

Paradoxical fate and biological action of peroxynitrite on human platelets

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ABSTRACT Peroxynitrite (ONOO⁻), which is formed from the reaction of nitric oxide (NO) and superoxide (O₂⁻), has been suggested to be responsible for some of the cytotoxic effects of these molecules. When protonated, ONOO⁻ gives rise to hydroxyl (OH[•]) and nitrogen dioxide (NO₂) radicals, which are capable of inducing tissue damage. We have investigated the effects of ONOO⁻ on human platelets *in vitro* in order to explore the potential of this oxidant to contribute to tissue damage. ONOO⁻ caused aggregation of washed platelets and reversed the inhibition of aggregation induced by *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), prostacyclin, and indomethacin. However, in platelet-rich plasma, ONOO⁻ not only did not possess proaggregatory properties but acted as an inhibitor of platelet aggregation. This reversal of the aggregatory effect of ONOO⁻ could also be achieved in washed platelets by adding low concentrations of plasma, human serum albumin, or glutathione and was inhibited by hemoglobin. An analysis of the reaction products of ONOO⁻ and glutathione revealed the presence of both NO and *S*-nitrosoglutathione in quantities sufficient to account for the antiaggregatory effects observed. Thus the fate and therefore the actions of ONOO⁻ in biological systems are critically dependent on the biological environment in which this oxidant is present.

Nitric oxide (NO) synthesized from L-arginine is known to be an important mediator in several physiological systems. However, when generated in large quantities for long periods, mainly during immunological reactions, NO is cytostatic/cytotoxic for invading microorganisms and cancer cells and might be also damaging for the tissues that generate it (1).

The mechanism of NO-induced cytostasis/cytotoxicity includes direct inhibition of mitochondrial respiration, aconitase activity, and DNA synthesis (2). However, another potential route of NO toxicity is through its interaction with other molecules, mainly superoxide anion (O₂⁻). It was originally demonstrated that NO generated from vascular cells or from leukocytes interacts with O₂⁻, leading to the reduction of its vasorelaxant and platelet antiaggregatory actions (3–5). The product of the reaction between these two free radicals is the peroxynitrite anion (ONOO⁻), which has also been shown to be formed *in vitro* by activated macrophages (6–8). This powerful oxidant, when protonated, decomposes rapidly, resulting in the formation of OH[•] and NO₂, both of which are tissue-damaging agents.

Although the formation of ONOO⁻ has not been directly demonstrated *in vivo*, the potential relevance of this biochemical pathway in conditions such as atherosclerosis and chronic inflammation led us to investigate the biological actions of ONOO⁻. We selected platelets as a biological system because of their involvement in some of these pa-

thologies and also because they can be isolated and their response assessed *in vitro* under very clearly defined conditions. We have found that ONOO⁻ has paradoxical effects on platelets, showing both pro- and antiaggregatory actions, which depend on the environment in which this oxidant is present.

MATERIALS AND METHODS

Preparation of Human Platelet-Rich Plasma (PRP) and Washed Platelets (WP). Human blood was collected and PRP and WP (2.0–2.5 × 10⁸ platelets per ml) were prepared as described (9). Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 720 × *g* for 10 min. Platelet aggregation was studied in a platelet-ionized calcium aggregometer (Chrono-Log, Havertown, PA) using both WP and PRP.

Platelet Aggregation in WP. The effect of ONOO⁻ (20–200 μM) or decomposed ONOO⁻ (200 μM at pH 7.4; see below) on platelet aggregation was studied for 3 min. *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 3 or 10 μM), prostacyclin (3 or 10 nM), indomethacin (0.3 or 1 μM), catalase (500 units/ml), superoxide dismutase (180 units/ml), desferrioxamine (100 μM), mannitol (10 mM), diethylenetriaminepentaacetic acid (DTPA) (20 μM), Arg-Gly-Asp-Ser (RGDS) (0.1–3 mM), or EGTA (5 mM) was incubated with WP for 1–2 min before addition of ONOO⁻ (150–200 μM) or decomposed ONOO⁻.

