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Interactions of peroxynitrite and other nitrating substances with human platelets: the role of glutathione and peroxynitrite permeability

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Abstract

Platelets labeled with 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA) and stimulated with 50–400 nM peroxynitrite ($ONOO^-$) produced a rapid increase of the fluorescence signal at 523 nm with good linearity and reproducibility. Platelet fluorescence was inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), suggesting that HCO_3^-/Cl^- transporter mediated $ONOO^-$ transport into the platelets. Exposure of platelets to potassium superoxide, hydrogen peroxide, and sodium nitroprusside at concentrations of up to 100 μM did not generate a fluorescence signal. We also studied other nitrating compounds to establish the specificity of the DCF-DA-labeled platelet $ONOO^-$ assay. A rapid increase of fluorescence was observed when sodium hypochlorite (0.15 to 0.75 mM) was added to platelets suspended in a buffered nitrite solution. Exposure of platelets to NO_2, nitroglycerin, and tetranitromethane produced a slow sustained increase of fluorescence. Endogenous glutathione appeared to be an essential factor in the generation of fluorescence by $ONOO^-$ and other nitrating compounds. We further studied other conditions that increased platelet fluorescence. Stimulation of platelets with thrombin (1 U/mL) produced a rapid increase in fluorescence that corresponded to the formation of 20.5 nmol $ONOO^-$ per 10^7 cells, whereas stimulation with collagen and arachidonic acid was without effect. Hypoxia of platelets for 20 and 40 min followed by 5 min of reoxygenation doubled the fluorescence from these platelets compared with control platelets. Thus, thrombin produced an effect that was likely due to the formation of $ONOO^-$ in platelets, whereas hypoxia–reoxygenation was likely to cause the formation of an active nitroglutathione-like molecule.

Keywords: Platelets; Peroxynitrite; Nitroglycerin; Glutathione; Hypoxia–reoxygenation; Fluorometry

1. Introduction

Activated platelets produce relatively large amounts of superoxide (O_2^-) and hydroxyl ($^\bullet OH$) radicals [1–3]. These species can cause platelet aggregation that is mediated, in part, by products of lipid peroxidation originating from the attack of $^\bullet OH$ on fatty acids [3]. Platelets also biosynthesize NO [4] by means of the constitutive and inducible forms of NOS [5–7]. Because NO reacts with O_2^- to form $ONOO^-$, a potent oxidant and nitrating molecule, circulating platelets might potentially contribute to tissue damage by releasing $ONOO^-$ in sites more remote to the site of injury and their activation. Previous work has established that $ONOO^-$ modulates platelet function [8–12], which largely depends on the dose applied and the cellular milieu. In our previous study, $ONOO^-$ inhibited platelet aggregation induced by arachidonic acid at the effective concentration of 5.8 μM [12]. This inhibition was caused by a fairly selective nitration of critical tyrosine residues of the cyclooxygenase enzyme. At concentrations below 50 μM, $ONOO^-$ did not induce platelet aggregation [8,12]; however, at concentrations greater than 50 μM, the aggregatory effect of $ONOO^-$ was detectable [8]. Radomski and coworkers [8] have reported that $ONOO^-$ stimulates the aggregation of washed platelets and reverses the inhibition of aggregation induced by sodium nitroprusside, prostacyclin, and indomethacin. $ONOO^-$-induced aggregation is not due to the formation of H_2O_2, O_2^-, and $^\bullet OH$ radicals since scavengers of these oxidants or chelators of transition metals did not affect aggregation [8]. $ONOO^-$ counteracts platelet-inhibitory mechanisms induced by stimulation of cyclic GMP and cyclic AMP, and

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Abbreviations: $ONOO^-$, peroxynitrite anion and its conjugated form; DCF-DA, 2′,7′-dihydrodichlorofluorescein diacetate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; L-NAME, L-nitroarginine methyl ester; DEM, maleic acid diethyl ester; DCFH, 2′,7′-dihydrodichlorofluorescein; $GSNO_2$, *S*-nitroglutathione; NaOCl, sodium hypochlorite; NO, nitric oxide; and NOS, nitric oxide synthase.

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by inhibition of thromboxane A_2 (TxA_2) formation. Proaggregatory action of $ONOO^-$ can be prevented by RGDS, a GPIIb-IIIa receptor antagonist, or by EGTA, a calcium-chelating agent that may interfere with the exposure of this receptor. In platelet-rich plasma, $ONOO^-$ loses its proaggregatory properties and inhibits platelet aggregation largely because it forms various nitrosothiols such as GSNO that release NO and inhibit aggregation [8,10]. 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 [8]. Thus, it appears that the fate and the actions of $ONOO^-$ in biological systems critically depend on the biological environment in which this oxidant is present. Other effects involve nitration of platelet protein tyrosine residues, a process that impairs platelet function [11,12]. Pretreatment of platelets with $ONOO^-$ decreases thrombin-induced tyrosine phosphorylation, P selectin expression, serotonin secretion, and aggregation. $ONOO^-$ exposure and tyrosine nitration decreases platelet sensitivity to thrombin but does not inhibit tyrosine phosphorylation and other platelet responses [11]. In addition, $ONOO^-$ inhibits cyclooxygenase and stimulates the formation of new lipids including *trans*-fatty acids [12]. In the present study, we further characterized the formation and transport of $ONOO^-$ in the human platelet and found that a substance with fluorogenic and oxidative properties similar to those of $ONOO^-$ was produced by stimulation of platelets with thrombin and carbon monoxide, whereas hypoxia–reoxygenation generated an oxidant that was likely to be a product of glutathione with a nitrating molecule, NO_2 or $ONOO^-$.

