Effect of Peroxynitrite on Passive K⁺ Transport in Human Red Blood Cells

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Key Words

Peroxynitrite • K⁺ transport • Oxygen • Volume • pH • Protein phosphatase

Abstract

Peroxynitrite is generated *in vivo* by the reaction between nitric oxide, from endothelial and other cells, and the superoxide anion. It is therefore pertinent to examine its effects on the membrane permeability of red blood cells. Treatment of human red blood cells with peroxynitrite (nominally 1 mM) markedly stimulated passive K⁺ permeability. The main effect was on a Cl⁻-independent K⁺ pathway, which remains unidentified. Although K⁺-Cl⁻ cotransport (KCC) was stimulated, this was dependent on saline composition, being inhibited by physiological levels of glucose (IC₅₀ 4 mM), and also by sucrose and MOPS. Effects on the Cl⁻-independent K⁺ pathway were less dependent on saline composition, and were not inhibited by amiloride, ethylisopropylamiloride, dimethylamiloride or gadolinium. Na⁺-K⁺-2Cl⁻ cotransporter was inhibited whilst there was little effect on the Gardos channel (Ca²⁺-activated K⁺ channel). Peroxynitrite was markedly more effective in oxygenated cells than

deoxygenated ones. Treatment with peroxynitrite *per se* did not affect initial cell volume. Anisotonic swelling modestly increased the Cl⁻-independent K⁺ influx, but did not affect peroxynitrite-stimulated KCC. Decreasing extracellular pH from 7.4 to 7.2 or 7.0 increased KCC stimulation, whilst the Cl⁻-independent component of K⁺ transport was lowest at pH 7.2. Finally, protein phosphatase inhibition with calyculin A (100 nM) inhibited KCC, implying that, as with other KCC stimuli, peroxynitrite acts via decreased protein phosphorylation; pre-treatment with calyculin A also inhibited the Cl⁻-independent component of K⁺ transport. These findings are relevant to the actions of peroxynitrite *in vivo*.

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Introduction

A number of oxidants have been shown to increase the passive K⁺ permeability of red blood cells [1-3]. This action is important because, as the predominant cation, efflux of K⁺ will result in cell shrinkage, with concomitant rheological effects[4]. It is relevant *in vivo* since oxidants

can accumulate under certain pathological conditions[5], an example would be heavy metal poisoning. The effect of oxidants may also be exacerbated should the antioxidant pathways of the red blood cell become compromised. These include principally reduced glutathione (GSH) and ascorbate [5, 6], which may be depressed by inhibition of the pentose-phosphate pathway, eg glucose 6-phosphate dehydrogenase deficiency, or inadequate ingestion of ascorbate.

Peroxynitrite represents a powerful oxidant. It is generated in the circulation *in vivo* by the reaction between nitric oxide from endothelial cells, and the superoxide anion [7]. Peroxynitrite is cell permeable and known to react with nucleic acid [8], phospholipids, sulphhydryl groups and tyrosine residues. Although its biological half-life is short [7], because nitric oxide is released from endothelial cells, it is generated directly in the circulation and will therefore have potential access to the red blood cells. It is therefore pertinent to examine the effects of peroxynitrite on the membrane permeability of red blood cells. Indeed, a recent preliminary report has shown that peroxynitrite increases K^+ permeability in human red blood cells [9] but did not identify definitively the pathway(s) involved. Nor did it address any potential interactions between peroxynitrite-induced transport and other stimuli.

The major passive K^+ pathway of normal red blood cells is probably the Cl^- -dependent K^+ transport which is mediated mainly via the K^+ - Cl^- cotransporter (KCC, probably KCC1). KCC activity is controlled by protein kinases and phosphatases [10]. It is also sensitive to physiological stimuli such as cell volume, pH and O_2 tension. The Cl^- -independent component of K^+ transport is represented mainly by the Ca^{2+} -activated K^+ channel (the Gardos channel, probably SK4 [11, 12]). In addition, however, there is a basal K^+ permeability of uncertain identity, but which includes K^+/Na^+ or H^+ exchange [13]. More recently a non-selective cation pathway has been described in cells treated with oxidants or osmotic shock [14-16]. In normal red blood cells, however, all these pathways have low activity under physiological conditions: KCC is largely quiescent but can be stimulated by low pH or increased cell volume [17]; the Gardos channel is inactive because the plasma membrane Ca^{2+} pump maintains intracellular $[Ca^{2+}]$ below the level required to activate the channel [18]; K^+/Na^+ or H^+ exchange requires non-physiological conditions (eg low ionic strength) to become manifest; and, finally, the non-selective cation channel is cryptic.