In a separate series of experiments, the effect of ONOO⁻ was studied on the inhibition of collagen-induced platelet aggregation induced by maximally effective (80–95%) concentrations of SNAP (3 or 10 μM), prostacyclin (3 or 10 nM), or indomethacin (0.3 or 1 μM). In this case, the inhibitors were incubated for 1 min with WP; then ONOO⁻ (150–200 μM) or decomposed ONOO⁻ was incubated for 3 min before addition of collagen (2–5 μg/ml). Finally, the effects of coincubations of ONOO⁻ with homologous PPP, human serum albumin (HSA), glutathione (GSH), or glutathione disulfide (GSSG) were studied on platelet aggregation induced by collagen (2–5 μg/ml). PRP (0.01–1%; vol/vol) or HSA (0.12–0.6 mg/ml) was incubated with platelets for 2 min before addition of ONOO⁻ (150–200 μM) and for 3–5 min before stimulation of platelets with collagen. GSH or GSSG (0.075–37.5 mM in 0.5 M sodium phosphate, pH 7.4) was preincubated with ONOO⁻ (5 mM) or decomposed ONOO⁻ for 1 min at room temperature and then added to the platelets (final concentrations, 0.75–375 and 50 μM, respectively) for 1 min before aggregation was induced by collagen. In some experiments, hemoglobin (5 μM) was preincubated with platelets for 1 min before addition of ONOO⁻ or the incubate. For statistical analysis, changes in platelet aggregation were

Abbreviations: PRP, platelet-rich plasma; WP, washed platelet(s); PPP, platelet-poor plasma; GSH, glutathione; GSNO, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; DTPA, diethylenetriaminepentaacetic acid; HSA, human serum albumin; GSSG, glutathione disulfide.

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expressed as percentage of the response to collagen (2–5 $\mu\text{g/ml}$).

Platelet Aggregation in PRP. Platelets were incubated for 1 min with ONOO⁻ (20–200 μM) or decomposed ONOO⁻ (200 μM at pH 7.4) in the absence or presence of hemoglobin (5 μM) and aggregation was induced by collagen (2–5 $\mu\text{g/ml}$).

Platelet Morphology. The effect of ONOO⁻ on platelet morphology was studied by light and electron microscopy. The WP samples were incubated in the aggregometer in the presence or absence of ONOO⁻ (200 μM), decomposed ONOO⁻, and SNAP (3 μM), and the effect of collagen (2 $\mu\text{g/ml}$) was recorded. For light microscopy studies, the platelets (50 μl) were fixed by using an equal volume of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) on a glass slide and examined by differential interference contrast microscopy with a Zeiss Axiophot photomicroscope. For transmission electron microscopy, platelets were prepared as described (10) and examined in a Philips CM10 transmission electron microscope.

Platelet P-Selectin Release. WP samples were prepared as described (11). The release of P-selectin, a marker for the platelet release reaction, was investigated by enzyme immunoassay.

Measurement of NO. ONOO⁻ (1 mM) or the products of its reaction with GSH or GSSG (1–3 mM in 0.5 M sodium phosphate, pH 7.4; 1 min at room temperature) were analyzed by chemiluminescence for generation of NO. The detection limit was in the parts per trillion range (12).

Measurement of S-Nitrosoglutathione (GSNO). The products of the reaction of ONOO⁻ (0.05–1 mM) for 1 min at room temperature with PPP or with GSH or GSSG (1 mM in 0.5 M sodium phosphate, pH 7.4) were analyzed for the presence of GSNO by reverse-phase high-performance liquid chromatography without prior sample processing (13). The detection limit for GSNO was 0.1 μM .

