Free Radical Research, Volume 38 Number 6 (June 2004), pp. 581–589

Taylor & Francis
healthsciences

Nitrite Generates an Oxidant Stress and Increases Nitric Oxide in EA.hy926 Endothelial Cells

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Accepted by Professor V. Darley-Usmar

(Received 16 February 2004; In revised form 27 February 2004)

Nitrite is a breakdown product of nitric oxide that in turn is oxidized to nitrate in cells. In this work, we investigated whether reactive oxidant species might be generated during nitrite metabolism in cultured EA.hy926 endothelial cells. Nitrite was taken up by the cells in a time- and concentration-dependent manner and oxidized to nitrate, which accumulated in cells to concentrations almost 10-fold those of nitrite. Conversion of low millimolar concentrations of nitrite to nitrate was associated with increased oxidant stress in the cells. This manifested as increased oxidation of dihydrofluorescein in tandem with depletion of both GSH and ascorbate. Further, loading cells with ascorbate or treatment with desferrioxamine prevented nitrite-induced dihydrofluorescein oxidation. Nitrite within cells also increased the fluorescence of 4-amino-5-methylamino-2′,7′-difluorofluorescein and inhibited the activity of cellular glyceraldehyde 3-phosphate dehydrogenase, which are markers of intracellular nitrosation reactions. Intracellular ascorbate partially prevented both of these effects of nitrite. Although ascorbate can reduce nitrite to nitric oxide at low pH, in endothelial cells loaded with ascorbate, its predominant effect at high nitrite concentrations is to prevent potentially damaging nitrosation reactions.

Keywords: Nitrite; Nitric oxide; Nitrosation; Ascorbic acid; Dihydrofluorescein; EA.hy926 endothelial cells

INTRODUCTION

Nitric oxide (NO) has important actions in the vascular bed, including regulation of vascular tone and resulting blood pressure, and modulation of platelet function.[1] Further, NO produced by the vascular endothelium inhibits virtually all of the processes that are involved in the pathogenesis of atherosclerosis, including monocyte adhesion, platelet aggregation, endothelial permeability, and vascular smooth muscle cell proliferation.[2] Accordingly, endothelial dysfunction due to inadequate NO is considered a precursor for atherosclerosis.[1,3] The availability of NO for vascular functions is determined not only by its rate of generation in endothelial cells, but also by its metabolism. Regarding the latter, NO is consumed through its interaction with iron centers in its target enzymes,[4] by reaction with hemoglobin in erythrocytes,[4] or by direct oxidation to nitrite by molecular oxygen.[5]

Nitrite, which is also derived from the diet and from symbiotic bacteria in the gastrointestinal tract,[6] is found in low concentrations in plasma ($\sim 0.45\,\mu M$) compared to its oxidation product, nitrate ($40-60\,\mu M$).[7,8] Although most nitrite in the circulation is scavenged by its reaction with oxyhemoglobin in erythrocytes to form nitrate,[9] it can also be oxidized or reduced by several enzyme systems. For example, nitrite has long been known to undergo two sequential one-electron oxidations to nitrate by the catalase/H_2O_2 system.[10,11] More recently, it has been shown that lactoperoxidase and probably other heme-based peroxidases (including catalase) can also mediate a one-electron oxidation of nitrite to form a radical species, probably nitrogen dioxide.[6] At the other end of the spectrum, reduction of nitrite to NO is catalyzed by xanthine oxidase and

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760410001688366

several other heme containing enzymes,[12–14] and ascorbic acid can carry out the reduction directly.[15] These reactions are known to proceed most rapidly at low pH and at low oxygen tension,[16,17] conditions likely to be present only during severe tissue ischemia. Nevertheless, nitrite has long been known to relax arterial smooth muscle under more physiologic conditions,[18] which probably reflects its reduction by one of these mechanisms to NO or an NO-equivalent. However, the extent to which cells convert nitrite to NO or to potentially toxic radicals is unknown.