In this report, we use $^{86}Rb^+$ as a K^+ congener to investigate the effect of peroxynitrite on passive K^+ permeability in human red blood cells, focussing on its interaction with other important regulators including cell volume, pH, O_2 tension and the protein phosphatase inhibitor calyculin A.

Materials and Methods

Chemicals

A23187, amiloride, bumetanide, clotrimazole, dimethylamiloride, ethylisopropylamiloride, glucose, inosine, MOPS, ouabain, salts and sucrose were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Peroxynitrite and calyculin A were purchased from Calbiochem (Nottingham, UK) and $^{86}Rb^+$ was from DuPont-NEN (Stevenage, UK).

Sample collection and handling

Blood samples were obtained by venepuncture of healthy donors (genetically HbAA), with permission under ethical consent, and collected into heparinized syringes. Samples were kept at 4°C until use within 48h.

Solutions

The standard saline (MBS) comprised (in mM): NaCl 100, Na_2HPO_4 / NaH_2PO_4 50, pH 7.4 at 37°C; 290 ± 5 mosmol.kg⁻¹ H_2O . Following control experiments, glucose was omitted because of its reaction with peroxynitrite, but in these cases, cells were pre-incubated at 37°C for 60 min with glucose and inosine (both 10 mM) to ensure ATP repletion, until just prior to treatment. They were then washed (three times) in glucose- and inosine- free saline prior to treatment with peroxynitrite and measurement of K^+ influx. They were not deprived of metabolic substrates for more than 15 min at 37°C. In some experiments, MOPS was used as the buffer instead of phosphate, in which case saline composition was (in mM): NaCl 145, MOPS 10, pH 7.4 at 37°C. For experiments in which Cl^- dependence of K^+ influx was examined, NO_3^- -containing salts replaced those containing Cl^- (four washes). Anisotonic cell swelling was achieved by adding distilled water. Extracellular pH was altered by addition of HNO_3 or NaOH. For experiments to examine effects on Gardos channel activity, extracellular Ca^{2+} (2 mM $Ca(NO_3)_2$) was added to the standard saline. Stock solutions of bumetanide (1 mM) were prepared daily in 100 mM Tris base and used at a final concentration of 10 μ M. Stock solutions of ouabain (10 mM) were prepared in distilled water and used at a final concentration of 100 μ M. Stocks of calyculin A (10^{-5} M) were prepared in 20% DMSO / 80% water, frozen until required, and used at final concentrations of 100 nM. Clotrimazole stocks (10 mM) were made in DMSO and used at a final concentration of 5 μ M. Peroxynitrite was purchased as a solution (200 mM in 1.15 M NaOH). It was stored under N_2 at -70°C. It was usually added to give an initial concentration of 1 mM, pH 7.2.

Tonometry

To equilibrate at the requisite O_2 tension, red cell suspensions were placed in tonometers (Eschweiler, Kiel, Germany) at 40% haematocrit (Hct) and flushed with warm, humidified gas mixtures, supplied at the appropriate O_2 tension (see [19] for details). Samples were then diluted 10-fold into saline, again pre-equilibrated at the required O_2 tension, prior to addition of peroxynitrite and measurement of influx.

K^+ influx

To determine the activity of the K^+ transport pathways, K^+ influx was measured over 10 min at 37°C using $^{86}Rb^+$ as a congener for K^+ (see [20]), added in 150 mM KNO_3 to give a final $[K^+]$ of 7.5 mM. Cells were taken from the tonometers and diluted 10-fold into saline. Where appropriate, salines were made anisotonic through addition of water or titrated to different pHs. Ouabain (100 μM) and bumetanide (10 μM) were present in all experiments to obviate any K^+ transport through the $Na^+-K^+-ATPase$ and the $Na^+-K^+-2Cl^-$ cotransporter, respectively, except for experiments to measure NKCC activity (when influx was measured in the presence of $Cl^- \pm$ bumetanide). Either microhaematocrit determination or the cyanohaemoglobin method was used to measure the haematocrit (Hct). Cl^- -independent K^+ influx was measured in the absence of Cl^- ; KCC activity was assayed as Cl^- -dependent K^+ influx; NKCC as the bumetanide-sensitive K^+ influx (in the presence of Cl^-) and the Gardos channel activity as the clotrimazole-sensitive K^+ influx.

Cell volume

Cell water content was assessed using the wet weight / dry weight method of Borgese *et al* [21] and expressed as $ml.g^{-1}$ dry cell solids (d.c.s.).

Statistics

Results are presented as single observations representative of at least 3 others, or as means $\pm$ S.E.M. of n observations. Where appropriate, comparisons were made using Student's t test.