Synthesis of ONOO⁻. ONOO⁻ was synthesized as described and the concentration was determined spectrophotometrically ($\epsilon_{302} = 1670 \text{ M}^{-1}\text{cm}^{-1}$) (6, 14). Supplementation of the platelet suspension with 5 mM sodium phosphate (pH 7.4) was made where necessary in order to avoid alkalization. This concentration of buffer had no effect on platelet aggregation and dilutions of ONOO⁻ were made into water before addition to preparations of platelets. Control experiments were performed with decomposed ONOO⁻, which was prepared by incubation at room temperature in 0.5 M sodium phosphate (pH 7.4) for 5 min.

Reagents. Human hemoglobin was prepared by the method of Paterson *et al.* (15). Collagen (Hormon-Chemie, Munich); Tyrode's salt solution (GIBCO); SNAP, GSNO, and prostacyclin sodium salt (Wellcome); indomethacin, catalase, superoxide dismutase, mannitol, DTPA, EGTA, HSA, GSH, GSSG, and RGDS (all from Sigma); desferrioxamine (CIBA-Geigy); and P-selectin enzyme immunoassay kit (British Biotechnology, Oxford, U.K.) were obtained from the sources indicated.

Statistics. Results are means \pm SEM of at least three separate experiments. They were compared by analysis of variance and $P < 0.05$ was considered as statistically significant.

RESULTS

Aggregation of Washed Platelets by ONOO⁻. ONOO⁻, but not decomposed ONOO⁻ (20–200 μM), caused a partial but significant (maximum of $22\% \pm 3\%$ at 200 μM) and concentration-dependent increase in light transmission (Fig. 1A). This increase was due to the formation of platelet aggregates, as seen by light microscopy (Fig. 2A and C). Most of the intracellular granules appeared intact upon examination by electron microscopy (Fig. 2B and D) and release of P-selectin

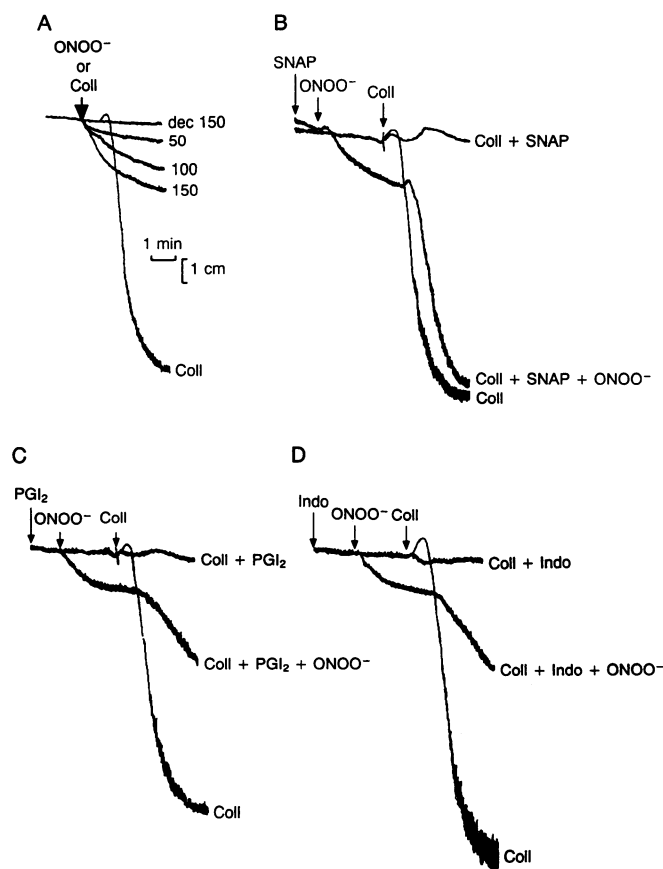


FIG. 1. Effects of ONOO⁻ on WP. (A) ONOO⁻-induced platelet aggregation. Superimposed traces of the result of incubating WP with ONOO⁻ (50–150 μM), decomposed ONOO⁻ (dec 150; 150 μM), or collagen (Coll; 2 $\mu\text{g/ml}$). (B–D). Effects of ONOO⁻ on inhibition by SNAP (B), prostacyclin (C), and indomethacin (D) of collagen-induced aggregation of WP. Traces of the effects of SNAP (Coll + SNAP; 10 μM ; B), prostacyclin (Coll + PGI₂; 10 nM; C), and indomethacin (Coll + Indo; 1 μM ; D) on collagen-induced aggregation of WP (Coll; 2 $\mu\text{g/ml}$). ONOO⁻ (200 μM) abolished the effect of SNAP (Coll + SNAP + ONOO⁻) and partially reversed the effects of PGI₂ (Coll + PGI₂ + ONOO⁻) and indomethacin (Coll + Indo + ONOO⁻). Traces are representative of 3–10 similar experiments.