We previously studied the uptake and reactions of nitrite in human erythrocytes.[19] In that work we found the expected prominent reaction of nitrite with oxyhemoglobin to form methemoglobin and nitrate. We also found that intracellular ascorbate reacted with nitrite or its breakdown products, and that ascorbate in turn decreased methemoglobin formation due to nitrite. We concluded that pharmacologic concentrations of nitrite generate an oxidant stress in erythrocytes, which was partially prevented by ascorbate. In the present work, we evaluated nitrite uptake and metabolism in relation to ascorbate in cultured endothelial cells. There were several reasons for choice of this cell type for study, including absence of the confounding effects of hemoglobin, exposure to nitrite in the vascular bed, and relevance to endothelial dysfunction and atherosclerosis. We studied EA.hy926 cells, which are derived from human umbilical vein endothelial cells and retain many of the unique characteristics of endothelial cells.[20–22] We found that nitrite taken up by EA.hy926 cells causes an oxidant stress that manifests as increased dihydrofluorescein oxidation and that nitrite also generates reactive species that mediate nitrosation reactions. Ascorbic acid inhibits both of these effects.

MATERIALS AND METHODS

Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied the dehydroascorbic acid (DHA), tetrapentyl ammonium bromide, sodium nitrite, *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (Hepes), and sodium ascorbate. Dihydrofluorescein diacetate, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) were obtained from Molecular Probes, Inc. (Eugene OR). Both diacetate derivatives were initially dissolved in dimethylsulfoxide, such that the final dimethylsulfoxide concentration in the incubation was 0.2%.

Cell Culture and Preparation for Assays

EA.hy926 cells were kindly provided by Dr Cora Edgell (University of North Carolina, Chapel Hill, NC). Cell culture was carried out using Dulbecco's minimal essential medium that contained 20 mM D-glucose and 10% (v/v) fetal bovine serum, as prepared by the Cell Culture Core of the Vanderbilt Diabetes Research and Training Center. The culture medium contained HAT supplement (Sigma/Aldrich Chemical Co., St. Louis, MO), which provided 5 mM hypoxanthine, 20 μM aminopterin and 0.8 mM thymidine in culture. Cells were cultured to confluence for 18–24 h before use in an experiment.

Assay of Intracellular Ascorbate, GSH, Nitrite and Nitrate

Following incubation as indicated in 6-well plates, the medium was aspirated, and the cells were gently rinsed twice with 2 ml of ice-cold Krebs-Ringer Hepes buffer (KRH). KRH consisted of 20 mM Hepes, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$ and 1.4 mM $CaCl_2$, pH 7.4. The medium was removed and the cell monolayer was treated with 0.1 ml of 25% metaphosphoric acid (w/v), scraped from the plate and diluted with 0.35 ml of a buffer containing 0.1 M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0. The lysate was microfuged at 3°C for 1 min at 13,000*g*. Aliquots of the supernatant were taken for assay of ascorbic acid as previously described[23] using high performance liquid chromatography with electrochemical detection, except that tetrapentylammonium bromide was used as the ion pair reagent. GSH was assayed on paired aliquots by the method of Hissin and Hilf.[24] Nitrite was measured by the method of Saville,[25] except that ammonium sulfamate and mercuric chloride were omitted. The sensitivity of the assay was 0.5 μM, based on nitrite standards. Nitrate was measured in the same samples after reduction of nitrate to nitrite using the copper–cadmium method of Cortas and Wakid.[26] Intracellular concentrations of ascorbate, GSH, nitrite, and nitrate were calculated based on an intracellular water space in EA.hy926 cells of 3.6 ± 1.2 μl/mg protein, as previously measured in these cells.[27]