2. Materials and methods

2.1. Chemicals

DIDS, DEM, L-NAME, and thrombin (from bovine plasma) were purchased from the Sigma Chemical Co. DCF-DA was purchased from Molecular Probes. Nitroglycerin (0.4 mg sublingual tablets) was purchased from Parke-Davis. Nitrogen dioxide was from the Matheson Tri-Gas Co. All other reagents were of research grade quality. $ONOO^-$ was synthesized in a quenched-flow reactor as previously described [9]. Hydrogen peroxide was removed from the solution by the addition of granular manganese oxide, and $ONOO^-$ concentrations were determined spectroscopically by measuring the absorbance at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$). Dilutions of $ONOO^-$ in 0.1 M NaOH were made shortly before use to achieve the desired concentrations.

2.2. Platelet preparation

Fresh platelet concentrate from healthy human donors (N = 5) who had not taken any medication known to influence platelet function was purchased from Hudson Valley Blood Services. Platelet concentrate (10 mL) was subject to centrifugation at 1100 *g* for 15 min at 21°. The pellet was resuspended in 5 mL of buffer A, which contained NaCl (140 mM), KCl (2 mM), $NaHCO_3$ (14 mM), glucose (5.5 mM), $MgCl_2$ (1 mM), HEPES buffer (5 mM), 0.2% BSA, 0.2 μM prostaglandin E_1 (PGE_1), and 1.5 μg/mL of apyrase, with the pH adjusted to 6.4. Platelets were counted by using a PS 540 electronic counter (Coulter Electronics). Platelets were centrifuged again at 1100 *g* for 15 min at 21°, and the pellet was resuspended in buffer B (same as buffer A but without BSA, PGE_1, and apyrase, pH = 7.4); the final platelet concentration was 1×10^9 cells/mL.

2.3. Fluorescence measurements

In a typical experiment, 5 mL of platelet suspension in buffer B was incubated with DCF-DA (final concentration 50 μM) for 30 min, and then the platelets were washed two times to remove extracellular DCF-DA and finally resuspended in 5 mL of buffer B. The platelet suspension (100 μL) was added to 2.9 mL of the working solution (90 mM NaCl, 5 mM KCl, 50 mM sodium phosphate buffer at pH 7.4) in a fluorescence cuvette so that the final concentration of platelets was 3×10^7 cells/mL. The plastic disposable cuvettes showing no background absorbance and emission in the working wavelength were purchased from Fisher. The cuvettes were placed on a magnetic microplate for continuous stirring with a remote speed control set at 900 rpm. A Teflon™-coated 7-mm metal bar was used to initiate stirring of the cells. The cuvettes were thermostated at 37° with a temperature controller. Fluorescence measurements were conducted using a Shimadzu fluorospectrophotometer (model RF-5000). The cuvette cells were irradiated with an excitation wavelength of 503 nm, and the emission was recorded at 523 nm. Both the emission and excitation lights were passed through a 10-nm monochromator slit. The $ONOO^-$ solution (in 0.1 N NaOH) was injected slowly into the platelet suspension with a precision micro gas-tight syringe attached to a 30-cm flexible narrow needle (0.25 mm i.d.). Aliquots of $ONOO^-$ solution were delivered near the bottom of the cuvette and outside the light path. Control experiments were performed with $ONOO^-$ decomposed in phosphate buffer (pH 7.4) for 5 min and with vehicle solvents. Addition of control solutions did not produce fluorescence in platelets and did not change the pH of buffer B. In experiments where the $ONOO^-$ transport was characterized, aliquots of DCF-DA-treated platelets (3 mL) resuspended in buffer B were incubated separately for 30 min at room temperature with the following inhibitors: DIDS (300 μM), DEM (7 mM) and L-NAME (300 μM). Control samples with inhibitor solvents (DMSO, ethanol at 1 μL/mL) and without additives were incubated in parallel. After incubation, the platelets were rinsed and resuspended in 1 mL of buffer B and submitted for fluorescence measurements. A nitroglycerin tablet was dissolved

Table 1
Comparison of fluorescence and equivalent peroxynitrite concentrations generated by DCF-DA-labeled human platelets[a]

Treatment	Fluorescence (units)	$ONOO^-$ (nmol/10^7 cells)
Hypoxia–reoxygenation (40 min/5 min)	32.3 ± 7	256 ± 56
Air (40 min)[b]	16.1 ± 4	128 ± 32
NO_2 (47 μM)[c]	14.2	113.3
Tetranitromethane[d]	23.8	189.1
Tetranitromethane[d] + DEM[e]	10	80
NaOCl[f]	0	0
NaOCl[f] + nitrite (50 mM)	19.4 ± 8	154.6 ± 64
Nitroglycerin[g]	85	675.2
Nitroglycerin[g] + DEM[e]	4	32.4
Thrombin (1 U/mL)	2.5 ± 0.1	20.5
Carbon monoxide[h]	2.4	19.7
SNP[i] (100 μM)	0	0
KO_2 (100 μM)	0	0
H_2O_2 (50 μM)	0	0
H_2O_2 (100 μM) + nitrite (50 mM)	0	0
Collagen (0.02%)	0	0
Arachidonic acid (10 μM)	0	0

[a] Fluorescence was measured 2–3 min after treatment; N = 3–5 for measurements where SEM is given.
[b] Hypoxia control.
[c] 10 μL delivered in a gas-tight syringe.
[d] 4 μL, 0.3 mM.
[e] DEM (1 μL/mL, final concentration, 7 mM).
[f] 10 μL of a 3% solution in 0.1 N NaOH.
[g] 0.4 mg table dissolved in 3 mL resulting in final concentration of 6.3 μM.
[h] Bubbled at ~1 mL/min for 0.3 min.
[i] Sodium nitroprusside.

in buffer B, and 10–100 μL was added to a platelet suspension. Other chemicals as listed in Table 1 were added, using the microsyringe, to the cuvette of platelet suspension placed in the fluorospectrophotometer.