Results

Effect of peroxynitrite on passive K^+ transport

Preliminary experiments were carried out using saline containing 10 mM glucose and MOPS as a buffer, which is standard in many laboratories including our own. Peroxynitrite was added either as a single bolus (1 mM initial concentration), or serially (100 μM every min during the 10 min influx period) because of its lability at physiological pH. The oxidant action of peroxynitrite was immediately apparent from the generation of methaemoglobin.

In a typical experiment, in the presence of bumetanide (10 μM), K^+ influx increased from 0.22 ± 0.02 $mmol.(l\ cells.h)^{-1}$ in the presence of Cl^- and

0.20 ± 0.02 in its absence, to 3.42 ± 0.31 and 3.32 ± 0.23 , respectively (both $p < 0.0005$; means $\pm$ S.E.M., $n = 3$). We examined effects of peroxynitrite on the Gardos channel (the Ca^{2+} -activated K^+ channel). In control conditions, in the presence of 2 mM extracellular Ca^{2+} , the clotrimazole-sensitive influx was 0.13 ± 0.21 $mmol.(l\ cells.h)^{-1}$, declining to -0.01 ± 0.53 with peroxynitrite; when the Gardos channel was stimulated by raising intracellular Ca^{2+} through incubation for 10 min with A23187 (2 μM), the clotrimazole-sensitive influx was 32.33 ± 2.91 in the absence of peroxynitrite to 29.36 ± 2.24 in its presence (means $\pm$ S.E.M., $n = 3$; $p > 0.15$). Under these conditions, therefore, peroxynitrite markedly increased the Cl^- -independent K^+ transport, but had little effect on the activity of the Gardos channel or KCC.

The previous report describing peroxynitrite-induced K^+ transport used phosphate-buffered saline in the absence of glucose [9]. When these conditions were replicated, stimulation of Cl^- -independent K^+ influx was similar to that described above, whilst that of Cl^- -dependent pathway increased (Figure 1; $p < 0.02$). Nevertheless, the magnitude of the Cl^- -independent influx remained about 3-fold greater, showing the greater effect of peroxynitrite on this pathway. Decomposed peroxynitrite (left overnight at room temperature in air) was without effect (Figure 1).

We also examined effects on the bumetanide-sensitive K^+ influx, in the presence of Cl^- , as a measure of $Na^+-K^+-2Cl^-$ cotransport activity: bumetanide-sensitive influx was 0.39 ± 0.04 $mmol.(l\ cells.h)^{-1}$ in control cells, and 0.16 ± 0.07 in the presence of peroxynitrite (means $\pm$ S.E.M., $n = 5$; $p < 0.02$).

A series of experiments was carried out to investigate the interaction of glucose with peroxynitrite using phosphate-buffered saline. KCC activity was inhibited by the presence of glucose with an IC_{50} of about 4 mM ($54 \pm 4\%$ reduction; Figure 2). By contrast, the Cl^- -independent K^+ pathway remained elevated in glucose, eg 4.79 ± 0.41 $mmol.(l\ cells.h)^{-1}$ in 5 mM glucose. Thus at physiological levels of glucose, the main stimulatory effect of peroxynitrite on passive K^+ flux (via the Cl^- -independent pathway) would remain, notwithstanding the marked decrease in stimulation of KCC activity. In similar experiments, it was found that MOPS and sucrose also inhibited peroxynitrite-induced stimulation of KCC (data not shown). Peroxynitrite stimulation of KCC therefore was dependent on the composition of the saline. All remaining experiments were carried out in phosphate buffered saline in the absence of glucose.

Fig. 1. Effect of peroxynitrite on passive K^+ transport in human red blood cells. Red blood cells were incubated with peroxynitrite (1 mM) in air and K^+ influx measured in the presence and absence of Cl^- (substituted with NO_3^-). Decomposed peroxynitrite (left in air at room temperature overnight) was also tested. Glucose and inosine were omitted from the saline, which was buffered with phosphate. Ouabain (100 μ M) and bumetanide (10 μ M) were present throughout. Histograms represent means $\pm$ S.E.M. for 4 separate ex-periments.

Interaction of peroxynitrite with O_2

Both the ability of peroxynitrite to oxidize target molecules and the activity of passive K^+ pathways may be influenced by O_2 tension [22, 23]. We therefore compared the effect of peroxynitrite in oxygenated and deoxygenated red blood cells. Results are shown in Figure 3. Under air, peroxynitrite had similar effects to those depicted in Figure 1 – marked stimulation of Cl^- -independent K^+ influx, to about 3 mmol.(l cells.h) $^{-1}$, and moderate stimulation of KCC activity, to about 1 mmol.(l cells.h) $^{-1}$. By contrast, in deoxygenated red blood cells, there was no activation of KCC ($p < 0.03$ cf air) and stimulation of the Cl^- -independent pathway was much reduced ($p < 0.02$ cf air). These results show that the effect of peroxynitrite on passive K^+ permeability varies with O_2 tension.

