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Peroxynitrite can affect platelet responses by inhibiting energy production

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Peroxynitrite ($ONOO^-$) strongly inhibits agonist-induced platelet responses. However, the mechanisms involved are not completely defined. Using porcine platelets, we tested the hypothesis that $ONOO^-$ reduces platelet aggregation and dense granule secretion by inhibiting energy production. It was found that $ONOO^-$ (25–300 μM) inhibited collagen-induced dense granule secretion (IC_{50} = 55±7 μM) more strongly than aggregation (IC_{50} = 124±16 μM). The antiaggregatory and antisecretory effects of $ONOO^-$ were only slightly (5–10%) reduced by 1*H*-[1,2,4]-oxadiazolo-[4,3-α]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylate cyclase. In resting platelets $ONOO^-$ (50–300 μM) enhanced glycolysis rate and reduced oxygen consumption, in a dose dependent manner. The $ONOO^-$ effects on glycolysis rate and oxygen consumption were not abolished by ODQ. The extent of glycolysis stimulation exerted by $ONOO^-$ was similar to that produced by respiratory chain inhibitors (cyanide and antimycin A) or an uncoupler (2,4-dinitrophenol). Stimulation of platelets by collagen was associated with a rise in mitochondrial oxygen consumption, accelerated lactate production, and unchanged intracellular ATP content. In contrast to resting cells, in collagen-stimulated platelets, $ONOO^-$ (200 μM) distinctly decreased the cellular ATP content. The glycolytic activity and oxygen consumption of resting platelets were not affected by 8-bromoguanosine 3′,5′-cyclic monophosphate. Blocking of the mitochondrial ATP production by antimycin A slightly reduced collagen-induced aggregation and strongly inhibited dense granule secretion. Treatment of platelets with $ONOO^-$ (50–300 μM) resulted in decreased activities of NADH:ubiquinone oxidoreductase, succinate dehydrogenase and cytochrome oxidase. It is concluded that the inhibitory effect of $ONOO^-$ on platelet secretion and to a lesser extent on aggregation may be mediated, at least in part, by the reduction of mitochondrial energy production.

Keywords: aggregation, glycolysis, mitochondria, peroxynitrite, porcine platelets, secretion

INTRODUCTION

Peroxynitrite ($ONOO^-$, oxoperoxonitrate (-1)) is a potent oxidant and nitrating agent formed by the rapid reaction between superoxide ($O_2^{\bullet-}$) and nitric oxide (NO) (Beckman & Koppenol, 1996; Murphy *et al.*, 1998). Formation of large quantities of $ONOO^-$ is expected to occur during ischemia-reperfusion injury, sepsis, neurodegenerative diseases and inflammatory processes where NO and $O_2^{\bullet-}$ production is elevated (Radi *et al.*, 2001). The main source of $ONOO^-$ in blood stream are activated circulating inflammatory cells, e.g. neutrophiles and monocytes (Ischiropoulos *et al.*, 1992). Activated platelets may also release small amounts of $ONOO^-$ since they have been proposed to generate both NO and $O_2^{\bullet-}$ (Malinski *et al.*, 1993; Marcus *et al.*, 1997; Boulos *et al.*, 2000). Appearing in the blood stream, $ONOO^-$ is therefore likely to interact with platelets. *In vitro* micromolar concentrations of $ONOO^-$ are able to affect some platelet responses (Moro *et al.*, 1994; Boulos *et al.*, 2000; Nowak & Wachowicz, 2001). However, the results of those studies are conflicting. It has been reported that $ONOO^-$ may either strongly reduce platelet responses or behave as an inducer of their aggregation (Moro *et al.*, 1994; Brown *et al.*, 1998). The mechanisms underlying interactions of $ONOO^-$ with platelets are not clear. It was hypothesized that

Abbreviations: 8-BrcGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; ODQ, 1*H*-[1,2,4]-oxadiazolo-[4,3-α]quinoxalin-1-one; PRP, platelet-rich plasma; SGC, guanylate cyclase.

