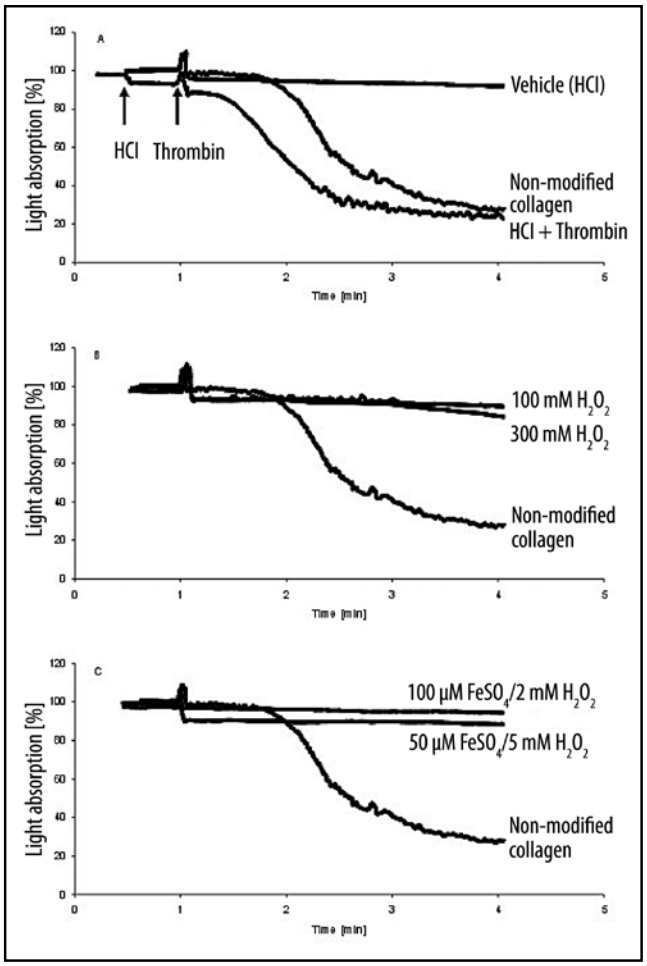

Figure 2. Effect of non-modified and oxidatively modified collagens on platelet aggregation. Aggregation was continuously monitored as a decrease in light absorption; representative curves are shown. Panel A: Comparison of the effect of non-modified collagen with thrombin and vehicle (HCl). Panel B: The effect of collagen oxidatively modified with hydrogen peroxide. Panel C: The effect of collagen oxidatively modified with hydroxyl radical generated in the system of ferrous sulphate and hydrogen peroxide.

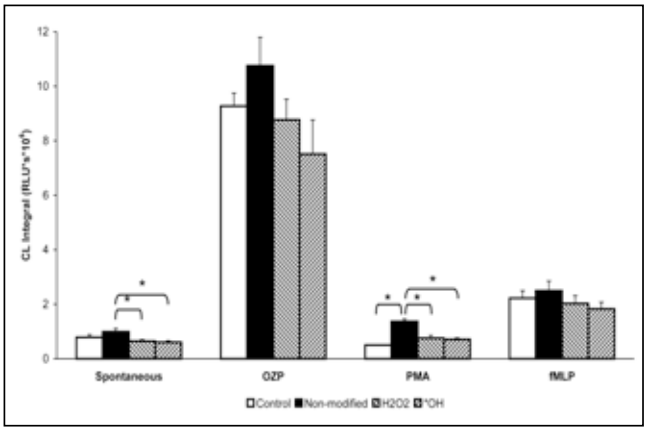

Figure 3. The effects of non-modified and oxidatively modified collagen samples on spontaneous and OZP-, PMA-, and fMLP-activated oxidative burst of human whole blood phagocytes expressed as integrals of luminol-enhanced chemiluminescence. The data represent mean ± SEM, n = 8. Asterisks show the statistically significant differencies at $p < 0.05$.

The nativity of the collagen structure is of crucial importance for the expressed platelet aggregation effect (Puett *et al.* 1973). According to Jaffe & Deykin (1974), an architectural requirement exists for the initiation of the platelet aggregation process. A soluble collagen preparation, consisting of the microfibrillar form of the protein, contains the minimum structural unit and is effective. Any modification of side groups affects the aggregation ability of collagen. According to Wilner *et al.* (1968), the most important are the positively charged functional groups – in particular, the Lys-derived ε-amino groups. Their blockage by deamination, N-acetylation, treatment with dinitrofluorbenzene or trinitrobenzene sulfonic acid resulted in an over 90% reduction in platelet aggregating activity. According to Wang *et al.* (1978), monomeric collagen that had been modified by deguanidation, methylation or succinilation, i.e., by a set of reactions blocking the free amino groups, failed to polymerize in physiological conditions and did not induce platelet aggregation. But, if collagen fibrils were already formed, the status of the arginyl residues became more important for the platelet aggregation ability. Chesney *et al.* (1983) have confirmed these observations and have shown that negatively charged groups (carboxyl groups) in the collagen molecule do not play a key role in collagen induced platelet aggregation. The decrease of free amino groups, as reported by Komsa-Penkova *et al.* (2000), as a consequence of the oxidizing procedures applied, is in agreement with the above-mentioned observations and explains the loss of platelet aggregation ability in the oxidized collagen preparations.

Collagen is also known to be able to prime the respiratory burst of phagocytes. In contrast to the effect of oxidatively modified collagen, pre-treatment of collagen by collagenase resulted in a higher stimulatory capacity towards phagocytes when compared to non-digested collagen (Castillo-Briceno *et al.* 2009).

It is assumed that the oxidative modifications that we have performed *in vitro* have, in principle, a similar effect on the collagen structure because they have one and the same target. Speculatively, this might mean that the oxidative burst of phagocytes *in vivo* may affect, in a similar manner, the collagen structure. In this sense, the presence or extra introduction of antioxidative substances is extremely important for the scavenging of the excessive free radicals.

The hypothesis that collagen, platelets, and professional phagocytes cooperate to modulate the oxidative burst of phagocytes and the extent of oxidative stress was proved. Reactive oxygen species produced by activated professional phagocytes were able to modify collagen, a major constituent of the extracellular matrix. On the other hand, oxidatively modified collagen lost its activating properties towards platelets and phagocytes.

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