2.4. Hypoxia–reoxygenation

Nitrogen (~1 mL/min) was bubbled into a 3-mL suspension of DCF-DA-labeled platelets (4×10^7 cells/mL) through a narrow flexible plastic tube equipped with a glass capillary for 20 and 40 min. This was followed by 5 min of reoxygenation with air. Fluorescence was monitored during the reoxygenation period and shortly thereafter.

2.5. Statistical analyses

Results are expressed as means ± SEM, with N equal to the number of platelet donors used for separate measurements. Comparisons between groups were made by ANOVA and Student's *t*-test with a Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was used to determine statistical significance.

3. Results

A sensitive and specific fluorescence assay made it possible to study the formation and permeability of $ONOO^-$ in the human platelet. Exposure of DCF-DA-labeled platelets

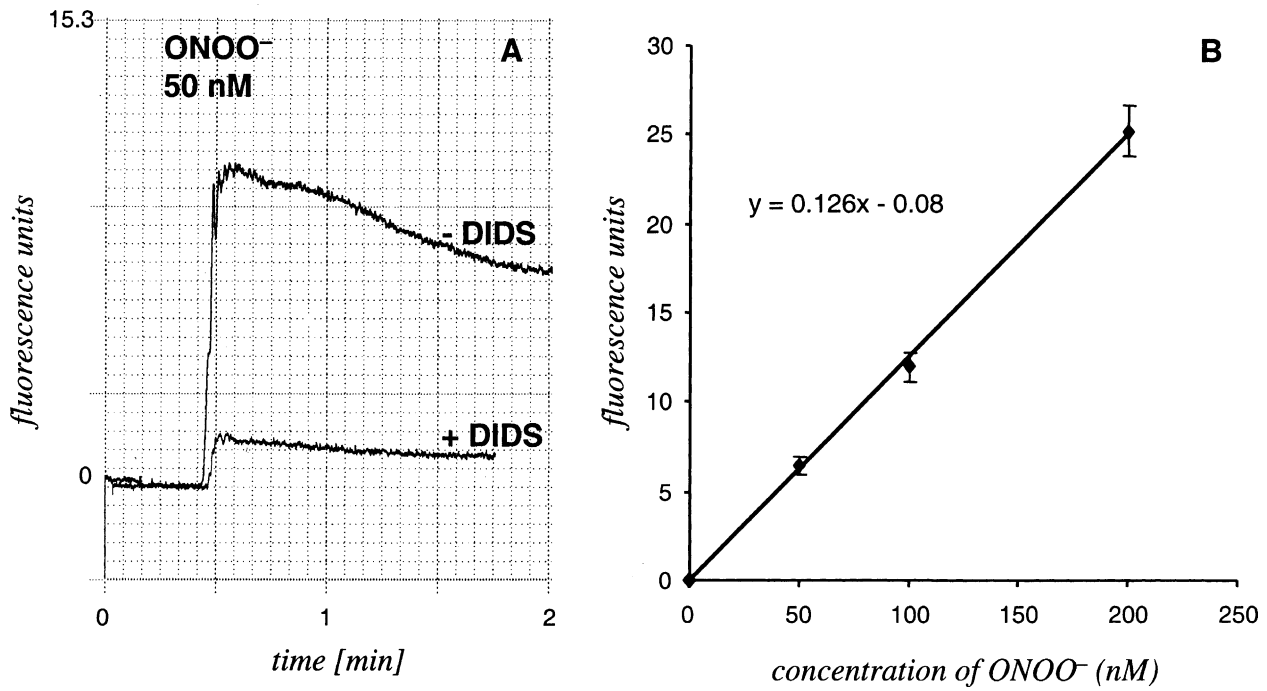

Fig. 1. Detection of fluorescent light emitted by DCF-DA-labeled platelets. (A) Effect of DIDS (300 μM), an inhibitor of HCO_3^-/Cl^- anion transporter, on fluorescence induced by peroxynitrite ($ONOO^-$, 50 nM) at 523 nm (at an excitation wavelength of 503 nm). DIDS slowed the transport of $ONOO^-$ across the platelet membrane by >88%. (B) A linear correlation obtained between the concentration of $ONOO^-$ and fluorescence was used to calculate the amount of $ONOO^-$ from fluorescence measurements (mean ± SEM, N = 3).

