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# The formation of nitric oxide donors from peroxynitrite

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1 Administration of peroxynitrite ($ONOO^-$, 30–300 μM) caused relaxation of rabbit aortic strips superfused in series in a cascade. The compound responsible for this effect had a half-life greater than 20 s and could not therefore be either nitric oxide (NO) or $ONOO^-$ which have half-lives in the order of 1–2 s under these conditions. However the relaxation was inhibited by oxyhaemoglobin, suggesting that the compound could be converted to NO in the vascular tissues or in the superfusate.

2 The products of the reaction between $ONOO^-$ and Krebs buffer containing 11 mM glucose, but not glucose-free Krebs buffer, caused relaxation of the bioassay tissues. These data suggest that stable NO donor(s) were formed from the reaction of $ONOO^-$ with glucose. We therefore prepared these NO donor(s) by the reaction of glucose solutions with $ONOO^-$ in order to characterize their pharmacological actions both in the cascade bioassay and on platelets and to study their ability to release NO.

3 These reaction product(s) caused relaxation in the cascade and inhibition of platelet aggregation. Both effects were dependent on the concentration of D-glucose, were equally effective if L-glucose was used as a reactant and were reversed by oxyhaemoglobin.

4 The products of the reaction between $ONOO^-$ and glucose or other biological molecules containing an alcohol functional group, such as fructose, glycerol, or glyceraldehyde, released NO in the presence of $Cu^{2+}$ and L-cysteine.

5 These results indicate that $ONOO^-$ reacts with sugars or other compounds containing an alcohol functional group(s) to form NO donor(s) with the characteristics of organic nitrate/nitrites. This may represent a further detoxification pathway for $ONOO^-$ *in vivo*.

**Keywords:** Nitric oxide; peroxynitrite; vascular wall; platelets; glucose; oxidative stress

## Introduction

Peroxynitrite ($ONOO^-$) is a powerful oxidant which is formed by the reaction between NO and superoxide ($O_2^-$, Beckman *et al.*, 1990). Formation of $ONOO^-$ has been proposed as a mechanism to explain the cytotoxic effects of NO and $O_2^-$ (Beckman *et al.*, 1990). Peroxynitrite has pro-aggregatory effects on platelets (Moro *et al.*, 1994), impairs the actions of several vasodilators on the coronary circulation (Villa *et al.*, 1994), modifies low density lipoprotein to an atherogenic form (Graham *et al.*, 1993) and may be formed in the atherosclerotic plaque (Beckman *et al.*, 1994). However, we have shown that $ONOO^-$ reacts with glutathione to form an S-nitrosothiol able to regenerate NO and have proposed that this could represent a detoxification pathway for this oxidant (Moro *et al.*, 1994). Exposure of isolated or intact vasculature to $ONOO^-$ results in relaxation (Liu *et al.*, 1994; Wu *et al.*, 1994; Villa *et al.*, 1994) and it has been suggested that this is due to the nitrosylation of tissue thiols (Wu *et al.*, 1994). During the course of our investigation of the relaxant effects of $ONOO^-$ on three tissues superfused in series in a cascade we found that this compound induced relaxation of all the assay tissues, suggesting that stable NO donor(s) had been generated. We therefore decided to investigate the nature of these molecules, their ability to release NO and their pharmacological effects on both vascular strips and platelets.

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## Methods

### *Cascade bioassay*

The cascade bioassay was set up using rabbit thoracic aortae which were denuded of endothelium and cut into spiral strips as described previously (Gryglewski *et al.*, 1986). Unless otherwise stated, Krebs buffer containing 11mM glucose was used in these experiments. Following submaximal (approximately 80%) pre-contraction of tissues with the thromboxane $A_2$-mimetic, U46619 (30 nM), vascular responses were measured with an auxotonic transducer (Harvard Bioscience). The sensitivity of the tissues was adjusted so that similar relaxations to the same concentration of glyceryl trinitrate ($ED_{30}$ GTN = 50 nM) were observed in each tissue. Responses were expressed as percentage of the maximal relaxation to GTN. Peroxynitrite, or the product of the reaction of $ONOO^-$ with the compounds indicated, was added at a rate of 25 μl over a period of 30 s to the superfusate (flow rate of 5 ml $min^{-1}$). This resulted in a dilution of approximately 1/100 from the stock solutions. Additions were made so that there was a delay of 3 s before they reached the first tissue. Under these conditions the basic solutions of $ONOO^-$ were effectively buffered by the superfusate, which showed no change in pH. In some experiments, oxyhaemoglobin (5 μM) was included in the superfusate prior to or during the addition of $ONOO^-$ or its reaction products. The sensitivity of the bioassay tissues to the standard dose of GTN (50 nM) remained unchanged for the duration of the experiments (approximately 2–3 h).