Interaction of peroxynitrite with cell volume and pH

Cell volume and pH also represent important modulators of passive K^+ permeability in red blood cells. Cell water content was unaffected by peroxynitrite (1.84 $\pm$ 0.02 ml.g $^{-1}$ d.c.s. in control cells, 1.80 $\pm$ 0.02 with peroxynitrite, means $\pm$ S.E.M., $n = 14$; N. S.). Saline osmolality was reduced to produce anisotonic cell swelling in cells treated with 1 mM peroxynitrite. The effect on

K^+ permeability is shown in Figure 4. Cl^- -independent K^+ influx increased progressively as saline osmolality was decreased (Figure 4; $p < 0.001$ at 237 mOsm.kg $^{-1}$). By contrast, KCC activity was unaffected by reduction in osmolality to 275 (5% reduction in osmolality) and 257 (11%) mOsm.kg $^{-1}$. At 237 mOsm.kg $^{-1}$ (18%), KCC activity was reduced but not significantly ($p > 0.20$).

The interaction of peroxynitrite and saline pH is shown in Figure 5. In these experiments, cells were treated with peroxynitrite (1 mM) at pH 7.2. They were then divided into three aliquots for K^+ influx determination at pH 7, 7.2 and 7.4. Cl^- -independent K^+ influx was lowest at pH 7.2 and increased as saline pH was raised to pH 7.4 ($p < 0.002$) or lowered to pH 7.0. By contrast, KCC activity showed a different relationship with pH, being reduced as pH was increased ($p < 0.04$).

Effect of inhibitors of passive K^+ permeability on peroxynitrite-treated red blood cells

Peroxynitrite-induced K^+ influx was also examined in cells treated with the protein phosphatase inhibitor calyculin A (100 nM). Results are shown in Figure 6. In cells pretreated with calyculin A before addition of peroxynitrite, KCC activity was abolished ($p < 0.02$), whilst the magnitude of Cl^- -independent K^+ influx was markedly reduced ($p < 0.02$). In cells treated with calyculin A after

Fig. 2. Effect of glucose on peroxynitrite-induced $\text{K}^+\text{-Cl}^-$ cotransport in human red blood cells. Red blood cells were treated with peroxynitrite (1 mM) in the absence or presence of glucose (3, 4 or 5 mM). Only the Cl^- dependent K^+ influx (influx $\pm \text{Cl}^-$) is shown, in the presence of ouabain (100 μM) and bumetanide (10 μM). Histograms represent means $\pm$ S.E.M. for 3 separate experiments.

Fig. 3. Effect of oxygen tension on peroxynitrite-induced K^+ transport in human red blood cells. Oxygenated or deoxygenated red blood cells were incubated with peroxynitrite (1 mM) in air and K^+ influx measured in the presence and absence of Cl^- (substituted with NO_3^-). Ouabain (100 μM) and bumetanide (10 μM) were present throughout. Histograms represent means $\pm$ S.E.M. for 4 separate experiments.

peroxynitrite, KCC activity was reduced whilst Cl^- -independent K^+ influx was increased, but both changes were insignificant. These experiments indicate that a functional protein phosphatase is required for the stimulatory effect of peroxynitrite on KCC but not on the Cl^- -independent K^+ pathway.

In the final series of experiments, the action of several cation channel inhibitors was examined on

peroxynitrite-induced passive K^+ fluxes. There was no inhibition by amiloride (1.1 mM), ethylisopropylamiloride (111 μM), dimethylamiloride (111 μM) or gadolinium (167 μM), data not shown. Whilst gadolinium inhibits stretch-activated pathways, amiloride and its derivatives would block the non-selective cation channel, recently described [14-16].

Fig. 4. Effect of cell volume on peroxynitrite-induced K^+ transport in human red blood cells. Red blood cells were incubated with peroxynitrite (1 mM) in air and K^+ influx measured in the presence and absence of Cl^- (substituted with NO_3^-), at 4 different osmolalities (300, 275, 257 and 237 mOsm.kg $^{-1}$) to swell cells anisotonicly. Ouabain (100 μ M) and bumetanide (10 μ M) were present throughout. Histograms represent means $\pm$ S.E.M. for 3 separate experiments.