$ONOO^-$ may affect platelets through the nitration of protein tyrosine residues (Brown *et al.*, 1998; Boulos *et al.*, 2000), but it soon appeared that nitrotyrosine-containing proteins are found also in the cells activated by collagen, i.e. in the absence of exogenous peroxynitrite (Naseem *et. al.*, 2000). It has also been proposed that in the presence of naturally occurring SH-containing compounds (e.g. glutathione) $ONOO^-$ can be converted to nitrosothiols, which may act as NO donors. Thus produced NO was suggested to inhibit platelet responses *via* the stimulation of soluble guanylate cyclase (Brown *et al.*, 1998). It was also reported that $ONOO^-$ might affect platelets nonspecifically *via* peroxidation of membrane lipids (Nowak & Wachowicz, 2001).

Studies performed on cells other then platelets have shown that $ONOO^-$ may strongly diminish mitochondrial respiration (Radi *et al.*, 2002). Platelets possess mitochondria and oxidative energy production in resting (unstimulated) cells provides up to 50% of the total ATP formed (Holmsen, 1981). Metabolically, platelets are relatively active and have a total ATP turnover rate of about 3–8 times that of resting mammalian muscle (Akkerman 1978; Akkerman *et al.*, 1978; Niu *et al.*, 1996). Unstimulated platelets use the energy in the processes enabling them to be permanently in the responsive state. In activated platelets metabolic energy is required mainly for secretion and aggregation (Holmsen, 1981). Therefore the inhibition of platelet energy production may have critical implications for their responses (Holmsen *et al.*, 1982; Verhoeven *et al.*, 1984; Guppy *et al.*, 1995). Consequently, the present study was performed to test the hypothesis that $ONOO^-$ may affect platelet responses through the inhibition of energy production.

MATERIALS AND METHODS

Chemicals. Antimycin A, 2,4-dinitrophenol (DNP), 1*H*-[1,2,4]-oxadiazolo-[4,3-α]quinoxalin-1-one (ODQ), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Munich, Germany).

Synthesis of $ONOO^-$. Peroxynitrite was synthesized by the reaction of acidified $NaNO_2$ (1.2 M) with H_2O_2 (1.4 M) in a quenched flow reactor (Koppenol *et al.*, 1996). The excess of H_2O_2 was removed by passing of the product over a column filled with granular manganese dioxide. Stock solutions containing at least 200 mM $ONOO^-$ were prepared and stored at –70°C. The concentration was determined prior to each experiment by measuring the absorbance at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}cm^{-1}$). Decomposed $ONOO^-$ was obtained by allowing the compound to decay in 0.5 M phosphate buffer (pH 7.4) at 25°C for 30 min.

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP). Blood was collected in the local slaughterhouse from adult pigs. The blood was withdrawn by direct carotid catheterization and collected into 3.8% (w/v) sodium citrate, one volume per nine volumes of blood. PRP was obtained by centrifugation of the blood at 200 × *g* for 20 min at room temperature. PPP was obtained by further centrifugation of PRP at 2700 × *g* for 10 min.

Preparation of platelet concentrate. Blood collected into ACD (0.075 M citric acid, 0.085 M sodium citrate and 0.11 M glucose) solution (one volume per six volumes of blood) was used to prepare platelet concentrate (Tomasiak *et al.*, 2004).

Preparation of platelet homogenates. Platelet homogenate was prepared from fresh blood as described previously (Tomasiak *et al.*, 2004).

Assay of platelet aggregation. Platelet aggregation was followed turbidimetrically by recording the light transmission through a stirred platelet suspension in the plastic cuvette of an aggregometer (Elvi, Logos, Milan, Italy) at 37°C (Born & Cross, 1963). All experiments were performed at least in quadruplicate using six different platelet preparations.

Simultaneous measurement of platelet aggregation and ATP secretion. The platelet release reaction was monitored simultaneously with optical aggregation using lumiaggregometer (Chrono-log Corp., Havertown, PA, USA). All procedures were conducted as described previously (Tomasiak *et al.*, 2004). The chemiluminescence signal was converted to nanomoles ATP per 10^8 cells by comparison with an ATP standard curve.