was not detected (Table 1). Inhibitors of platelet aggregation such as SNAP, prostacyclin, and indomethacin did not affect ONOO⁻-induced platelet aggregation (Fig. 1B–D; $n = 3$ –6). ONOO⁻-induced aggregation was not modified by catalase, superoxide dismutase, mannitol, desferrioxamine, or DTPA (data not shown, $n = 3$ for each). In contrast, ONOO⁻-induced aggregation was completely inhibited by EGTA (5 mM; $n = 3$) and by RGDS ($\text{IC}_{50} = 0.67 \pm 0.11 \text{ mM}$; $n = 3$).

To assess the effects of ONOO⁻ on the SNAP-, prostacyclin-, and indomethacin-dependent inhibition of collagen-induced platelet aggregation, WP were treated with ONOO⁻ (150–200 μM) in the presence of these compounds. ONOO⁻ completely reversed the antiaggregatory effects of SNAP (Figs. 1B and 2E and G), whereas the effects of indomethacin and prostacyclin were reversed, respectively, by $51\% \pm 6\%$ and $48\% \pm 7\%$ ($n = 3$; Fig. 1C and D). Moreover, ONOO⁻, but not decomposed ONOO⁻, reversed SNAP-induced inhibition of the platelet release reaction (Table 1; Fig. 2F and H).

Inhibition of Platelet Aggregation by ONOO⁻ in PRP and in WP in the Presence of PPP and Thiols. ONOO⁻ (20–200 μM) did not induce aggregation in platelets suspended in PRP. Instead, it inhibited collagen-induced platelet aggregation ($\text{IC}_{50} = 102 \pm 31 \mu\text{M}$; $n = 3$), an effect that could be prevented by preincubation with hemoglobin (5 μM). This response