### *Washed platelet suspensions*

Human blood was collected and washed platelet (WP) suspensions ($2.0–2.5 \times 10^8$ platelets $ml^{-1}$) were prepared as described previously (Radomski & Moncada, 1983).

### *Platelet aggregation*

Platelet aggregation was studied in a Platelet Ionised Calcium Aggregometer (Chronolog). The mixtures of glucose and $ONOO^-$ were incubated with WP for 1 min prior to the addition of collagen (0.75–1.5 $\mu g\ ml^{-1}$). In some experiments, oxyhaemoglobin (5 $\mu M$) was preincubated with platelets for 1 min before the addition of the reaction products of $ONOO^-$. For statistical analysis, changes in platelet aggregation were expressed as percentage of the response to the minimum concentration of collagen required for maximal aggregation (0.75–1.5 $\mu g\ ml^{-1}$).

### *Peroxynitrite preparation*

Peroxynitrite was synthesized by the reaction of acidified $NaNO_2$ (1.8 M) with $H_2O_2$ (2.1 M) and quenched with NaOH (4.2 M) (Blough & Zafiriou, 1985). To prepare decomposed $ONOO^-$ (dec $ONOO^-$), the addition of NaOH to the $H_2O_2$/$NaNO_2$ mixture was delayed for 3 min, after which no $ONOO^-$ was present (Villa *et al.*, 1994). Excess hydrogen peroxide was removed from the $ONOO^-$ preparation by passage down a manganese dioxide column (Beckman *et al.*, 1990). Typical concentrations of $ONOO^-$ after synthesis were 200–350 mM. Dilutions were made into water before addition to the superfusate for the cascade bioassay or to the platelet preparation.

### *Preparation of the reaction products of $ONOO^-$ and glucose or other alcohols*

For the cascade bioassay, the platelet aggregation experiments and NO determination by chemiluminescence, the products of the reaction of glucose with $ONOO^-$ were prepared by incubating $ONOO^-$ (20 mM) in Krebs solution with or without glucose, or $ONOO^-$ (20 mM) with D-glucose (1–300 mM) or L-glucose (100 mM) in 50 mM phosphate buffer (final pH 9–9.5) for 1 min at room temperature. The pH of the reaction mixture was adjusted to 7.4 with HCl (1 mM). In another series of experiments, the products of the reaction between $ONOO^-$ (20 mM) and D-glucose (100 mM) were kept on ice for 180 min protected from light. The dilution of the reaction mixtures on addition to the cascade or platelet suspensions was typically 1/100.

### *Detection of NO*

For the chemical characterization of the NO-releasing properties of compounds formed from the reaction of $ONOO^-$ with alcohols, NO was measured electrochemically using a Clark-type electrode (Diamond General Corporation) which was calibrated using anaerobic solutions of pure NO gas in water. $ONOO^-$ (20 mM) was incubated with D-glucose (0.25–1 M), D-fructose (1 M), glycerol (1 M) or DL-glyceraldehyde (1 M) in 0.5 M phosphate buffer (final pH 9–9.5) for 4 min on ice, then a 3 fold excess of buffer (pH 7.4) was added to ensure that all the $ONOO^-$ was decomposed. The mixture was then added to the electrode chamber (1/10) in Tyrode's solution (pH 7.2, 37°C), after which $CuSO_4$ (10 $\mu M$) and L-cysteine (1 mM) were added 2 and 3 min respectively after the addition of the mixture, and the maximal release of NO measured. In some experiments, 50 $\mu M$ oxyhaemoglobin was added to the chamber 1 min before the addition of the reaction products.