Measurement of serotonin secretion. Secretion was determined by the release of [^{3}H]serotonin using a procedure described previously (Tomasiak *et al.*, 2004).

Measurement of glycolysis in intact platelets. Platelets (2×10^9 platelets/ml) suspended in dialyzed plasma were incubated at 37°C in plastic vessels (50 ml conical Falcon tubes) with gentle stirring under the atmosphere of 95% O_2 + 5% CO_2. Every 5 min the pH of the incubation mixture was checked and, if necessary, corrected by the addition of 0.2 M NaOH (total volume of NaOH added never exceeded 5% of the volume of platelet suspension). Incubation was started by the addition of glucose to the final concentration of 10 mM and was carried out for 30 min. It was stopped by the addition of 3 volumes of cold 6% (w/v) perchloric acid. Lactate was measured in deproteinized and neutralized extract (Gutmann & Wahlefeld, 1985). The formation of lactate was found to be linear during 30 min of incubation.

Measurement of glycolysis in cell-free system. Platelets obtained from 2 l of blood were suspended in 20 ml of dialyzed plasma. The concentrate was cooled to 0°C, supplemented with dithiothreitol (1.5 mM final concentration) and disrupted sonically. Immediately after sonication samples were used for the measurement of glycolytic activity (Tomasiak *et al.*, 2004).

Measurement of the respiration rate. Respiration was measured polarographically at 37°C with a Clark-type oxygen electrode (Yellow Springs, USA, model YSI 4004) in a closed plastic vessel of 2 ml.

Measurement of intracellular ATP. After incubations platelet suspensions (1 ml) were supplemented with 3 ml of ice cold 6% (w/v) perchloric acid, sonicated and left at 0°C for 20 min. The extracts were centrifuged to remove protein and neutralized with ice cold 6 M KOH/0.5 M morpholine sulphonic acid. ATP content was determined in neutralized cellular extracts by the luciferase-luciferin assay (Holmsen *et al.*, 1972).

Assays of succinate dehydrogenase, NADH:ubiquinone oxidoreductase, cytochrome *c* oxidase activities were performed as described previously (Tomasiak *et al.*, 2004).

Data analysis. Data reported in this paper are the mean (±S.D.) of the number of determinations indicated (n). Statistical analysis was performed by the Student's *t*-test and elaboration of experimental data by the use of Slide Write plus (Advanced Graphics Software, Inc. Carlsbad, CA, USA.)

RESULTS

Figure 1 illustrates the results of experiments in which the effect of increasing concentrations of $ONOO^-$ on collagen-induced platelet aggregation and dense granule secretion was studied. Dense granule secretion was determined using two independent methods, i.e. by luminometry which measured the ATP (nonmetabolic pool of nucleotide normally stored in dense granules) released from dense granules, and an isotopic method which determined the release of [^{3}H]serotonin (stored in dense granules) from the platelets preloaded with tritiated serotonin. As it is seen, a two-minute incubation of platelets with $ONOO^-$ (25–300 μM) inhibited, in a dose dependent manner, the aggregation and dense granule secretion induced by a threshold concentration of collagen. The estimated IC_{50} values for aggregation, ATP release and serotonin secretion were 124±18, 55±7, and 58±8 μM, respectively. Total inhibition of aggregation and secretion was observed in the presence of 250–300 μM $ONOO^-$.

Experiments shown in Table 1 were performed to compare the susceptibility of (collagen-induced) platelet aggregation and secretion to the inhibition by $ONOO^-$ and a respiratory chain blocker, antimycin A. Platelet responses were measured in PRP samples using a lumiaggregometer. Aggregation and secretion of ATP from platelet dense granules were recorded simultaneously in the same platelet sample. Treatment of platelets with 100 μM $ONOO^-$ resulted in a distinctly stronger inhibition of secretion (about 60%) than aggregation (about 30%). Similar results were obtained following the blocking of mitochondrial energy production by antimycin A. In this case pretreatment with 10 μM antimycin A produced 25% and 75% inhibition of aggregation and secretion, respectively.