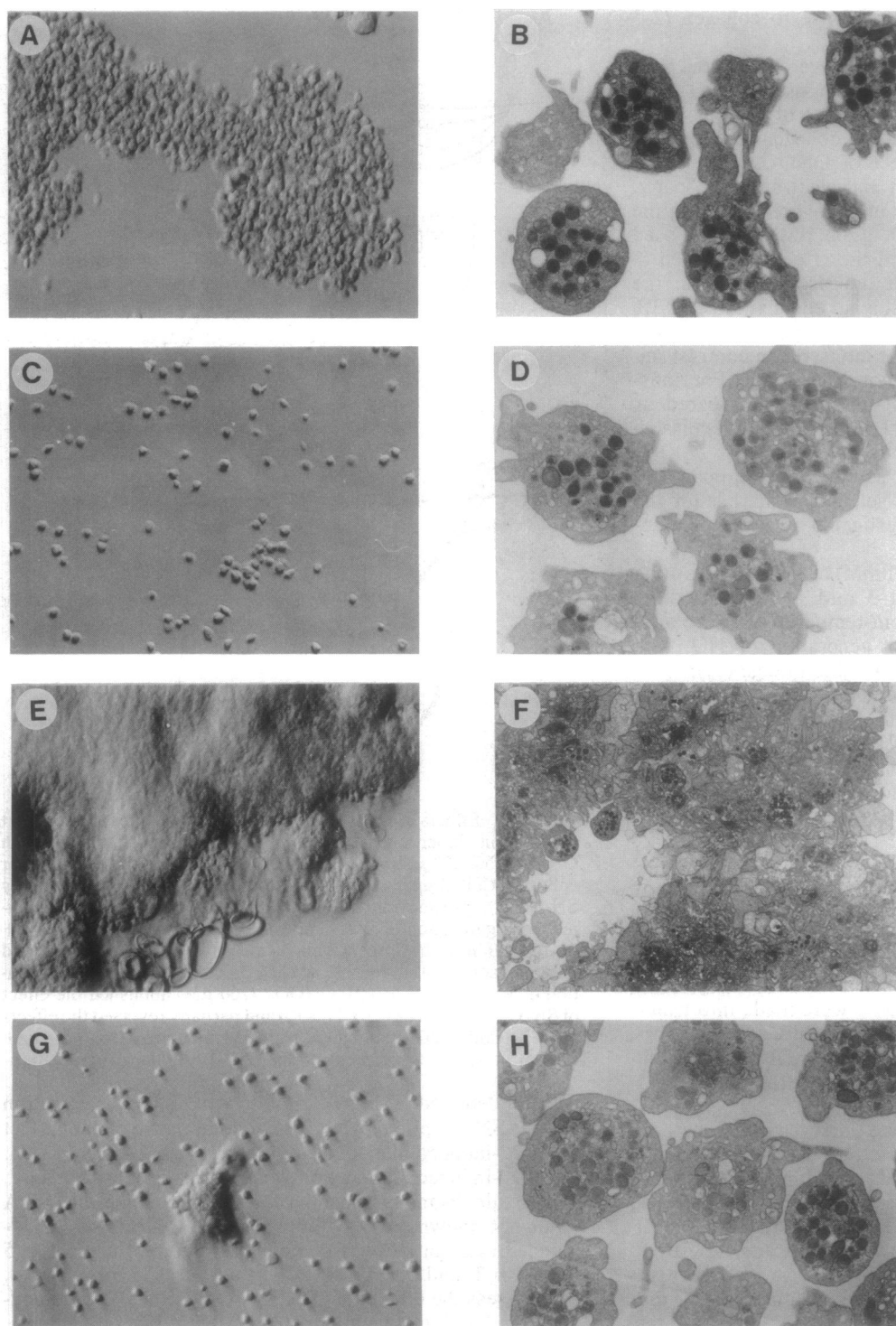


FIG. 2. Effect of ONOO^- on platelet morphology. Differential interference contrast (Left) and transmission electron microscopy (Right) were used to study platelet morphology. ONOO^- (200 μM) resulted in the formation of aggregates (A) but not in degranulation (B), while decomposed ONOO^- (200 μM ; C and D) had no effect. ONOO^- (200 μM ; E and F) but not decomposed ONOO^- (200 μM ; G and H) reversed SNAP (3 μM)-induced inhibition of collagen (2 $\mu\text{g}/\text{ml}$)-induced aggregation. [A, C, E, and G, contrast microscopy, $\times 550$; B, D, F, and H, electron microscopy; $\times 8625$ (B, D, and H); $\times 2850$ (F)].

could be reproduced in WP in a concentration-dependent manner by addition of PPP (0.01–1%) (Fig. 3A) or by addition of HSA (0.12–0.6 mg/ml) to WP prior to ONOO^- (Fig. 3B). Furthermore, the products of the reaction of ONOO^- with GSH but not GSSG also resulted in inhibition of aggregation ($\text{IC}_{50} = 4.4 \pm 0.5 \mu\text{M}$ GSH; $n = 3$) (Fig. 3C). The inhibitory effects of PPP, HSA, and the reaction products of ONOO^- with GSH were prevented by hemoglobin (Fig. 3).

NO and GSNO Formation from ONOO^- and GSH. There was very little formation of NO during the spontaneous decomposition of ONOO^- (1 mM) in phosphate buffer at pH 7.4, as detected by chemiluminescence. However, in the presence of GSH but not GSSG, significant amounts of NO were formed (Fig. 4A). In addition, the incubation of ONOO^-

(0.05 mM) with GSH but not GSSG (1 mM) resulted in concentration-dependent formation of GSNO (Fig. 4B), with a maximal yield of 1–2%. The addition of ONOO^- to plasma, however, did not produce detectable amounts of GSNO.