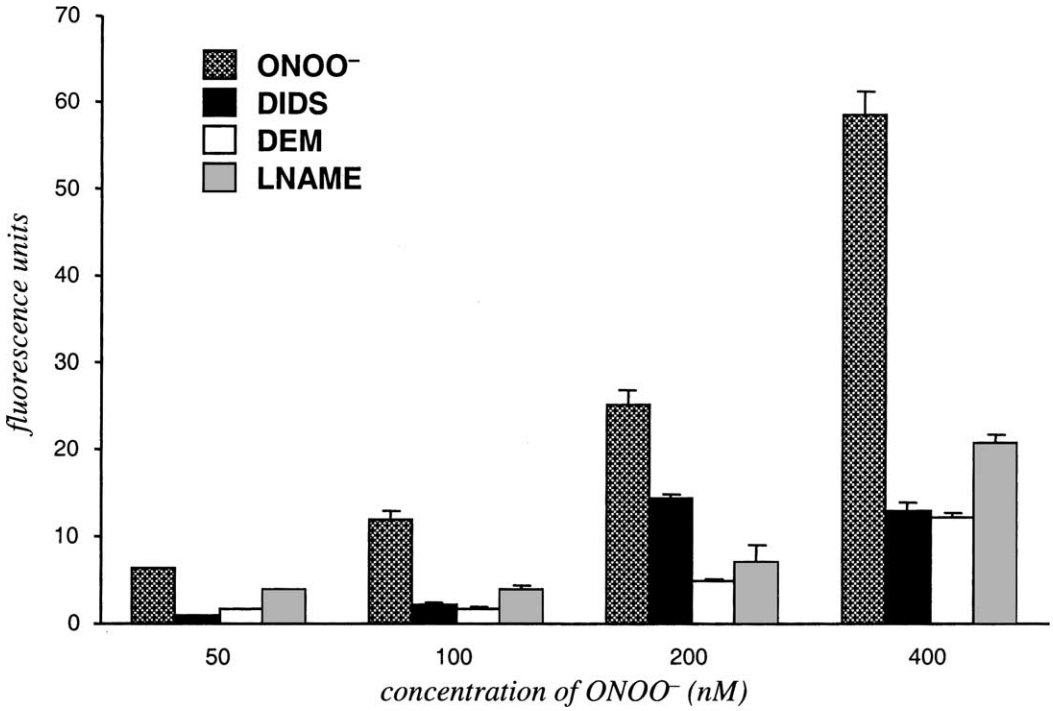

Fig. 2. Inhibition of $ONOO^-$-generated fluorescence. DIDS (300 μM), DEM (7 mM), and L-NAME (300 μM) reduced fluorescence in DCF-DA-labeled platelets. Bars represent average values of fluorescence detected following the addition of $ONOO^-$ (mean ± SEM, N = 3).

to $ONOO^-$ (50–400 nM) produced a concentration-dependent linear increase of the fluorescence signal at 523 nm upon excitation at 503 nm (Fig. 1). The fluorescence could be observed in a platelet suspension with the final $ONOO^-$ concentration as low as 50 nM (Fig. 1). A linear regression between the fluorescence intensity and $ONOO^-$ concentration (50–200 nM) allowed calculating the $ONOO^-$ concentration from the intensity of the fluorescent light emitted from platelets (Fig. 1B). The generation of fluorescence from DCF-DA-labeled platelets appeared to be specific for $ONOO^-$ because the addition of hydrogen peroxide (1–50 μM), sodium nitroprusside (1–100 μM), or potassium superoxide (1–100 μM) did not generate fluorescence (Table 1).

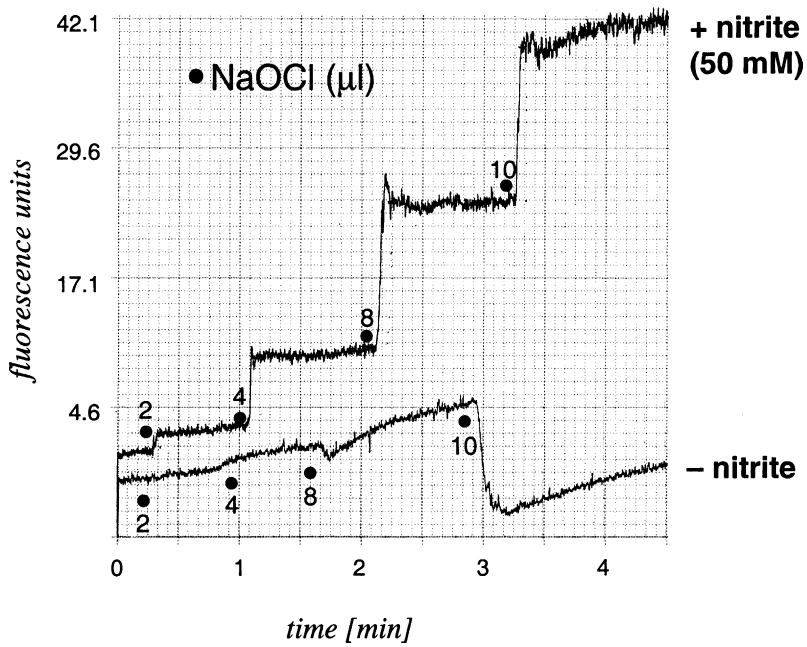

Fig. 3. Effect of nitrite (50 mM) on fluorescence induced by the addition of NaOCl (3% solution in 0.1 N NaOH) to platelets. Dots indicate addition time and volume of NaOCl solution.

3.1. Transport of $ONOO^-$

We further characterized the permeability of $ONOO^-$ across the platelet membrane. A specific inhibitor of anion transport, DIDS, decreased by 42–78% the fluorescence generated by $ONOO^-$ (50–400 nM) (Figs. 1A and 2). The generation of fluorescent light by $ONOO^-$ was also inhibited by DEM (7 mM), which inhibited $ONOO^-$-induced fluorescence by 80–86%, suggesting that glutathione participated in the generation of fluorescence from $ONOO^-$. L-NAME, an inhibitor of NOS, also decreased the generation of $ONOO^-$ fluorescence by 64–71%, indicating that L-NAME competes with $ONOO^-$ transport or that the generation of NO is important for $ONOO^-$-mediated increase of fluorescence in DCF-DA-labeled platelets.