Measurement of Intracellular Oxidant Stress and NO Generation due to Nitrite

Reactive oxygen species generated within cells in response to nitrite were measured as oxidation of intracellular dihydrofluorescein using 96-well plates and a fluorescence microtiter plate reader exactly as recently described.[27] Intracellular NO generation was measured by two methods. In the first, NO was detected following reaction with DAF-FM

and measured by an increase in fluorescence in the fluorescence microtiter plate reader. As with measurement of oxidant stress using dihydrofluorescein, DAF-FM diacetate (5 μM) was loaded into cells in a 96-well plate for 30 min at 37°C in KRH buffer containing 5 mM D-glucose. The cells were rinsed 3 times in 0.2 ml of 37°C KRH, followed by treatments noted in the figure legends. The plate was then loaded into a fluorescence microtiter plate reader (Fluostar Galaxy, BMG Labtechnologies, Cork, Ireland) and was incubated at 37°C for 33 min with measurement of the fluorescence in each well every 4 min. The excitation wavelength was 480 nm, and the emission wavelength was 520 nm. The fluorescence readings for each well were normalized to the initial reading at time zero. In some experiments, the slope of the linear portion of the time course was calculated and divided by the initial slope in a sample that did not contain nitrite.

Intracellular nitrosation was also measured by the ability of nitrite inhibit cellular glyceraldehyde 3-phosphate dehydrogenase (G3PDH), as described by Padgett and Whorton.[28] After treatments as noted, EA.hy926 cells in 6-well plates were rinsed 3 times with 2 ml of KRH, and covered with 0.5 ml of ice cold 10 mM sodium pyrophosphate buffer (pH 7.5) containing 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride. The cells were scraped from the plate and transferred to microfuge tubes on ice. The lysate was sonicated using the microtip probe of a Branson Model 250 sonicator with the following settings: intensity setting = 3, duty cycle setting # 3 and 10 bursts/tube. The sonicated lysate was microfuged for 10 min at 14,000*g* at 3°C and the supernatant was assayed for activity of glyceraldehyde 3-phosphate dehydrogenase. The assay buffer contained 10 mM sodium pyrophosphate, 12 mM sodium arsenate, 1 mM NAD^+, and 1 mM glyceraldehyde 3-phosphate, pH 8.4. The assay was carried out at 23°C in a Beckman DU-640 recording spectrophotometer by adding an aliquot of lysate with mixing to the assay buffer in a cuvette and following the change in the rate of absorbance at 340 nm. The linear rate of increase in absorbance was calculated and the rate of NAD^+ reduction was determined with an extinction coefficient of 6.22 mM/cm.

Other Methods and Statistical Analysis

AFR generation from ascorbate and ascorbate oxidase was measured spectrophotometrically by its absorbance at 360 nm, using an extinction coefficient of 3.3 mM/cm.[29] Protein was measured by the method of Bradford.[30] Results are shown as mean ± standard error. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences between treatment groups were assessed by one or two-way analysis of variance with *post-hoc* testing using Dunnett's test.

RESULTS

Nitrite Uptake and Oxidation to Nitrate by EA.hy926 Cells

To determine whether nitrite enters the cells and is metabolized, intracellular concentrations of nitrite and nitrate were measured. Nitrite was present in the cells at the earliest time point measured, and was taken up by EA.hy926 cells in a time-dependent but non-linear manner (Fig. 1). A five-fold increase in the initial extracellular nitrite concentration increased nitrite in the cells, but only by about 2-fold (Fig. 1). This lack of direct correspondence between the loading concentration of nitrite and its concentration in cells is evident in the concentration-response studies shown in Fig. 2A. Here, it was necessary to use relatively high nitrite concentrations to see significant increases in intracellular nitrite. The rise in intracellular nitrite concentrations at a nitrite loading concentration of 10 mM was halved when the cells were pre-loaded with ascorbate by incubation with 0.5 mM DHA (square, Fig. 2A). Intracellular nitrate concentrations were also measured in these experiments and were about 10-fold those of nitrite, even in the absence of added nitrite (Fig. 2B). Intracellular nitrate concentrations increased linearly with increases in extracellular nitrite from an initial intracellular concentration of 1 mM. As with nitrite, loading the cells with ascorbate before incubation with nitrite halved the rise in intracellular nitrate due