Fig. 5. Effect of extracellular pH on peroxynitrite-induced K^+ transport in human red blood cells. Red blood cells were pre-incubated with peroxynitrite (1 mM) at pH 7.2. They were then washed into saline at 3 different extracellular pHs (7.0, 7.2 and 7.4) before measuring K^+ influx in the presence and absence of Cl^- (substituted with NO_3^-). All procedures were carried out in air. Ouabain (100 μ M) and bumetanide (10 μ M) were present throughout the influx period. Histograms represent means $\pm$ S.E.M. for 3 separate experiments.

Discussion

The findings presented in this paper further our understanding of how peroxynitrite affects passive K^+ transport in human red blood cells. We confirm that passive

K^+ permeability was stimulated by peroxynitrite. We demonstrate that the main stimulatory action was via a Cl^- -independent K^+ pathway whose identity remains uncertain. We show that NKCC was inhibited by peroxynitrite, with little effect on the Gardos channel.

Fig. 6. Effect of the protein phosphatase inhibitor calyculin A on peroxynitrite-induced K^+ transport in human red blood cells. Red blood cell samples were divided into three aliquots. Control cells were incubated with peroxynitrite (1 mM) in air and K^+ influx measured in the presence and absence of Cl^- (substituted with NO_3^-). The other two aliquots were either treated with calyculin A (100 nM) for 10 min before addition of peroxynitrite (Calyculin / $ONOO^-$) or treated with peroxynitrite before addition of calyculin A ($ONOO^-$ / Calyculin). Ouabain (100 μ M) and bumetanide (10 μ M) were present throughout the influx period. Histograms represent means $\pm$ S.E.M. for 3 separate experiments.

Stimulation of KCC activity did occur, but was modest compared to effects on the Cl^- -independent component of K^+ permeability. Increase in KCC activity was also particularly sensitive to the conditions used. Glucose (and other substances such as sucrose and MOPS) inhibited peroxynitrite-induced stimulation of both the Cl^- -independent K^+ flux and KCC activity. Oxygen tension and pH also modulated the response, whilst cell volume has little effect. Finally, functional protein phosphatase activity is required for stimulation of KCC. Although there is some variation with pH and cell volume, stimulation of Cl^- -independent K^+ transport is less sensitive to other interactions.

Peroxyntirite in vivo

Peroxyntirite is formed from nitric oxide and the superoxide anion [7]. It is a strong oxidant which has been proposed increasingly to have biological relevance. Physiologically, it may be generated by macrophages to facilitate killing of bacteria. In addition, overproduction could result in oxidative damage to the host. It may participate in a number of pathological conditions involving oxidation, such as ischaemic/reperfusion injury, neurodegenerative diseases, cardiovascular disorders and severe inflammatory conditions.

The reactions of peroxyntirite are complex and remain incompletely understood [7]. Prevailing environmental conditions modulate its reactivity. In particular, although stable at alkaline pHs, at physiological

pH, it is protonated (forming peroxyntirous acid, which has a pK_a of about 6.8) and rapidly decomposes to the inactive nitrate. Although its physiological half life is short (< 0.1 s), it is capable, nevertheless, of reacting with various target molecules. Thus, in solution, it has been shown to oxidise a number of molecules including GSH [22, 24], proteins [25, 26], lipids [27] and nucleic acids [7, 8]. In the presence of CO_2 , peroxyntirite will also rapidly form nitrosoperoxocarbonate [28]. Nevertheless, and notwithstanding the antioxidant defences of plasma [29], or the presence of CO_2 [28], peroxyntirite does enter red blood cells by both free diffusion (as undissociated acid) and a DIDS-sensitive pathway via the anion exchanger (as $ONOO^-$) [30]. It may also enter presumably via anion channels but this has not been investigated. Inside the red blood cell, peroxyntirite reacts with oxyhaemoglobin, to form metHb, and nitrates aromatic compounds such as tyrosine residues [28, 31]. It also oxidises thiols, such as GSH and $-SH$ residues of proteins [30]. In the absence of reactive targets, peroxyntirite slowly reacts with O_2 decomposing to nitrite and nitrate [7].