3.2. Other oxidants and nitrating compounds

To further study the mechanisms by which DCF-DA-labeled platelets produce fluorescence, the effects of other

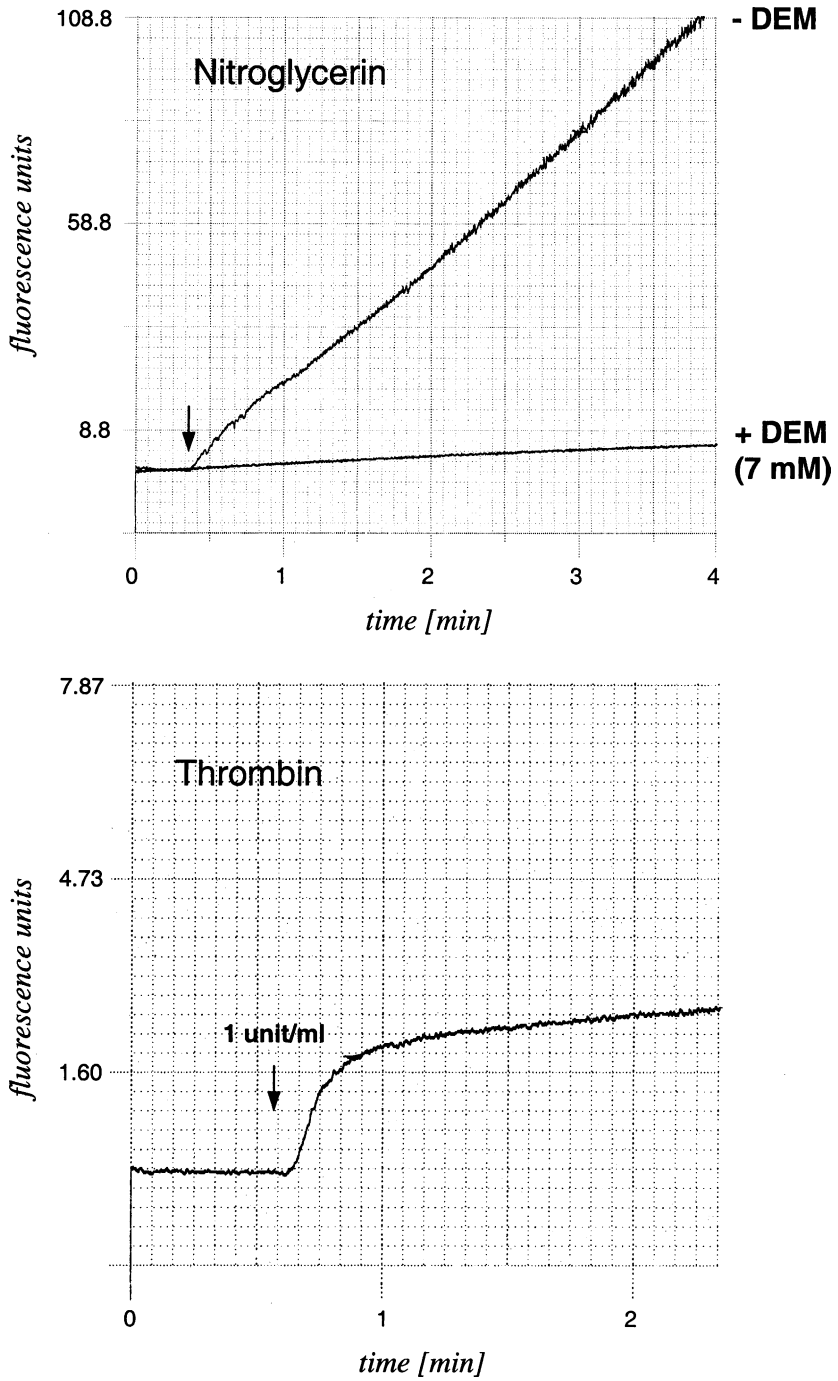

Fig. 4. Fluorescence profiles in the DCF-DA-labeled platelets. Nitroglycerin (6.3 μM) produced a steady increase of a fluorogenic compound in a manner sensitive to DEM (7 mM) (top panel), whereas thrombin (1 U/mL) caused a rapid increase similar to that produced by $ONOO^-$ (bottom panel).

compounds known to have nitrating and/or oxidative properties were studied and compared with $ONOO^-$. We found two types of fluorescence signal responses. One response was similar to that generated by $ONOO^-$; it was characterized by a rapid onset and then a sustained signal with a tendency to decrease with time. This type of response was also observed when NaOCl (3% in 0.1 N NaOH, 2–10 μL) was added to platelets suspended in the buffer also containing 50 mM nitrite (Fig. 3). Under these conditions, NO_2Cl (nitryl chloride) was probably produced. The amount of NO_2Cl generated in this experiment was difficult to determine, but it was likely to be in the nanomolar range based on the strength of the fluorescence signal as compared to the signal generated by $ONOO^-$, assuming similar oxidation potential towards 2′,7′-dihydrodichlorofluorescein. Nitrite and NaOCl in the absence of platelets as well as NaOCl in the absence of nitrite did not generate fluorescence (Fig. 3). Interestingly, in the absence of nitrite a decrease of fluorescence intensity probably resulted from "bleaching" of the dye by NaOCl. The other type of response was observed with nitroglycerin and NO_2 (Fig. 4 and Table 1). The fluorescence signal was sustained and did not appear to reach a plateau within the time of the experiment. Gaseous NO_2 delivered at a concentration of 47 μM/min produced a similar increase of the fluorescence signal. Nitroglycerin (0.4 mg tablet dissolved in 3 mL, final concentration of 6.3 μM) also produced a sustained increase of fluorescence (Fig. 4). The nitroglycerin-induced fluorescence was inhibited almost completely by DEM (Fig. 4, Table 1). Tetranitromethane (4 μL, 0.3 mM) produced a sustained and continuous increase of fluorescence over 5 min that was nearly abolished by DEM. Table 1 summarizes the data of our fluorescence assay, which was designed to detect the formation of $ONOO^-$ in platelets. Curiously, CO toxicity has been known to induce protein nitration probably via $ONOO^-$ formation [13]. Exposure of DCF-DA-labeled platelets to CO confirmed the formation of an $ONOO^-$-like oxidant that induced fluorescence (Table 1). CO did not produce fluorescence in a cell-free system.