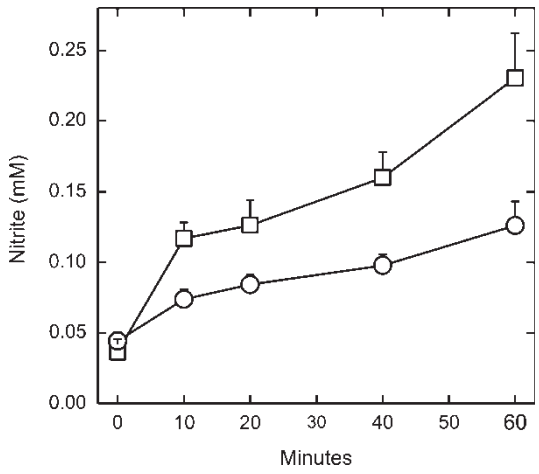

FIGURE 1 Time course of nitrite uptake by EA.hy926 cells. Cells that had been rinsed free of culture medium were incubated at 37°C in KRH containing 5 mM D-glucose and either 0.8 mM (circles, $N = 8$ experiments) or 4 mM nitrite (squares, $N = 6$ experiments) for the times indicated, followed by three rinses in ice-cold KRH and assay of intracellular nitrite. Results are shown as mean ± SE.

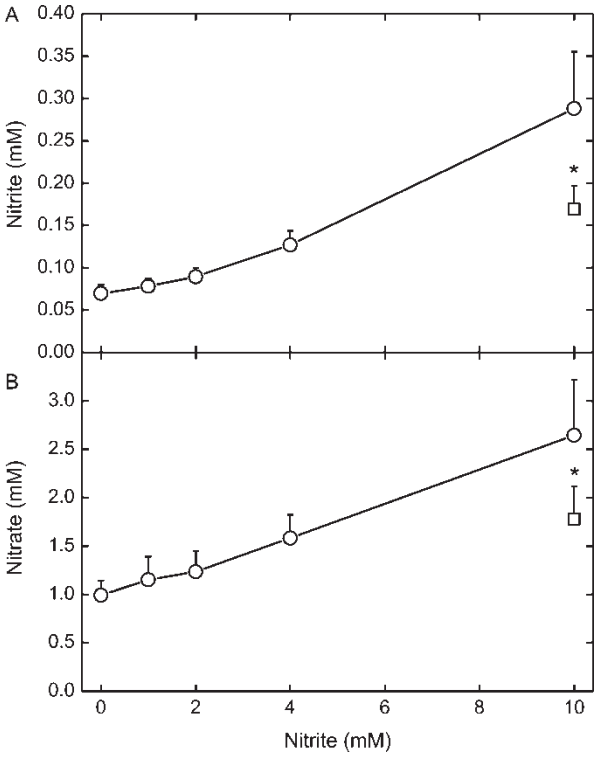

FIGURE 2 Concentration-dependence of nitrite uptake and conversion to nitrate by EA.hy926 cells. Rinsed cells were incubated at 37°C in KRH that contained 5 mM D-glucose and the indicated concentration of nitrite. After 15 min, the cells were rinsed twice in ice-cold KRH and taken for assay of intracellular nitrite (Panel A) and nitrate (Panel B). The single squares in each panel show the results in cells that had been pre-incubated at 37°C for 15 min with 0.5 mM DHA before addition of nitrite. An asterisk indicates $P < 0.05$ compared to the corresponding sample without DHA at the same initial nitrite concentration. Results are shown as mean ± SE from 6 experiments.