In order to measure NO release under conditions similar to those used to assess their biological effects, the more sensitive chemiluminescence method was used. Briefly, 1 ml of Tyrode's solution was pre-warmed at 37°C, and placed in a flat-bot-

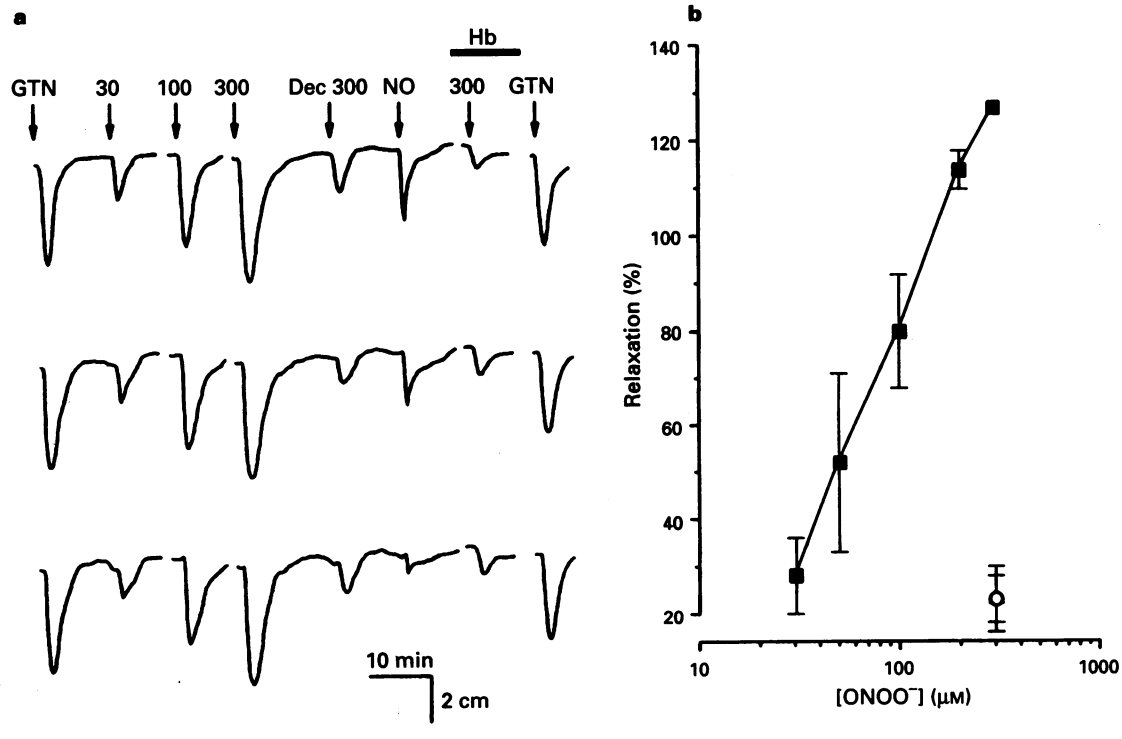


**Figure 1** Effect of $ONOO^-$ on vascular strips in the cascade bioassay. Three endothelium-denuded rabbit thoracic aortae were superfused in a cascade bioassay with Krebs buffer containing 11 mM glucose. The tissues were separated by a 3 s delay in the cascade. The responses of the tissues to a 30 s superfusion of (a) $ONOO^-$, (30–300 $\mu M$) and its decomposition products (300 $\mu M$, Dec 300) and the relaxation to a bolus injection of NO (4 $\mu M$, 15 s) are shown. Oxyhaemoglobin (5 $\mu M$, Hb) was added to the superfusion medium as indicated. Traces are representative of 3 similar experiments. (b) The concentration-dependent relaxation of vascular strips by $ONOO^-$ (■, 30–300 $\mu M$), the effect of the decomposition products of 300 $\mu M$ $ONOO^-$ (○) and the effect of oxyhaemoglobin (5 $\mu M$, Δ) on the relaxation caused by 300 $\mu M$ $ONOO^-$ are shown. Responses are plotted as a function of the nominal concentrations of $ONOO^-$ superfused over the tissues and results are calculated as percentages of the vasorelaxation caused by 50 nM GTN. The data represent the mean ± s.e.mean of 3 independent experiments.