Peroxyntirite and Cl^- - independent K^+ transport

We show here that the main effect of peroxyntirite on passive K^+ transport in human red blood cells is via a Cl^- -independent passive K^+ permeability. Stimulation of this pathway is several fold that of KCC activity – see below. Notwithstanding, the molecular identity of this pathway is not clear. In other cells, peroxyntirite has been

shown either to stimulate (cerebral arteriolar ATP-dependent K^+ conductance [32, 33]) or inhibit (basolateral renal K^+ channels [34] or Ca^{2+} -activated K^+ channel of coronary arterioles [35]) passive K^+ transport. In our experiments, the absence of Cl^- , the presence of bumetanide and ouabain, obviate the participation of the two cation-chloride cotransporters (KCC and NKCC) and the Na^+/K^+ pump. In addition, from our results, the Gardos channel (the Ca^{2+} -activated K^+ channel) appears to be slightly, but not significantly, inhibited by peroxynitrite (as in coronary arterioles [32]), and NKCC also. The lack of effect of amiloride and its derivatives, and inhibition by shrinkage, indicate that the pathway is probably different to the non-selective cation channels, previously observed in response to oxidative damage and osmotic stress [14-16]. Other possible K^+ pathways include the K^+/H^+ or Na^+ exchanger [13, 36]. In fact, it shares some properties with this pathway at physiological ionic strengths - ie partial inhibition by calyculin A, insensitivity to amiloride and slight inhibition as pH is lowered from 7.4 to 7.2 [36]. It would appear, therefore, that protein phosphorylation plays some role in regulating this component of the K^+ flux. Further experiments are required, however, to ascertain whether the two permeabilities are, in fact, mediated by the same transport protein. It is also possible that oxidation of membrane proteins and / or lipids induces the formation of an abnormal K^+ pathway. Notwithstanding, the interaction of peroxynitrite with this K^+ pathway shows markedly different characteristics to that with KCC – see below. Thus it is increased by both cell swelling and by elevation of pH from 7.0 or 7.2 to 7.4. It is largely unaffected by glucose, but it is partially inhibited by deoxygenation. Furthermore, stimulation of the Cl^- -independent K^+ pathway is less sensitive to prior treatment of cells with calyculin A ($p < 0.02$) and stimulated by post treatment (N.S.).

Peroxynitrite and K^+ - Cl^- cotransport

Pervious reports of stimulation of a Cl^- -dependent K^+ transport by peroxynitrite did not exclude effects on NKCC, nor did they abolish Na^+/K^+ pump activity [9]. We therefore show here that peroxynitrite stimulates KCC activity, in the presence of bumetanide to abrogate any flux via NKCC. By contrast, we demonstrate that peroxynitrite inhibits the bumetanide-sensitive K^+ influx, likely to be via NKCC. Furthermore, we demonstrate that pre-treatment with the serine-threonine protein

phosphatase inhibitor calyculin A [37, 38] abrogated stimulation by peroxynitrite, whilst calyculin A treatment after peroxynitrite also reduced its efficacy. Since blocking serine-threonine dephosphorylation prevents KCC stimulation, the mechanism of action of peroxynitrite must involve either inhibition of the serine-threonine protein kinase proposed to inhibit KCC activity [39] or stimulation of the conjugate phosphatase. Peroxynitrite treatment is known to increase tyrosine phosphorylation in red blood cells, notably of band 3 [40]. The mechanism is thought to involve both inhibition of protein tyrosine phosphatases (PTPs) and stimulation of protein tyrosine kinases (PTKs). Pharmacological evidence suggests that upstream to the serine-threonine phosphorylation step controlling KCC activity, there is a stimulatory tyrosine phosphorylation (which stimulates the serine-threonine dephosphorylation step) [41, 42]. We propose that this is the site of action of peroxynitrite on KCC activity.

We have also extended our investigation of the action of peroxynitrite on KCC activity to show that effects are very dependent on saline composition and O_2 tension. Glucose, sucrose and MOPS all inhibited peroxynitrite-induced KCC activity. These solutes all contain hydroxyl residues, which may be nitrosylated by peroxynitrite, thereby decreasing reactivity with the red blood cell. Normal plasma glucose levels may protect red blood cells against activation of KCC by peroxynitrite, although the same is not true of the Cl^- -independent K^+ pathway. On the other hand, nitrotyrosine levels are not altered in diabetes mellitus [43]. We also show that peroxynitrite did not stimulate KCC in deoxygenated red blood cells. The proposed reaction scheme of peroxynitrite with haemoglobin requires O_2 and is the likely cause [31].

Finally, we also investigated the interaction of peroxynitrite-induced KCC activity with cell volume and pH. Peroxynitrite treatment *per se* did not alter cell water content. In untreated cells, swelling or reduction in pH from 7.4 to 6.8-7.0 stimulate KCC. We found that cell volume did not affect KCC when activated by peroxynitrite, showing loss of response to cell volume, similar to that observed previously in sheep red blood cells with low GSH levels [44]. Conversely, reduction in pH did increase KCC activity. This may indicate relative protection of the normal pH response. It may also be due to the presence of residual peroxynitrite, however, with concomitant formation at lower pH of more peroxynitrous acid (pK_a around 6.8), thereby increasing the oxidative challenge to the cell. Nevertheless, the interaction of

peroxynitrite with KCC, therefore, shows markedly different characteristics to that with the Cl⁻-independent component of K⁺ permeability (see above).