DISCUSSION

We have shown that ONOO^- causes aggregation of WP, which is not associated with platelet release reaction. This seems to be a direct effect of ONOO^- and not due to the secondary formation of H_2O_2 , O_2^- , or OH^\cdot since it is not affected by scavengers of these oxidants or chelators of transition metals, which could have been involved in their generation. Furthermore, aggregation is not affected by

Table 1. Aggregation and corresponding release of P-selectin from WP

| | Aggregation, % of maximal | P-selectin release, ng/ml |
|---|------------------------------|------------------------------|
| Basal | 1 ± 1 | ND |
| ONOO ⁻ (100 μM) | 9 ± 2 | ND |
| ONOO ⁻ (200 μM) | 23 ± 3 | ND |
| Dec ONOO ⁻ (200 μM) | 1 ± 1 | ND |
| Collagen | 100 ± 0 | 201 ± 11 |
| Collagen + SNAP | 13 ± 3 | 67 ± 3 |
| Collagen + SNAP + ONOO ⁻ (200 μM) | 88 ± 7 | 114 ± 7 |
| Collagen + SNAP + dec ONOO ⁻ (200 μM) | 9 ± 2 | 80 ± 1 |

ONOO⁻, but not decomposed ONOO⁻ (Dec ONOO⁻), resulted in aggregation but not in the release of P-selectin. Both aggregation and P-selectin release were induced by collagen (2–5 μg/ml). These were inhibited by SNAP (3 or 10 μM). The inhibitory activity of SNAP was reversed by ONOO⁻ but not by its decomposition products. Basal, aggregation and P-selectin release in unstimulated platelets; ND, not detectable (<20 ng/ml). Results are means ± SEM of three experiments.

known inhibitors of platelet function such as the NO donor SNAP (13), prostacyclin (16), or indomethacin (17). Moreover, in the presence of ONOO⁻, the SNAP-dependent inhibition of collagen-induced platelet aggregation is abolished and those of both prostacyclin and indomethacin are reduced. In addition, ONOO⁻ reverses the SNAP-dependent inhibition of the collagen-stimulated release of P-selectin. This effect might be due to a direct reaction between ONOO⁻ and SNAP. However, this does not appear to be the case since the same results are obtained if SNAP is added after ONOO⁻ has decomposed in the platelet suspension. Thus, ONOO⁻ counteracts platelet-inhibitory mechanisms induced by stimulation of cGMP (18), cAMP (19), and by inhibition of thromboxane formation (20), respectively. However, the proaggregatory action of ONOO⁻ can be prevented by RGDS (21), a IIb/IIIa receptor antagonist, or by EGTA, a calcium-

chelating agent that interferes with the exposure of this receptor (22). These results are consistent with the possibility that ONOO⁻ causes platelet aggregation by stimulating directly the exposure of platelet receptors such as IIb/IIIa. This, however, remains to be investigated in detail.

While ONOO⁻ is clearly proaggregatory in WP, in PRP it inhibits platelet aggregation. In fact, very small quantities of plasma added to WP are sufficient to reveal this platelet-inhibitory action of ONOO⁻. It is, therefore, likely that ONOO⁻ is converted into a different entity in the presence of plasma. Several substances may be responsible for this action; plasma thiols are likely candidates since they are known to counteract the actions of oxidants (23). We examined, therefore, the interactions of albumin (a protein containing a free thiol) and GSH (a low molecular weight thiol) with ONOO⁻ and found that both can mimic the effect of plasma on ONOO⁻. Moreover, this effect is abolished by hemoglobin. These data suggest that ONOO⁻ is converted by thiols to NO and/or an NO donor, most probably *S*-nitrosoalbumin and/or GSNO (24, 25).