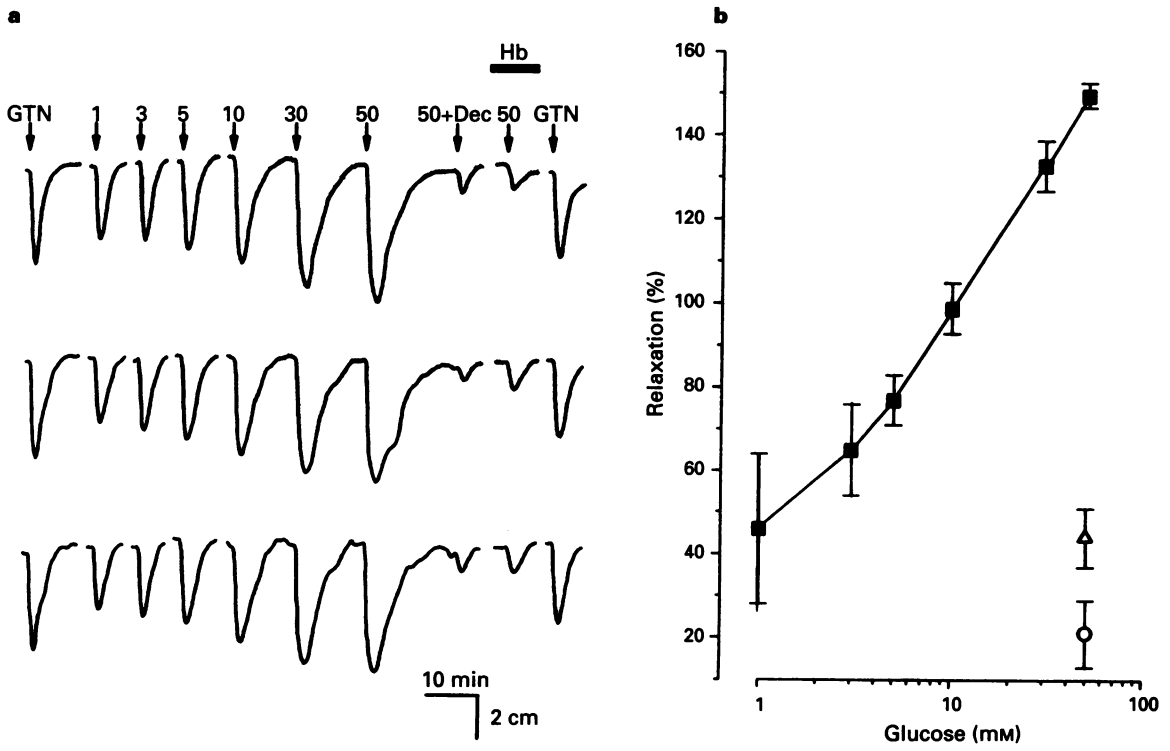


**Figure 2** Effect of the products of the reaction between D-glucose and $ONOO^-$ on vascular strips in the cascade bioassay. (a) The reaction products of $ONOO^-$ (20 mM) with D-glucose (1–50 mM) and decomposed $ONOO^-$ (20 mM) with 50 mM D-glucose (50+ Dec) were superfused over the three tissues in the cascade bioassay with an effective dilution of 1/100. The responses of the three tissues, which are separated by delay of 3 s in the cascade, are shown. Oxyhaemoglobin (5 μM, Hb) was added to the superfusion medium as indicated. Tracings are representative of 3 similar experiments. (b) The concentration-dependent relaxation of vascular strips by the reaction products of $ONOO^-$ (20 mM) with D-glucose (■, 1–50 mM), the effect of the reaction between decomposed $ONOO^-$ (20 mM) with 50 mM D-glucose (○) and the effect of 5 μM oxyhaemoglobin on the relaxation caused by the product of $ONOO^-$ and 50 mM D-glucose (Δ) are shown. Responses are plotted as a function of the concentration of D-glucose in the reaction mixture and results are calculated as percentages of the vasorelaxation caused by 50 nM GTN. The data represents the mean ± s.e.mean of 3 independent experiments.

tomed plastic test tube capped with a septum seal through which two needles were inserted. The solution was constantly stirred at room temperature and additions made through the septum. Nitrogen gas ($N_2$) was blown onto the surface of the solution through one of the needles, and the effluent gas exited via the other needle into the chemiluminescence system to determine NO. The products of the reaction of glucose with $ONOO^-$ were prepared exactly as decribed above for addition to platelets and diluted 1/100 before NO release was promoted by the addition of L-cysteine (1 mM) and $CuSO_4$ (10 μM). Standard curves were calculated from the peak height of the response with anaerobic solutions of pure NO gas in water.

### *Reagents*

Human oxyhaemoglobin was prepared by the method of Paterson *et al.* (1976). Prostacyclin sodium salt ($PGI_2$) (Wellcome), 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619, Calbiochem), glyceryl trinitrate (Du Pont Pharmaceuticals), Tyrode's salt solution (Gibco), collagen (Hormon-Chemie), L-glucose (Koch-Light Ltd), DL-glyceraldehyde (Aldrich), $CuSO_4$, D-glucose, D-fructose, glycerol (BDH) and L-cysteine (Sigma) were obtained from the sources indicated.