Conclusion

This work details some of the effects of peroxynitrite on the main passive K⁺ pathways of the human red blood cell. For Cl⁻-independent K⁺ pathway, stimulation is very pronounced, phosphorylation is involved to some extent but overall, the effect of peroxynitrite is less affected by other manipulations (such as changing volume or pH).

For KCC, there are considerable interactions with other factors, notably glucose, O₂ tension and pH – these may be relevant for determining its effects on red blood cell KCC activity *in vivo*. Protein dephosphorylation is required for stimulation of KCC. These findings are important for understanding the effects of peroxynitrite *in vivo*.

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References

- Lauf PK: Kinetic comparison of ouabain-resistant K:Cl fluxes (K:Cl [co]-transport) stimulated in sheep erythrocytes by membrane thiol oxidation and alkylation. *Mol Cel Biochem* 1988;82:97-106.
- Olivieri O, Bonollo M, Friso S, Girelli D, Corrocher R, Vettore L: Activation of K⁺/Cl⁻ cotransport in human erythrocytes exposed to oxidative agents. *Biochim Biophys Acta* 1993;1176:37-42.
- Muzyamba MC, Speake PF, Gibson JS: Oxidants and regulation of KCl cotransport in equine red blood cells. *Am J Physiol* 2000;279:C981-C989.
- Ellory JC, Gibson JS, Stewart GW: Pathophysiology of abnormal cell volume in human red cells. *Contrib Nephrol* 1998;123:220-239.
- Edwards CJ, Fuller J: Oxidative stress in erythrocytes. *Comp Haematol Int* 1996;6:24-31.
- Sies H: Oxidative stress: oxidants and antioxidants. *Exper Physiol* 1997;82:291-295.
- Beckman JS, Koppenol WH: Nitric oxide, superoxide anion and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* 1996;271:C1424-C1437.
- King PA, Jamison E, Strahs D, Anderson VE, Brenowitz M: 'Footprinting' proteins on DNA with peroxonitrous acid. *Nucleic Acids Res* 1993;10:2473-2478.
- Grzelak A, Mazur J, Bartosz G: Peroxynitrite activates K⁺-Cl⁻ cotransport in human erythrocytes. *Cell Biol Int* 2001;25:1163-1165.
- Gibson JS, Ellory JC: K⁺-Cl⁻ cotransport in vertebrate red cells. In: Bernhardt I, Ellory JC, eds. *Red Cell Membrane Transport in Health and Disease*. Berlin: Springer Verlag. 2004. pp 197-220.
- Gardos G: The function of calcium and potassium permeability of human erythrocytes. *Biochim Biophys Acta* 1958;30:653-654.
- Hoffman JF, Joiner W, Nehrke K, Potapova O, Foye K, Wickrema A: The hSK4 (KCNN4) isoform is the Ca²⁺-activated K⁺ channel (Gardos channel) in human red blood cells. *Proc Nat Acad Sci USA* 2003;100:7366-7371.
- Bernhardt I, Kummerow D, Weiss E: K⁺(Na⁺)/H⁺ exchange in human erythrocytes activated under low ionic strength conditions. *Blood Cells Mol Dis* 2001;27:108-111.
- Huber SM, Gamper N, Lang F: Chloride conductance and volume-regulatory nonselective cation conductance in human red blood cell ghosts. *Pflugers Arch* 2001;441:551-558.
- Durant C, Huber SC, Lang F: Oxidation induces a Cl(-)-dependent cation conductance in human red blood cells. *J Physiol* 2002;539:847-855.
- Lang F, Lanf KS, Wieder T, Myssina S, Birka C, Lang PA, Kaiser S, Kempe D, Durant C, Huber SM: Cation channels, cell volume and the death of an erythrocyte. *Pflugers Arch* 2003;447:121-125.
- Hall AC, Ellory JC: Evidence for the presence of volume-sensitive KCl transport in 'young' human red cells. *Biochim Biophys Acta* 1986;858:317-320.
- Lew VL, Tsien RY, Miner C, Boockchin RM: Physiological [Ca²⁺]_i level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature* 1982;298:478-481.
- Speake PF, Roberts CA, Gibson JS: Effect of changes in respiratory blood parameters on equine red blood cell K-Cl cotransporter. *Am J Physiol* 1997;273:C1811-C1818.
- Dunham PB, Ellory JC: Passive potassium transport in low potassium sheep red cells: dependence upon cell volume and chloride. *J Physiol* 1981;318:511-530.
- Borgese F, Motais R, Garcia-Romeu F: Regulation of Cl-dependent K⁺ transport by oxy-deoxyhemoglobin transitions in trout red cells. *Biochim Biophys Acta* 1991;1066:252-256.
- Beckman JS, Chen J, Ischiropoulos H, Crow JP: Oxidative chemistry of peroxynitrite. *Neurosci* 1994;233:229-240.
- Gibson JS, Cossins AR, Ellory JC: Oxygen-sensitive membrane transporters in vertebrate red cells. *J Exp Biol* 2000;203:1395-1407.
- Quijano C, Alvarez B, Gatti RM, Augusto O, Radi R: Pathways of peroxynitrite oxidation of thiol groups. *Biochem J* 1997;322:167-173.
- Ischiropoulos H, Al-Mehdi AB: Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett* 1995;364:279-282.
- Pietraforte D, Salzano AM, Marino G, Minetti M: Peroxynitrite-dependent modifications of tyrosine residues in hemoglobin. Formation of tyrosyl radical(s) and 3-nitrotyrosine. *Amino Acids* 2003;25:341-350.