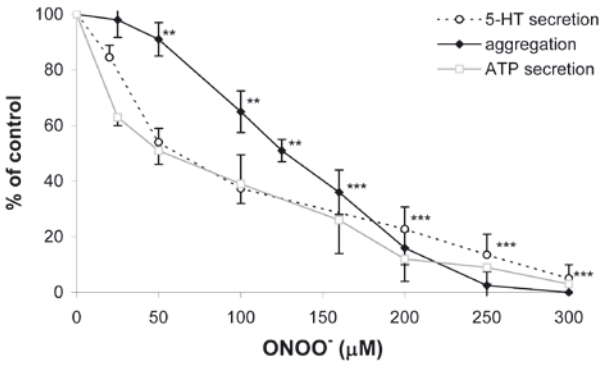

Figure 1. The effect of $ONOO^-$ treatment on platelet aggregation and dense granule secretion.
Aliquots (0.5 ml) of PRP or washed platelets were incubated for 2 min at 37°C without (control) or with $ONOO^-$ added to the final concentration as indicated. The extent of platelet aggregation (◆), ATP secretion (□) and release of [^{3}H]serotonin (○) were measured 3 min after the addition of the threshold concentration of collagen (15–20 μg/ml). Decomposed $ONOO^-$ (300 μM) exerted no observable effect on collagen-induced aggregation and dense granule secretion. Aggregation, ATP secretion and [^{3}H]serotonin release were monitored as under Methods. All experiments were performed at least in quadruplicate using five different platelet preparations. Means ±S.D. are presented. The differences between the control and $ONOO^-$-treated platelets are significant. $^{**}P<0.05$; $^{***}P<0.01$.

To estimate whether the inhibitory effect of $ONOO^-$ on platelet responses is mediated by NO-stimulated guanylate cyclase (sGC) we studied the effect of ODQ, an inhibitor of sGC, on the antiaggregatory and antisecretory action of $ONOO^-$ in collagen-stimulated platelets. As is seen in Table 1 (panel B) concentrations of ODQ nearly completely reducing the antiaggregatory effect of sodium nitroprusside (NO donor) only slightly abolished the $ONOO^-$ effect on secretion (about 5%) and aggregation (about 11%).

The results presented in Table 2 show that the rate of glycolysis in intact platelets was increased by $ONOO^-$ (25 to 300 μM), respiratory chain blockers (cyanide, antimycin A), an uncoupler (2,4-dinitrophenol), and following activation of platelets by collagen. Decomposed $ONOO^-$ (300 μM) did not

Table 1. The effect of $ONOO^-$, sodium nitroprusside, and antimycin A on collagen-induced platelet aggregation and secretion

Experiment	Additions	Aggregation extent (% of control)	Secretion (% of control)
A	$ONOO^-$ (50 μM)	92.6 ± 6.8*	51.4 ± 6.3***
	$ONOO^-$ (100 μM)	66.9 ± 8.5***	38.4 ± 6.7***
	$ONOO^-$ (150 μM)	39.6 ± 9.2***	21.1 ± 6.4***
	Antimycin (5 μg/ml)	92.2 ± 7.6*	52.6 ± 6.1***
	Antimycin (10 μg/ml)	89.6 ± 8.1**	39.7 ± 7.1***
	Antimycin (20 μg/ml)	74.8 ± 7.1***	24.9 ± 5.2***
B	$ONOO^-$ (150 μM)	41.6 ± 9.4***	23.6 ± 4.6***
	$ONOO^-$ (150 μM)+ODQ (20 μM)	52.8 ± 7.5***	28.7 ± 4.8***
	SNP (25 μM)	44.4 ± 8.6***	n.d.
	SNP (25 μM) + ODQ (10 μM)	95.6 ± 4.4	n.d.