### *Statistics*

Results are mean ± s.e. mean of at least 3 separate experiments. Student's unpaired *t* test (GraphPad InStat, V2.04a, GraphPad Software, 1990–1993) was used to determine the significance of differences between means and $P<0.05$ was considered as statistically significant.

## Results

### *Effects of $ONOO^-$ on the cascade bioassay*

Addition of $ONOO^-$ (30–300 μM) to the superfusate at the top of the cascade, 3 s before it reached the first tissue, resulted in a concentration-dependent relaxation of bioassay tissues which was similar for all three detector strips and was inhibited by oxyhaemoglobin (5 μM, $n=3$, Figure 1a, b). Furthermore, relaxation induced by 30 μM $ONOO^-$ at the beginning of an experiment (32 ± 12%, $n=3$) was the same when this concentration was added again once the full concentration-response curve had been obtained (32 ± 11%, $n=3$, $P<0.05$). The highest concentration of decomposed $ONOO^-$ tested (300 μM) gave a small relaxation (23 ± 5%, $n=3$), which was negligible compared to 300 μM $ONOO^-$ (Figure 1) and was probably due to the effects of contaminating nitrite (Liu *et al.*, 1994).

These experiments suggested that a stable NO donor was formed from the reaction of $ONOO^-$ with the Krebs buffer. To investigate this in more detail, $ONOO^-$ (20 mM) was incubated in Krebs buffer without glucose to determine whether the sugar was the reactant leading to the formation of vasorelaxant substances. The products of the reaction between $ONOO^-$ (20 mM) and Krebs without glucose tested 1 min after preparation had little relaxant effect (18 ± 3%, $n=3$), whereas the products of the reaction of $ONOO^-$ with complete Krebs buffer led to vasorelaxation (95 ± 4%, $n=3$).

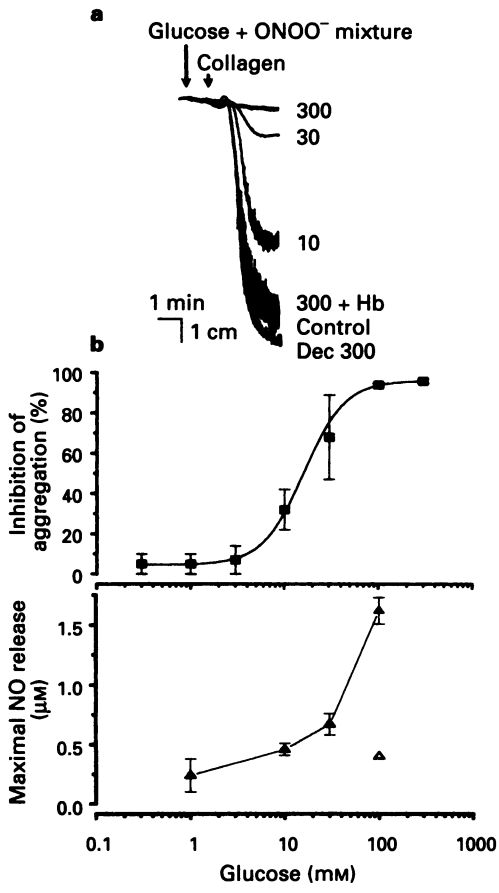


**Figure 3** Effect of the reaction product of $ONOO^-$ and D-glucose on collagen-induced platelet aggregation. (a) Inhibition by the reaction products of $ONOO^-$ (20 mM) and D-glucose (10–300 mM) of collagen-induced aggregation of WP (Coll; 1 $\mu g\,ml^{-1}$). Oxyhaemoglobin (300+Hb; 5 $\mu$M) abolished the anti-aggregatory effect of the product of the reaction between $ONOO^-$ and 300 mM D-glucose. The product of the reaction between 300 mM D-glucose and decomposed $ONOO^-$ (Dec 300; 300 $\mu$M) did not have any anti-aggregatory effect. Traces are representative of three different experiments. (b) The products of the reaction between $ONOO^-$ (20 mM) and D-glucose (0.3–300 mM) caused inhibition of collagen (0.75–1.5 $\mu g\,ml^{-1}$)-induced platelet aggregation (■). NO release from the products of glucose and $ONOO^-$ was measured by chemiluminescence (C). Samples were diluted 1/100 before NO release was promoted by the addition of L-cysteine (1 mM) and 1 min later $CuSO_4$ (10 $\mu$M). The NO released after the addition of both reagents to the sample is shown as function of glucose in the reaction mixture (▲) and for the reaction product with $ONOO^-$ and 100 mM glucose in the presence of cysteine alone (Δ). Data are mean ± s.e.mean, $n=3-7$.