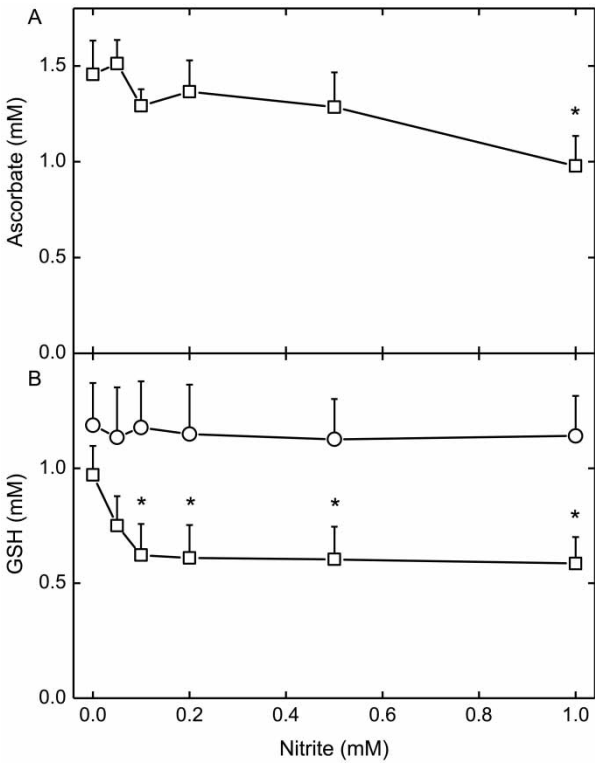

FIGURE 3 Nitrite effects on EA.hy926 cell ascorbate and GSH contents. Rinsed EA.hy926 cells were incubated at 37°C in KRH that contained 5 mM D-glucose and the indicated concentration of nitrite for 15 min. The incubation was continued following addition of either no DHA (circles) or 1 mM DHA (squares). After 15 min the intracellular ascorbate (Panel A) and GSH (Panel B) contents were determined. Results are shown from 5 experiments with DHA treatment, with an asterisk (*) indicating $P < 0.05$ compared to the sample that had no nitrite. Results are shown as mean ± SE from 3 experiments without DHA treatment.

to loading (square, Fig. 2B). In the absence of cells, nitrite was stable under the conditions of the uptake assay and not oxidized to nitrate (results not shown). This indicates that the observed increase in intracellular nitrate was due to transport of nitrite and subsequent oxidation to nitrate by the cells, and not to uptake of nitrate derived from oxidation of nitrite in the incubation medium. Further, the anion transport protein inhibitor DIDS at a concentration of 25 μM had no effect on the uptake of 4 or 10 mM nitrite (results not shown).

Nitrite and Reduction of DHA to Ascorbate by EA.hy926 Cells

An interaction between nitrite and ascorbate was further indicated by the finding that treatment of EA.hy926 cells with increasing concentrations of nitrite impaired their ability to reduce 1 mM DHA to ascorbate, although this only became significant at a nitrite concentration of 1 mM (Fig. 3A). Nitrite treatment had no effect on GSH concentrations in cells that did not contain measurable amounts of ascorbate (Fig. 3B, circles). Cells loaded with ascorbate alone by incubation with 1 mM DHA had a non-significant 16% decrease in intracellular GSH concentrations (Fig. 3B). However, treating cells with increasing amounts of nitrite followed by DHA treatment caused a significant decrease in the intracellular GSH concentration at 0.1 mM nitrite and higher (Fig. 3B, squares). These results suggest that ascorbate oxidation by nitrite is coupled to ascorbate recycling by GSH, and that nitrite treatment followed by DHA loading decreases the GSH reserve of EA.hy926 cells.

Generation of Oxidant Stress by Nitrite in EA.hy926 Cells

To confirm that the oxidation of GSH and ascorbate observed in the experiments shown in Fig. 3 was due to intracellular oxidant stress generated by nitrite, cells were loaded with dihydrofluorescein diacetate. Following removal of the acetate groups by intracellular esterases, dihydrofluorescein is trapped within cells and can be oxidized by intracellular