PRP (0.5 ml) was incubated at 37°C for 2 min, without (control) or with tested substances added to the final concentrations as indicated. Aggregation and secretion were initiated by the addition of a supra-threshold concentration of collagen (20 μg/ml). Optical aggregation and dense granule secretion were monitored simultaneously as in Methods. The data represent mean values ± S.D. of five experiments, each performed on separate platelet preparation (n=15). *$P<0.05$, **$P<0.01$, ***$P<0.005$.

affect glycolysis in intact platelets. The stimulatory effect of $ONOO^-$ on glycolysis was similar to that observed after the blocking of the respiratory chain by antimycin A or cyanide and to that produced by aggregating concentrations of collagen and was not abolished after the blocking of guanylate cyclase by ODQ. The rate of glycolysis in intact platelets was not affected following the treatment of the cells with 8-bromoguanosine 3′,5′-cyclic monophosphate, a lipid-soluble analog of cGMP (Corbin *et al*., 1986) and a specific stimulator of protein kinase G (Lincoln & Corbin, 1983)

Table 2. Glycolytic activity of intact platelets in the presence of $ONOO^-$, collagen, uncoupler of oxidative phosphorylation, respiratory chain blockers, and 8-BrcGMP

Addition	Lactate production (μmoles/min per 10^{11} cells)
None (control)	3.1 ± 0.2
25 μM $ONOO^-$	3.5 ± 0.3
50 μM $ONOO^-$	3.7 ± 0.4 *
100 μM $ONOO^-$	4.1 ± 0.5 **
200 μM $ONOO^-$	4.3 ± 0.4 **
300 μM $ONOO^-$	4.8 ± 0.5 ***
Decomposed $ONOO^-$ (300 μM)	3.2 ± 0.3
ODQ (20 μM)	3.2 ± 0.3
ODQ (20 μM) + 50 μM $ONOO^-$	3.6 ± 0.5 *
ODQ (20 μM) + 200 μM $ONOO^-$	4.4 ± 0.4 **
Collagen (20 μg/ml)	5.2 ± 0.3 ***
Antimycin A (10 μg/ml)	4.8 ± 0.2 ***
Cyanide (1 mM)	5.1 ± 0.3 ***
2,4-Dinitrophenol (1 mM)	5.5 ± 0.3 ***
8-BrcGMP (1 mM)	3.0 ± 0.3

Platelets (2×10^9 cells/ml) suspended in dialyzed plasma were incubated at 37°C for 30 min without (control) and with various substances added to the final concentrations as indicated. Further details in Materials and Methods. The numbers are means ±S.D. (n = 6) of one representative (out of six) experiment performed on single platelet preparation. Lactate production in the control varied from 2.7 to 3.8 μmoles × min^{-1} × 10^{-11} cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

To establish how $ONOO^-$ affects glycolysis in a cell-free system, i.e. in a system containing all the glycolytic enzymes and depleted of intact mitochondria, we measured the effect of $ONOO^-$ on lactate production in platelet homogenate. Platelet homogenate was used instead of a high-speed supernatant fraction (cytosol) since, as established by Akkerman (1978), 97% of the total hexokinase activity in platelets is membrane bound (mitochondria). Respiratory chain blockers (cyanide, antimycin A), uncouplers (2,4-dinitrophenol) and $ONOO^-$ (50–300 μM) did not affect the glycolysis rate measured in the cell-free system (not shown).

Table 3 shows that respiratory chain blockers (cyanide and antimycin A) and $ONOO^-$ (50–300 μM) inhibited the oxygen consumption by unstimulated platelets. The effect exerted by $ONOO^-$ was dose-dependent. The degree of this inhibition was similar to that observed in the presence of cyanide or antimycin A. In comparison to resting cells, collagen-stimulated platelets demonstrated two times faster oxygen consumption. Decomposed $ONOO^-$ (300 μM) did not affect the oxygen consumption by intact platelets. The inhibitory effect of $ONOO^-$ on respiration was not abolished following the blocking of guanylate cyclase by ODQ. In resting platelets a rise in the intracellular cGMP concentration evoked by a lipid-soluble analog (8-BrcGMP) did not modulate changes in oxygen consumption.