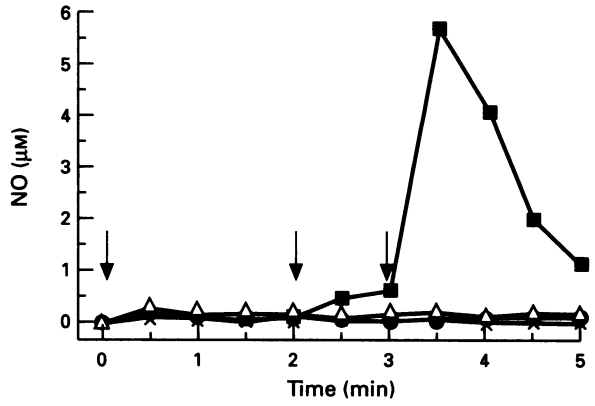


**Figure 4** Release of NO from the reaction product of D-glucose and $ONOO^-$. Peroxynitrite (20 mM) incubated with (■) or without (●), 1 M D-glucose and decomposed $ONOO^-$ (20 mM) incubated with 1 M D-glucose (Δ) were diluted 40 fold in the chamber of the NO electrode containing Tyrode solution (time = 0 min). In some experiments, oxyhaemoglobin (50 $\mu$M) was added to the electrode chamber 1 min before the addition of the product of the reaction of 20 mM $ONOO^-$ and 1 M D-glucose (X). After recording a stable baseline, 10 $\mu$M $CuSO_4$ was added (time = 2 min) followed 1 min later by 1 mM L-cysteine (time = 3 min).

**Table 1** Release of NO from $ONOO^-$-treated hydroxy compounds in the presence of cysteine and copper

| *Compound* | *Maximal concentration of NO release (μM)* |
|---|---|
| Peroxynitrite alone | 0 |
| Peroxynitrite + D-glucose 0.25M | 1.38 ± 0.11 |
| Peroxynitrite + D-glucose 0.5M | 3.45 ± 0.11 |
| Peroxynitrite + D-glucose 1M | 6.04 ± 0.13 |
| Decomposed peroxynitrite + D-glucose 1M | 0 |
| Peroxynitrite + fructose 1M | 5.63 ± 0.45 |
| Peroxynitrite + glycerol 1M | 2.32 ± 0.23 |
| Peroxynitrite + glyceraldehyde 1M | 0.44 ± 0.02 |

NO release was measured with the NO electrode. The products of the reaction of glucose and other sugars with $ONOO^-$ were prepared as described in the methods and diluted 1/10 before NO release was promoted by the addition of $CuSO_4$ (10 $\mu$M) and L-cysteine (1 mM). Standard curves were calculated from the peak height of the response using anaerobic solutions of pure NO gas in water and the minimum detection limit for NO was approximately 0.1 $\mu$M. Results are given as the mean ± s.e.mean for a minimum of 3 independent experiments.

### *Effect on the cascade bioassay and on platelet aggregation of the products of the reaction between glucose and $ONOO^-$*

The products of the reaction between D-glucose (1–50 mM) and $ONOO^-$ (20 mM) caused vasorelaxation of the cascade bioassay tissues, which was dependent on the glucose concentration in the reaction mixture (Figure 2 a, b). The effect was identical for the three tissues in the cascade (Figure 2a) and was inhibited in the presence of 5 $\mu$M oxyhaemoglobin ($n=3$, Figure 2a, b).

In addition to their vascular effects, the products of the reaction between D-glucose (0.3–300 mM) and $ONOO^-$ (20 mM) caused inhibition of collagen-induced platelet aggregation which was greater as the concentration of D-glucose in the reaction mixture was increased and was abolished in the presence of oxyhaemoglobin (5 $\mu$M, $n=3$, Figure 3a, b). Control experiments with decomposition products of $ONOO^-$ incubated with glucose showed no effect ($n=3$, Figure 3a). The inhibitory activity of the product formed in the reaction of D-glucose (100 mM) with $ONOO^-$ (94 ± 1%) decayed significantly 180 min after its preparation (49 ± 15%, $n=3$; $P<0.05$, mean ± s.e.mean). The products of the reaction between 100 mM L-glucose and 20 mM $ONOO^-$ also caused inhibition of platelet aggregation (93 ± 1%, $n=3$, $P>0.05$), which was not significantly different from that obtained when the same concentration of D-glucose was used as a reactant (94 ± 1%, $n=3$, $P>0.05$).