3.3. Platelet agonists

Platelets stimulated with thrombin at concentrations of 1 and 0.33 U/mL produced an increase of fluorescence equivalent to 20.5 nmol of $ONOO^-/10^7$ cells (Fig. 4, bottom panel) and 5.6 nmol $ONOO^-/10^7$ cells (not shown), respectively. Stimulation of platelets by collagen and arachidonic acid was without effect on fluorescence (Table 1). H_2O_2 with or without nitrite had no effect on the fluorescence signal (Table 1).

3.4. Hypoxia–reoxygenation

Platelets that were exposed to hypoxia for 20 min and then reoxygenated for 5 min (Fig. 5) showed a progressive increase in their fluorescence signal during reoxygenation compared with control cells, which were bubbled with air over the entire period (63 ± 9 vs 148 ± 13 nM $ONOO^-$, $N = 5$). A similar trend was observed when platelets were hypoxic for 40 min and reoxygenated (128 ± 16 vs. 256 ± 25 nM $ONOO^-$, $N = 5$). Thus, hypoxia followed by reoxygenation formed a substance in platelets that oxidized DCF-DA to a product that had fluorogenic properties similar to those of a product formed by exposure of

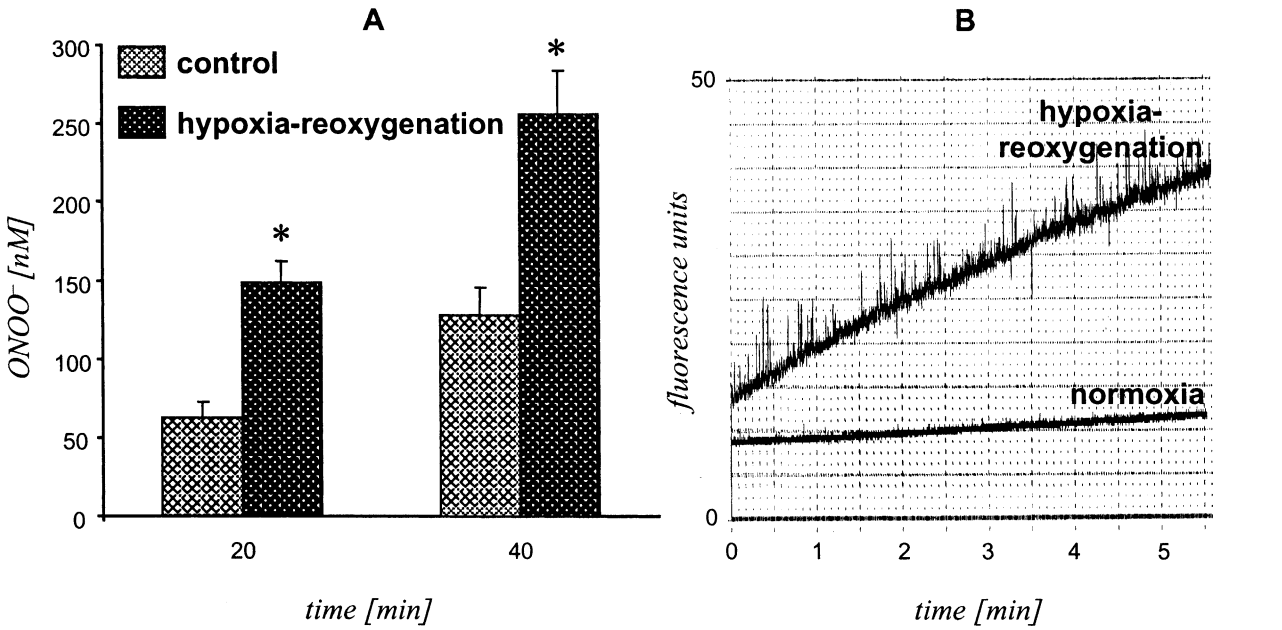

Fig. 5. Detection of hypoxia–reoxygenation-induced fluorescence in human platelets. (A) Hypoxia (20 and 40 min) followed by reoxygenation (5 min) caused a significant increase of fluorescence as compared with control normoxic platelets ($^* = P < 0.05$). Values are means ± SEM, $N = 5$. (B) Fluorescence (523 nm) recordings from DCF-DA-labeled platelets rendered hypoxic (40 min) and control normoxic. Top trace shows the increase of fluorescence during the reoxygenation period.

DCF-DA-labeled platelets to $ONOO^-$. As other oxidants did not generate fluorescence, it is likely that the increase of fluorescence by hypoxia–reoxygenation was the result of endogenous formation of $ONOO^-$ from NO and O_2^- in platelets.