Table 4 illustrates the results of experiments which were performed to estimate whether in platelets $ONOO^-$ affects intracellular ATP content. As is seen, $ONOO^-$ (100–300 μM) failed to affect distinctly the intracellular ATP content in resting cells and in platelets activated by collagen. By contrast, in collagen-stimulated platelets pretreated with 200 μM $ONOO^-$, a significant (about 30%) reduction of intracellular ATP content was observed. Decomposed

Table 3. Platelet oxygen consumption in the presence of ONOO⁻, respiratory chain inhibitors, 8-BrcGMP, and collagen

Addition	Oxygen consumption (nmol O_2/min per 10^{11} cells)
None	410 ± 50
Collagen (20 μg/ml)	800 ± 70 ***
Cyanide (1 mM)	48 ± 12 ***
Antimycin A (10 μg/ml)	70 ± 15 ***
8-BrcGMP (1 mM)	400 ± 50
50 μM $ONOO^-$	280 ± 50 **
100 μM $ONOO^-$	130 ± 40 ***
200 μM $ONOO^-$	80 ± 30 ***
300 μM $ONOO^-$	50 ± 30 ***
Decomposed $ONOO^-$ (300 μM)	390 ± 50
ODQ (20 μM) + 50 μM $ONOO^-$	260 ± 50 **
ODQ (20 μM) + 200 μM $ONOO^-$	90 ± 40 **

Oxygen consumption was measured polarographically with a Clark oxygen electrode, in a closed plastic vessel of 2 ml at 37°C. Aliquots (1 ml) of platelet concentrate and 1 ml of dialyzed plasma were added to the vessel to give the final concentration of 2 × 10^9 cells/ml. Measurements were started after 2 min preincubation and were carried out for 10 min. Additions to the measuring system were done 3 min after starting the recording of oxygen consumption. No exogenous glucose was added. The results of one representative experiment (out of five) are presented. Oxygen consumption in the control varied from 320 to 440 nmol × min^{-1} × 10^{-11} cells. **$P<0.05$, ***$P<0.001$.

Table 4. Effect of peroxynitrite on the total ATP content in resting and stimulated porcine blood platelets

Addition	ATP content (nanomoles per 10^8 cells)
None (control)	2.87 ± 0.49
100 μM $ONOO^-$	2.76 ± 0.41
200 μM $ONOO^-$	2.42 ± 0.56 *
300 μM $ONOO^-$	2.43 ± 0.21 *
Decomposed $ONOO^-$ (300 μM)	2.82 ± 0.38
Collagen (20 μg/ml)	2.69 ± 0.27
Collagen + 200 μM $ONOO^-$	1.91 ± 0.36 ***
Collagen + decomposed $ONOO^-$ (200 μM)	2.73 ± 0.31

Aliquots (1 ml) of PRP were incubated for 5 min at 37°C without (control) or with $ONOO^-$ added to the final concentration as indicated. ATP was measured in deproteinized and neutralized extracts as described under Methods. The data represent mean values ±S.D. of five experiments, each performed on separate platelet preparation (n=15). *$P<0.05$, ***$P<0.005$.

$ONOO^-$ did not affect the intracellular ATP concentration in resting or collagen-stimulated cells.

Table 5 demonstrates the results of experiments in which the effect of $ONOO^-$ on the activity of NADH:ubiquinone oxidoreductase, succinate dehydrogenase and cytochrome oxidase in platelet homogenate was studied. $ONOO^-$ (50–300 μM) reduced, in a concentration-dependent fashion, the activity of all the enzymes studied. The sensitivity of the enzymes to $ONOO^-$ increased in the following order: cytochrome oxidase < succinate dehydrogenase < NADH:ubiquinone oxidoreductase. Thus, 200 μM $ONOO^-$ inhibited cytochrome oxidase, succinate dehydrogenase and NADH:ubiquinone oxidoreductase by about 12%, 20% and 33%, respectively.

DISCUSSION

The results presented here show that $ONOO^-$ is a strong inhibitor of aggregation and secretion of porcine platelets. This is in agreement with previous reports (Moro *et al.*, 1994; Nowak & Wachowicz, 2001). It has been reported that activation of human platelets by high thrombin concentration is accompanied by the acceleration of lactate production and enhanced oxygen consumption (Akkerman & Holmsen, 1981; Niu *et al.*, 1996). Our results show that porcine platelets stimulated by collagen also produce more lactate and consume more oxygen indicating that both glycolytic and mitochondrial ATP production are necessary to support their responses. This implies that in platelets blocking of energy production is likely to diminish their responses.