### *Release of NO from the products of the reaction between $ONOO^-$ and D-glucose or other alcohols*

The decomposition of $ONOO^-$ (1 mM) was monitored spectrophotometrically at 302 nm in $H_2O$ (pH 9) and found to be accelerated 3.75 ± 0.023 fold (mean ± s.e.mean, $n=3$) by the presence of 20 mM glucose. When the adduct formed from the

reaction between $ONOO^-$ (20 mM and D-glucose (0.25–1 M) was added to the NO electrode chamber, no release of NO could be detected (Figure 4). The addition of $CuSO_4$ (10 $\mu$M) elicited a small increase in NO which was substantially enhanced on addition of L-cysteine (1 mM). The amount of NO released increased as a function of the concentration of D-glucose in the reaction mixture with $ONOO^-$ (Table 1). The NO signal in the electrode was inhibited by oxyhaemoglobin (50 $\mu$M, $n=3$, Figure 4). Incubating decomposed $ONOO^-$ (20 mM) with D-glucose (1 M), or the addition of $ONOO^-$ alone (20 mM), resulted in no detectable formation of NO in the electrode ($n=3$, Figure 4). Nitric oxide formation was also detected from the products of the reaction between $ONOO^-$ (20 mM) and other compounds containing the alcohol functional group such as fructose, glyceraldehyde and glycerol (all at 1 M, $n=3$, (Table 1). The NO-dependent response of the electrode was abolished in all cases by 50 $\mu$M oxyhaemoglobin ($n=3$, not shown).

In order to quantify the amounts of NO released during the biological experiments, the release of NO from the products of the reaction between $ONOO^-$ (20 mM) and D-glucose (0.3–300 mM) was measured by the more sensitive chemiluminescence method. In the presence of $Cu^{2+}$ (10 $\mu$M) and cysteine (1 mM), the amount of NO released was found to be dependent on the concentration of D-glucose in the reaction mixture (Figure 3c). The addition of cysteine alone elicited the release of a small amount of NO which is probably due to trace transition metal contamination of the Tyrode's buffer used in the experiment (Figure 3c). The release of NO obtained from the product of the reaction between $ONOO^-$ and 100 mM D-glucose ($1.65 \pm 0.24$ $\mu$M) decayed when the product was assayed 180 min after its preparation ($0.51 \pm 0.02$ $\mu$M, $n=3$, $P<0.05$).

## Discussion

Our results show that $ONOO^-$ reacts with glucose to form compounds which cause vasorelaxation and inhibit platelet aggregation. We have shown that $ONOO^-$ causes relaxation of bioassay tissues arranged in a cascade, confirming previous studies performed on bovine pulmonary arterial rings (Wu *et al.*, 1994), canine coronary arterial rings (Liu *et al.*, 1994) and coronary vasculature of the isolated rat heart (Villa *et al.*, 1994). However, in contrast to the results obtained in rat coronary vasculature (Villa *et al.*, 1994) $ONOO^-$ did not cause tachyphylaxis in this preparation. The reasons for these differences are unknown but could arise from the complete destruction of $ONOO^-$ through reaction with the medium or buffers which occupy a relatively large volume in the organ bath experiments or separate the tissues in the cascade. However, in the isolated rat heart, where $ONOO^-$ dependent damage was associated with tachyphylaxis, the oxidant was infused directly into the coronary vasculature, which may then have resulted in direct effects on the vasculature.

Consistent with an NO-dependent mechanism, the $ONOO^-$ induced vasorelaxation and inhibition of platelet aggregation was prevented by oxyhaemoglobin. However, the cascade bioassay revealed that the half-life of the vasorelaxant substance is longer than 20 s, since the responses do not change as it travels down the three tissues, which are separated from each other by a 3 s time delay. These responses cannot be ascribed either to $ONOO^-$ or NO, as these molecules have apparent half-lives of 2 and 4 s, respectively, under these conditions and vasorelaxation must, therefore, arise from the formation of a compound which can act as an NO donor (Palmer *et al.*, 1987; Beckman *et al.*, 1990). We have recently shown that $ONOO^-$ reacts with glutathione to form an S-nitrosothiol with NO-donating properties (Moro *et al.*, 1994). However, this reaction is unlikely to occur in the bioassay cascade since thiols are not present in the superfusate. Thiols are present in the tissues but the fact that the relaxation was identical in the three strips precludes a direct reaction with $ONOO^-$, which has a half life of approximately 2 s under these conditions. This suggests that the material was formed in the superfusion medium prior to its contact with the tissues. Since previous studies have shown that $ONOO^-$ reacts with a variety of organic molecules, including sugars and bicarbonate (Beckman *et al.*, 1990; Hogg *et al.*, 1992; Zhu *et al.*, 1992; Van der Vliet *et al.*, 1994), we tested this possibility and found that the reaction between D-glucose and $ONOO^-$ was responsible for the vasorelaxant effects we have observed.