4. Discussion

DCF-DA is a membrane-permeable non-fluorescent dye that is hydrolyzed by esterase(s), within the platelet cytoplasm, into a free acid, DCFH. $ONOO^-$ oxidizes DCFH to a strongly fluorescent dye [14,15], a process that is likely to involve a direct reaction of ONOOH with DCFH. ONOOH, a conjugate acid of $ONOO^-$ having a pK_a of 6.8 [16], can be generated in the platelet cytosol, which is more acidic (pH_i 6.9) relative to the extracellular milieu [17]. DCFH and its oxidized form are not membrane permeable; thus, the fluorescence observed by us originated from a dye accumulated within the cell cytoplasm as no dye was found in the medium of the washed cells. Detection of fluorescence from DCF-DA-labeled platelets indicated that $ONOO^-$ must have crossed the platelet membrane in order to reach the intracellular sites where DCFH was accumulated. Oxidation of the intra-platelet DCFH appeared to be a sensitive assay that allowed studying the effects of $ONOO^-$ in human platelets. The generated fluorescence was $ONOO^-$ specific with a sensitivity in the nanomolar range. In agreement with previous data [14], hydrogen peroxide, superoxide, and NO at concentrations below 100 μM failed to produce fluorescence. Previous studies have characterized the formation of superoxide and NO in platelets, which are then likely to generate $ONOO^-$ because these two radicals react with a high rate of $>10^9\ M^{-1}\ sec^{-1}$ [16].

A significant portion of extracellular $ONOO^-$ appeared to cross the platelet membrane by a mechanism involving an active transport system that was sensitive to DIDS, a specific inhibitor of anion transport. Because DIDS has been shown to be more specific towards HCO_3^-/Cl^- anion transporter, it is likely that $ONOO^-$ diffuses into platelets via a mechanism similar to that identified in erythrocytes [18]. Several recent studies have established that $ONOO^-$ crosses lipid membranes [18–20]. The permeability of $ONOO^-$ is comparable to that of water and approximately 400 times greater than that of superoxide [18]. Another factor that influenced $ONOO^-$-mediated fluorescence by DCF-DA-labeled platelets was DEM, an electrophilic compound, which conjugates glutathione by a glutathione *S*-transferase-catalyzed reaction. The DEM depletes cellular glutathione content as a result of efflux of the mixed disulfide ($GSS\text{-}C_2H_5$) from the cell [21]. Because removal of intracellular glutathione decreased fluorescence, it is possible that products of the reaction between $ONOO^-$ and glutathione, such as nitroso and nitroglutathione, participate in the generation of fluorescence [8,22,23]. Our previous work has described a reaction of GSH with $ONOO^-$ that produces $GSNO_2$, which spontaneously releases NO over a prolonged period of time [23]. Our current data are consistent with this observation because additional NO released from $GSNO_2$ might react with platelet superoxide generating additional $ONOO^-$ within cells that generated fluorescence. Alternatively, the $GSNO_2$-derived NO might react with $ONOO^-$ causing formation of other nitrogen oxides such as N_2O_3 [16], which could oxidize DCFH. We also observed that an inhibitor of NOS, L-NAME, decreased the fluorescence generated by $ONOO^-$. It is unlikely that L-NAME competed with the $ONOO^-$ transport, but rather additional effects of the intracellular form of $ONOO^-$, which might have reacted with endogenous NO to form more potent oxidants such as NO_2 and N_2O_3 [16], contributed to the generation of fluorescence. The post-transport intracellular form of $ONOO^-$ was probably further activated by cytosolic components into an oxidant of DCFH. In contrast, NO was not transformed into a fluorescence-producing oxidant. The lack of the effect of the NO donor, SNP, suggests that NO cannot oxidize DCFH into a fluorescent form. Superoxide levels in this experiment probably were insufficient to generate detectable amounts of $ONOO^-$. However, interestingly, endogenous NO appears to be required to some degree in the generation of fluorescence by $ONOO^-$, which is sensitive to L-NAME. Perhaps endogenous levels of NO were close to stoichiometric values for a reaction with the intracellular form of $ONOO^-$ or its decomposition product (e.g., $^\bullet NO_2$), as suggested by Fig. 6.

Our experiments have identified two types of fluorescence profiles. The first was produced by $ONOO^-$ with a characteristic sharp onset followed by a sustained signal. Thrombin, NO_2Cl (sodium hypochlorite plus nitrite), and CO produced this kind of a response. Thrombin has been known to induce the formation of O_2^- [1,3], but the release of NO by thrombin has not been conclusive [24,25]. In our study, thrombin was the platelet agonist that consistently, in a concentration-dependent manner, induced formation of an intra-platelet oxidant having a fluorescence profile similar to that of $ONOO^-$. Collagen and arachidonic acid did not share that property. Formation of a fluorescence-inducing oxidant, having properties similar to $ONOO^-$ in a hormonally stimulated cell system, suggests that such an oxidant may function as an intracellular signaling molecule. Although $ONOO^-$ has been suggested to function as a signaling mediator [26], further studies of the thrombin/platelet system might provide additional information about a mediator role of $ONOO^-$, some consequences of which could be phosphorylation and nitration of intracellular proteins, and loss of function [11,12].

While studying the conditions that generate fluorescence by DCF-DA-labeled platelets, we found that treatment of platelets with NaOCl in the presence of nitrite produced a sharp increase of fluorescence. This was likely due to the formation of nitryl chloride (NO_2Cl) within the platelets.