In addition, we have shown that NO and GSNO are formed in the reaction between ONOO⁻ and GSH. The mechanism of GSNO formation probably involves the homolytic cleavage of ONOO⁻ at neutral pH (26), yielding NO₂, which nitrosylates thiols (27). The release of NO from ONOO⁻ was detected only in the presence of GSH, presumably via formation of GSNO.

We did not detect formation of GSNO during incubation of ONOO⁻ in plasma. This may be due to the fact that the levels of GSH in plasma are in the low micromolar range (28). Since the yield of GSNO formation from ONOO⁻ and GSH is ≈1%, the amount of plasma GSNO that could be formed is below the detection limit of our assay (0.1 μM). Authentic GSNO inhibits platelet aggregation in plasma at concentrations <0.1 μM (25); thus, it is still possible that enough of this compound is formed to exert a biological action on platelets. In addition, *S*-nitrosylation of other thiols in plasma (e.g., albumin) could contribute to the inhibition of platelet aggregation during the reaction of ONOO⁻ with plasma.

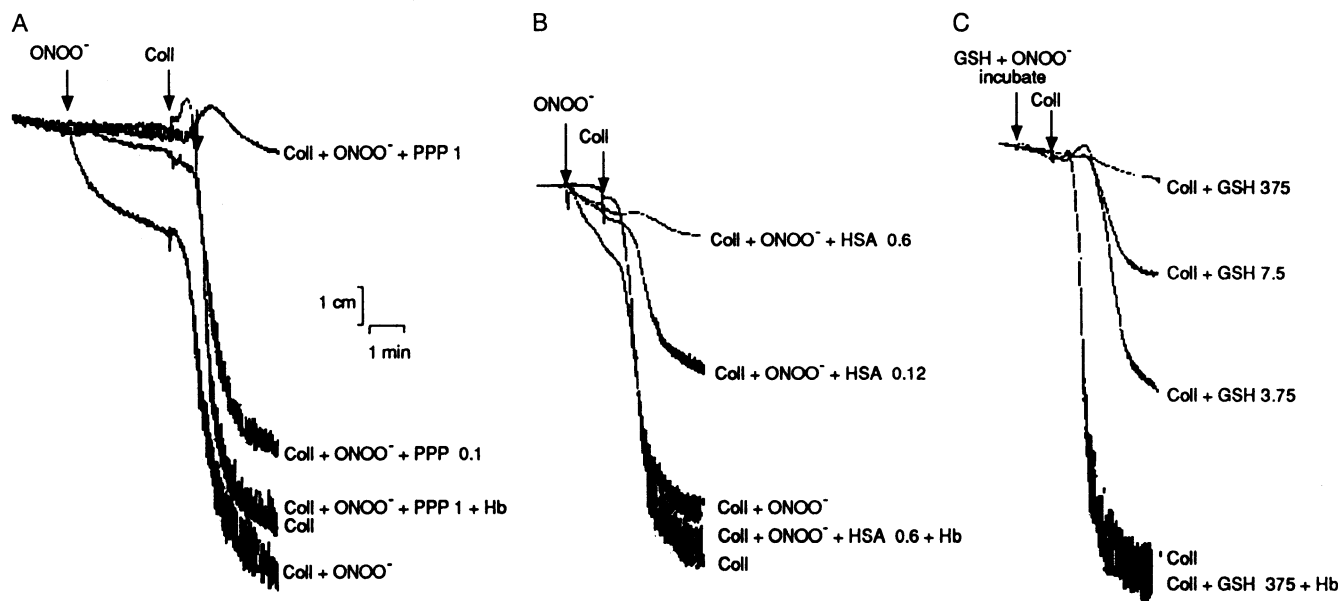


FIG. 3. Interactions between ONOO⁻ and PPP or thiols on collagen-induced aggregation of WP. (A and B) In the absence of plasma or human serum albumin, ONOO⁻ (200 μM) induced a partial aggregation that was further increased by collagen (Coll + ONOO⁻). This effect of ONOO⁻ was inhibited in a concentration-dependent manner by plasma (0.1% or 1%, Coll + ONOO⁻ + PPP 0.1 or 1) or by HSA (0.12 or 0.6 mg/ml, Coll + ONOO⁻ + HSA 0.12 or 0.6). Hemoglobin (5 μM) abolished the antiaggregatory effect of both 1% PPP (Coll + ONOO⁻ + PPP 1 + Hb) and HSA (0.6 mg/ml) (Coll + ONOO⁻ + HSA 0.6 + Hb). (C) Collagen-induced aggregation was inhibited by addition of an incubate of ONOO⁻ with GSH (3.75–375 μM) (Coll + GSH 3.75 or 7.5 or 375). The inhibitory action of the maximally effective concentration of GSH was abolished by hemoglobin (5 μM; Coll + GSH 375 + Hb). Traces are representative of three experiments.