The products of the reaction between glucose and $ONOO^-$ also caused vasorelaxation of the tissues in the cascade and inhibition of platelet aggregation. The reaction appears to be rapid and probably proceeds through a series of complex chemical processes which may involve a contribution from contaminating transition metals in the solutions. One possible mechanism for the formation of an NO donor is that in the scavenging of $ONOO^-$ by sugars a compound-derived alkoxyl radical is formed which terminates in a rapid reaction with nitrogen dioxide to form an organic nitrate. In support of this idea it has been shown that nitrogen dioxide is released on the reaction of sugars with $ONOO^-$ (Zhu *et al.*, 1992). Some of the compounds tested contain a carbonyl group which could also be involved in the reaction with $ONOO^-$. Although free radicals are involved in the reaction of sugars with $ONOO^-$, their short lifetime makes it highly unlikely that they contribute to the biological actions we are observing. However, the reaction of $ONOO^-$ *in vivo* will presumably include components of direct $ONOO^-$-dependent damage in addition to the formation of potentially cytoprotective NO donors, as we have found for its interaction with platelets and the coronary vasculature (Moro *et al.*, 1994; Villa *et al.*, 1994).

The product of the reaction between $ONOO^-$ and glucose caused significant release of NO only when exposed to both $Cu^{2+}$ and L-cysteine (Figures 3c, 4); a property shared with organic nitrates such as GTN or nitrites such as amyl nitrite (Feelisch & Noack, 1987; Chong & Fung, 1991). The precise mechanisms for this process are still unclear but could involve reaction of a thiol with an organic nitrite to form an S-nitrosocysteine intermediate (Feelisch, 1991), the decomposition of which is accelerated by copper ions (McAninly *et al.*, 1993). This hypothesis would also explain previous work showing a decrease in both relaxation and the formation of NO from pulmonary arteries on exposure to $ONOO^-$ when the preparation had been pre-treated with diethyl maleate, an agent that depletes tissue thiols (Wu *et al.*, 1994). It is not known whether metals or metalloenzymes are involved in the mechanisms leading to NO release in the tissues from organic nitrates such as GTN or the reaction product of $ONOO^-$ with glucose. This is currently difficult to probe experimentally since highly specific copper chelators which can enter cells are not available. It is important to note that the concentrations of NO released from the organic nitrate/nitrite in the presence of $Cu^{2+}$ and cysteine may be close to the maximum yields and substantially greater than that needed to inhibit platelet aggregation. Indeed, we were unable to detect NO release on mixing platelets with the product of glucose and $ONOO^-$ although inhibition of aggregation had clearly occurred (result not shown).

The generation of NO donors similar to that formed on reaction of $ONOO^-$ with D-glucose was found with other compounds containing the alcohol functional group such as L-glucose, D-fructose, DL-glyceraldehyde and glycerol. Moreover, the fact that the products resulting from the reaction of $ONOO^-$ with D- and L-glucose are equipotent suggests that the release of NO is extracellular, since L-glucose cannot be transported intracellularly.

In summary, our results show that $ONOO^-$ reacts with compounds containing an alcohol functional group to form an NO donor with characteristics of an organic nitrate/nitrite. The concentrations of glucose used in the physiological buffers are higher than those normally encountered in plasma but lower than the total concentration of polyhydroxy compounds with which $ONOO^-$ could conceivably react to form an NO donor. Our experiments also suggest that the previously re-

ported NO-dependent effects of $ONOO^-$ may in large part be ascribed to the formation of NO donors arising from its reaction with components in the media used for the experimental studies. Assuming that 100% of the molecules formed release NO in the presence of $Cu^{2+}$ and L-cysteine, our results indicate that the yield of the reaction must be at least 0.1%. The interesting question remains as to whether these reactions may also occur *in vivo*. If so, they may represent a further detoxification pathway for $ONOO^-$, resulting in products with potentially cytoprotective properties (Moro *et al.*, 1994; Villa *et al.*, 1994).

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