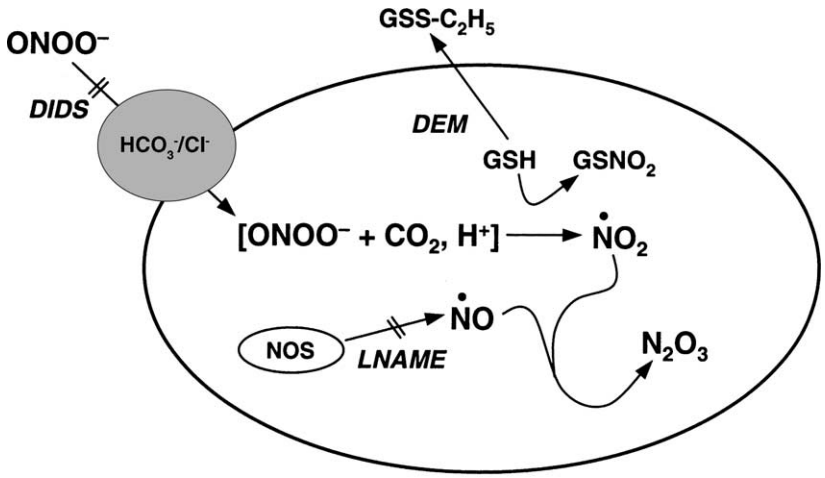

Fig. 6. Scheme illustrating interactions of $ONOO^-$ with platelets. A DIDS-sensitive HCO_3^-/Cl^- channel transports $ONOO^-$ into the platelet cytosol where CO_2 or protonation decomposes the intracellular form of $ONOO^-$ into $^{\bullet}NO_2$, which then reacts with NO by an L-NAME-dependent mechanism generating a potent oxidant, possibly N_2O_3. Removal of intracellular GSH by DEM reduces the ability of $ONOO^-$ to form a fluorescence-inducing oxidant intermediate, probably $GSNO_2$.

Previous reports have identified NO_2Cl in human neutrophils as a product of nitrite oxidation by myeloperoxidase [27,28]. Consistent with this mechanism, an unknown peroxidase within platelets appeared to oxidize nitrite to NO_2, which then reacted with HOCl to form NO_2Cl. Because the platelets were not contaminated by leukocytes and were normally nitrite free, it is unlikely that NO_2Cl was a consistent product in our system except for the experiments shown in Fig. 3. In agreement with a previous report showing that exposure of rats to CO causes nitration of brain protein tyrosine residues [29], our experiments suggest that exposure of platelets to CO can directly form an intracellular oxidant with properties similar to that of $ONOO^-$, probably by reducing oxygen tension and hypoxia.

The other type of fluorescence profile showed a relatively slow onset and was followed by a steady and continuous increase of fluorescence over several minutes without reaching a plateau within the time of the experiment. This response was produced by hypoxia–reoxygenation, nitroglycerin, tetranitromethane, and NO_2. Hypoxia for 20 and 40 min followed by reoxygenation (5 min) stimulated the formation of a substance having fluorogenic properties different from those of $ONOO^-$ but similar to those of nitroglycerin and NO_2. While the structure of the hypoxia–reoxygenation-derived oxidant could not be identified by comparison of the fluorescence profiles, it was unlikely to be $ONOO^-$. The lag period preceding the increase in fluorescence during the reoxygenation period suggested that the fluorescence-generating oxidant might have properties similar to that of nitroglycerin. In contrast, an oxidant derived from $ONOO^-$ would be expected to produce a steady increase in the fluorescence signal with a sharp onset. Interestingly, nitroglycerin-induced fluorescence was nearly completely abolished by the removal of intra-platelet glutathione by DEM. This suggested that platelets activated nitroglycerin to a fluorogenic oxidant, possibly through an adduct with glutathione. Keen *et al.* [30] have hypothesized that organic nitrates react with glutathione via a glutathione transferase-dependent mechanism producing an unstable glutathione sulfenyl nitrite that decomposed via non-enzymatic processes to oxidized glutathione (GSSG). We have previously studied [23] mechanisms of $GSNO_2$ formation and decomposition via its rearrangement into an unstable intermediate, glutathione sulfenyl nitrite (GSONO) and/or glutathione sulfinyl nitrite (GS(O)NO) [31]. Thus, a likely mechanism of fluorescence generation by nitroglycerin might involve the initial formation of NO_2 [32] or a similar nitrating molecule that can react with glutathione producing a nitrated glutathione, possibly via a rate-limiting glutathione transferase-dependent mechanism (Fig. 6). Such initially formed nitroglutathione might be isomerized into unstable glutathione sulfenyl or sulfinyl nitrite, which can either react with DCFH directly or decompose spontaneously to fluorogenic secondary oxidants. Formation of fluorescence by hypoxia–reoxygenation might follow similar steps with the initial formation of $ONOO^-$ within cells, possibly via superoxide formation and activation of NOS, followed by the nitration/nitrosylation of glutathione.

In summary, our experiments show that $ONOO^-$ is a reactive diffusible molecule that uses a complex transport mechanism to penetrate the platelet membrane. The reaction of $ONOO^-$ and other nitrating molecules (NO_2, nitroglycerin, tetranitromethane) with intracellular glutathione appears to be a critical step in the formation of secondary oxidants that generate fluorescence in DCF-DA-labeled platelets. Much more needs to be learned about these mechanisms. Further characterization of the intracellular form of $ONOO^-$ and its reaction with glutathione as well as identification of unstable sulfenyl/sulfinyl nitrites [33], which may function as a common intermediate generated by the reaction of intracellular thiols with a broad range of

nitrative oxidants and nitrovasodilators, might provide more insight into the complexity of the ancillary reactive oxidants derived from NO and superoxide. Nitrogen dioxide might play a key role in these processes [34,35], which, additionally, involve intracellular carbon dioxide, a catalyst for the decomposition of $ONOO^-$ to NO_2 [36,37].

Acknowledgments

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