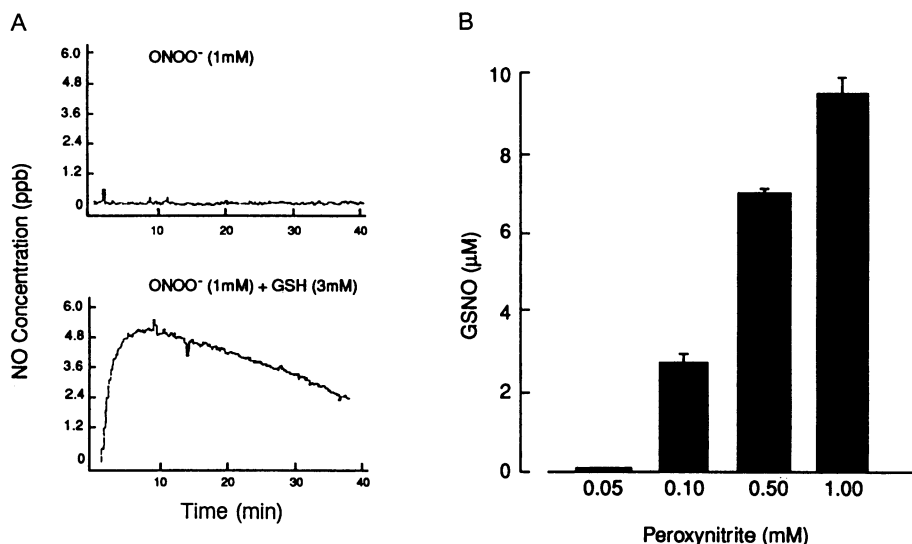


Fig. 4. Analysis of reaction products of GSH with ONOO⁻. Detection of NO by chemiluminescence (A) and of GSNO by HPLC (B). Results are means \pm SEM of three experiments.

In summary, our results demonstrating the paradoxical fate and actions of ONOO⁻ show that this compound cannot only be considered a cytotoxic agent in biological systems. Leaving aside the unresolved controversy about its generation *in vivo*, the reactivity of ONOO⁻ is such that, in many circumstances, it will lead to the formation of compounds with the capacity to generate NO, as we have clearly demonstrated. Indeed, it is likely that not only thiols, as we have shown, but other molecules might react with ONOO⁻ yielding products with NO-like activity. This series of reactions represents a very efficient defense mechanism against the potential toxicity of ONOO⁻. However, there are pathological conditions in which depletion of thiols may favor the direct interaction of ONOO⁻ with cell membranes, leading to tissue damage. Evidence suggests that oxidative damage and thiol depletion play a role in the pathogenesis of atherosclerosis (29–31). Furthermore, ONOO⁻ may be generated in the atherosclerotic plaque (32). Thus, the persistent production of oxidants, including ONOO⁻, may cause a depletion of thiols. This, in turn, would render tissues unprotected against the actions of ONOO⁻, leading to platelet aggregation, probably leukocyte activation, and progression of the atherosclerotic lesion. In this context, it is also worth investigating whether thiols, which have been claimed to be therapeutically useful in pathological conditions associated with oxidant damage, exert their protective actions via the mechanism that we have now described.

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