The present results show, for the first time, that under aerobic conditions exogenously added $ONOO^-$ stimulates glycolysis in intact porcine platelets. The degree of this stimulation was similar to that observed after the blocking of mitochondrial energy production by an uncoupler (dinitrophenol) or

Table 5. The effect of $ONOO^-$ on the activity of NADH:ubiquinone oxidoreductase, succinate dehydrogenase and cytochrome oxidase

Addition	NADH:ubiquinone oxidoreductase (μmoles/min per 10^{12} cells)	Succinate dehydrogenase (μmoles/min per 10^{12} cells)	Cytochrome oxidase (μmoles/min per 10^{12} cells)
None	18.0 ± 0.2	0.98 ± 0.05	3.11 ± 0.5
50 μM $ONOO^-$	17.6 ± 0.2	0.94 ± 0.05	3.07 ± 0.5
100 μM $ONOO^-$	13.5 ± 0.5 *	0.82 ± 0.04 **	2.94 ± 0.4
200 μM $ONOO^-$	11.1 ± 1.2 ***	0.79 ± 0.04 **	2.74 ± 0.5 **
300 μM $ONOO^-$	9.6 ± 2.1 ***	0.67 ± 0.07 **	2.21 ± 0.5***
Decomposed $ONOO^-$ (300 μM)	17.9 ± 0.3	0.96 ± 0.07	3.07 ± 0.4

Platelet homogenate was incubated for 20 min at 37°C with decomposed or native $ONOO^-$ added to the final concentration as indicated. Then the activities of NADH:ubiquinone oxidoreductase, succinate dehydrogenase and cytochrome oxidase were measured as described in Materials and Methods. Results are expressed as micromoles of oxidized substrate (NADH, succinate, cytochrome *c*)/min per 10^{12} cells. Mean values ± S.D. (n=15) are reported. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

by respiratory chain inhibitors (antimycin A or cyanide). At the same time $ONOO^-$, dinitrophenol, antimycin A and cyanide had no effect on the rate of glycolysis in the platelet homogenate in which functional (coupled) mitochondria were apparently not present. Since in platelets glycolysis and mitochondrial respiration are tightly functionally connected (Guppy *et al.*, 1995), this can be interpreted to mean that the stimulatory effect of $ONOO^-$ on glycolysis in intact platelets may be related to impairment of mitochondria. This interpretation is likely to be true since, as reported here, in porcine platelets $ONOO^-$ is able to reduce mitochondrial oxygen consumption and to inhibit NADH:ubiquinone reductase, succinate dehydrogenase and cytochrome oxidase. Interestingly, the concentrations of $ONOO^-$ inhibiting mitochondrial respiration and respiratory chain complexes were similar to those stimulating glycolysis in intact platelets.

To sum up, $ONOO^-$ is able to reduce platelet mitochondrial energy production through the inhibition of respiratory chain complexes I, II and IV.

The current observations indicate that in porcine platelets respiratory chain enzymes differ in their sensitivity to $ONOO^-$, which increases in the order: cytochrome oxidase < succinate dehydrogenase < NADH:ubiquinone reductase. This is consistent with the observation of Riobo *et al.* (2001) that in rat brain submitochondrial particles, complex I (NADH:ubiquinone reductase activity) is much more sensitive to peroxynitrite than complexes II and III.

Our results indicate that the $ONOO^-$ concentrations totally blocking mitochondrial respiration are not able to inhibit completely the activity of respiratory chain enzymes. This indicates that peroxynitrite may affect platelet mitochondria not only through the inhibition of respiratory chain complexes. Thus, other damaging effects of $ONOO^-$ on platelet mitochondria are also likely to take place.