- 27 Radi R, Beckman JS, Bush KM, Freeman BA: Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991;288:481-487.
- 28 Romero N, Denicola A, Souza JM, Radi R: Diffusion of peroxynitrite in the presence of carbon dioxide. *Arch Biochem Biophys* 1999;368:23-30.
- 29 Vasquez-Vivar J, Santos AM, Junqueira VBC, Augusto O: Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals. *Biochem J* 1996;314:869-876.
- 30 Denicola A, Souza JM, Radi R: Diffusion of peroxynitrite across erythrocyte membranes. *Proc Nat Acad Sci USA* 1998;95:3566-3571.
- 31 Romero N, Radi R, Linares E, Augusto O, Detweiler CD, Mason RP, Denicola A: Reaction of human hemoglobin with peroxynitrite. *J Biol Chem* 2003;278:44049-44057.
- 32 Wei EP, Konotos HA, Beckman JS: Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide and peroxynitrite. *Am J Physiol* 1996;271:H1262-H1266.
- 33 Wei EP, Konotos HA, Beckman JS: Antioxidants inhibit ATP-sensitive potassium channels in cerebral arterioles. *Stroke* 1998;29:817-823.
- 34 Lu M, Wang W-H: Reaction of nitric oxide with superoxide inhibits basolateral K⁺ channels in the rat CCD. *Am J Physiol* 1998;275:C309-C316.
- 35 Liu Y, Terata K, Chai Q, Kleinman LH, Gutterman DD: Peroxynitrite inhibits Ca²⁺-activated K⁺ channel activity in smooth muscle of human coronary arterioles. *Circul Res* 2002;91:1070-1076.
- 36 Weiss E, Lang H-J, Bernhardt I: Inhibitors of the K⁺(Na⁺)/H⁺ exchanger of human red blood cells. *Bioelectrochem* 2004;62:135-140.
- 37 Kaji DM, Tsukitani Y: Role of protein phosphorylation in activation of KCl cotransport in human erythrocytes. *Am J Physiol* 1991;260:C178-C182.
- 38 Starke LC, Jennings ML: KCl cotransport in rabbit red cells: further evidence for regulation by protein phosphatase type I. *Am J Physiol* 1993;264:C118-C124.
- 39 Dunham PB, Klimczak J, Logue PJ: Swelling activation of K-Cl cotransport in LK sheep erythrocytes: a three-state process. *J General Physiol* 1993;101:733-766.
- 40 Mallozzi C, Di Stasi AMM, Minetti M: Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J* 1997;11:1281-1290.
- 41 Cossins AR, Weaver YR, Lykkeboe G, Nielsen OB: Role of protein phosphorylation in control of K flux pathways of trout red blood cells. *Am J Physiol* 1994;267:C1641-C1650.
- 42 Flatman PW, Adragna NC, Lauf PK: Role of protein kinases in regulating sheep erythrocyte K-Cl cotransport. *Am J Physiol* 1996;271:C255-C263.
- 43 Wang XL, Rainwater DL, Leone A, Mahaney MC: Effects of diabetes on plasma nitrotyrosine levels. *Diabetic Med* 2004;21:577-580.
- 44 Lauf PK, Adragna NC, Agar NS: Glutathione removal reveals kinases as common targets for K-Cl cotransport stimulation in sheep erythrocytes. *Am J Physiol* 1995;269:C234-C241.