Activation of porcine platelets by collagen was not associated with a drop in the total cellular ATP content, despite the fact that aggregation and secretion are known to consume large quantities of metabolic energy (Holmsen *et al.*, 1982). This is because the accelerated oxidative and glycolytic ATP production have a capacity completely to meet the augmented (due to activation) energy demand. Also, no reduction in cellular ATP content was observed in resting platelets treated with $ONOO^-$. In this case accelerated glycolysis apparently had the capacity to fully compensate the lack of mitochondrial energy production and to meet the relatively small cellular ATP demand expected in resting cells. This is consistent with the observation that in platelets incubated under hypoxic conditions cellular ATP level remained unchanged (Akahori *et al.*, 1995). However, as is here reported, a distinct reduction of cellular ATP content was observed in activated (by collagen) platelets pretreated with $ONOO^-$. In this case even accelerated glycolytic ATP production failed to fully compensate for the impaired (by $ONOO^-$) mitochondrial ATP production and to meet the high metabolic energy demand of activated cells (Akkerman & Holmsen, 1981).

Does the $ONOO^-$-mediated blocking of mitochondrial energy production explain the inhibitory effect of this compound on *in vitro* platelet responses? As is shown here in porcine platelets, diminishing the mitochondrial energy production by antimycin A affects platelet secretion distinctly more strongly than aggregation. This is consistent with the suggestion that platelet responses have different metabolic energy requirements, which increase in the order: aggregation < dense and alpha granule secretion < lysosome secretion (Holmsen *et al.*, 1982; Verhoeven *et al.*, 1984; Morimoto & Ogihara, 1996). A comparison of the concentrations of $ONOO^-$ affecting mitochondrial respiration with those reducing platelet aggregation and secretion indicates that $ONOO^-$ cannot reduce significantly platelet aggregation through the inhibition of oxidative energy production. By contrast, the concentrations of $ONOO^-$ inhibiting platelet secretion and mitochondrial respiration were similar. This and the observation that $ONOO^-$ inhibits dense granule secretion more strongly than aggregation implies that in porcine platelets $ONOO^-$ can affect the secretory process through the inhibition of mitochondrial energy production.

To sum up, the inhibitory effect of $ONOO^-$ on platelet secretion, and to a lesser extent on aggregation, may be related to the reduction of mitochondrial energy production.

$ONOO^-$ was also reported to react with intracellular components containing SH-groups (e.g. glutathione) and to form nitrosothiols (Mayer *et al.*, 1995). Nitric oxide released from spontaneously decomposed nitrosothiols was proposed to activate soluble guanylate cyclase (sGC) (Mayer *et al.*, 1995) and to inhibit cytochrome oxidase (Brown, 2001). The rise in intracellular cGMP level thus produced (Danielewski *et al.*, 2005) and reduction of mitochondrial energy production (Tomasiak *et al.*, 2004) have been proposed to inhibit platelet activation. However, the results presented here indicate that soluble guanylate cyclase is not a predominant intracellular factor mediating the $ONOO^-$ action on platelets, although its involvement in the inhibition of aggregation (but not secretion) can not be completely excluded (Table 1). This is because blocking of the guanylate cyclase slightly affects $ONOO^-$ action on platelet aggregation and is practically without any effect on secretion. Guanylate cyclase cannot be in-

volved in the inhibition of energy production either, since both glycolysis and mitochondrial respiration were not affected by an artificially elevated intracellular cGMP concentration. An involvement of NO in the $ONOO^-$ action on platelet cytochrome oxidase is also unlikely. A comparison of the ODQ (a guanylate cyclase inhibitor) effect on the antiaggregatory action of sodium nitroprusside (NO donor) and $ONOO^-$ indicates that in platelets treated with peroxynitrite, guanylate cyclase is not maximally activated apparently due to a lower NO concentration. Taking into account that NO inhibits purified sGC (from brain cells) at concentrations 50–100 lower then that required to inhibit cytochrome oxidase (Bellamy *et al*., 2002), a nitric oxide-dependent $ONOO^-$ action on cytochrome oxidase is also excluded.

In conclusion, our data show that: 1) in porcine platelets $ONOO^-$ is able to diminish mitochondrial respiration through the inhibition of respiratory chain complexes I, II and IV; 2) the inhibitory effect of $ONOO^-$ on platelet secretion (but not aggregation) can be attributed, at least in part, to the reduction of mitochondrial energy production.

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