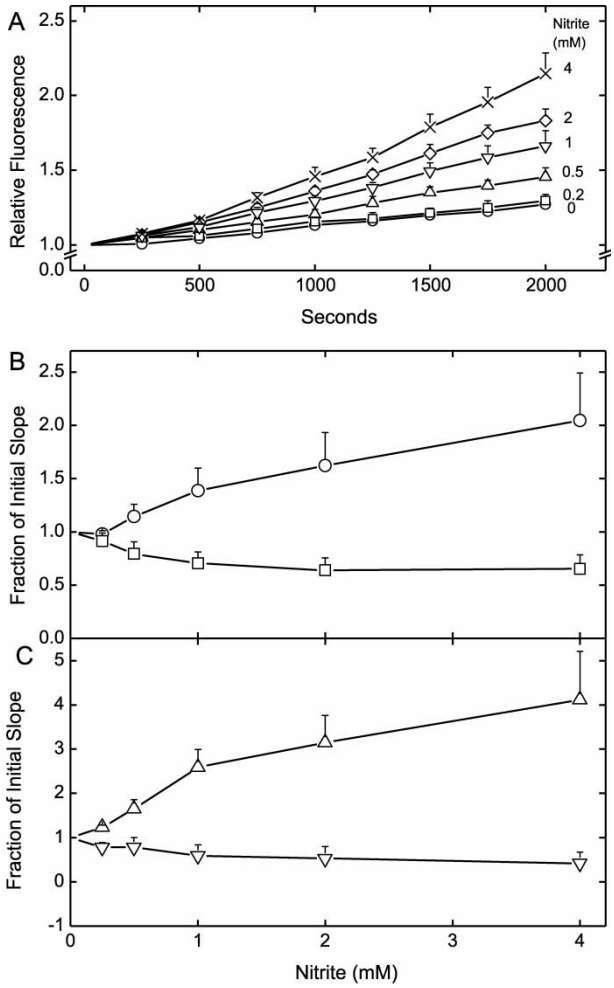

FIGURE 4 Nitrite induced dihydrofluorescein oxidation. Panel A. Rinsed EA.hy926 cells were incubated at 37°C in 96-well plates in KRH that contained 5 mM D-glucose and 20 μM dihydrofluorescein diacetate for 30 min in the dark. The cells were then rinsed three times with warm KRH and incubated in the microtiter plate reader at 37°C with KRH and 5 mM D-glucose and the indicated concentration of nitrite. Results are shown as mean ± SD from 4 wells of one plate. Panel B. EA.hy926 cells were loaded with dihydrofluorescein diacetate as in Panel A in the absence (circles) or presence of 1 mM DHA (squares) during the initial loading with dihydrofluorescein diacetate. Cells were treated with the indicated nitrite concentration as described in Panel A during acquisition of data. Panel C. Experiments were identical to Panel B, except pre-treatment was carried out in the absence (triangles) or presence of 0.1 mM desferrioxamine (inverted triangles) instead of DHA. Results in Panels B and C are presented as the normalized slopes of the linear portion of the time course curves (after 500 s) from 4 to 6 experiments (mean ± SE).

reactive oxygen species to fluorescin. The latter slowly leaks from the cells into the medium, and its fluorescence can be detected with a microtiter plate reader.[27] As shown in a representative experiment (Fig. 4A), treating EA.hy926 cells with increasing amounts of nitrite caused progressive increases in fluorescence over 33 min of incubation. Combining the results from several experiments, it was found that the slope of the most linear portion of the time course (after 500 s) increased with increasing nitrite concentrations (circles, Fig. 4B). Loading the cells with ascorbate by incubation with 1 mM DHA during incubation with dihydrofluorescein diacetate completely prevented the nitrite-induced increase in fluorescence, and even tended to decrease fluorescence at higher nitrite concentrations (Fig. 4B). This supports the notion that nitrite generates intracellular radical species that are scavenged by ascorbate. Similar inhibition of dihydrofluorescein oxidation was observed when cells were pretreated with 0.1 mM desferrioxamine (Fig. 4C), suggesting that intracellular iron is involved in the oxidation of dihydrofluorescein.

Nitrosation Reactions of Nitrite in EA.hy926 Cells

To determine whether nitrite also causes nitrosation within cells, the latter was measured as an increase in fluorescence in cells loaded with DAF-FM diacetate. As with dihydrofluorescein diacetate, the acetylated form of DAF-FM is taken up by the cells, deacetylated, and the negatively charged DAF-FM is retained in the cells. When this is nitrosated, a highly fluorescent product is generated that can be detected in the cells.[31] The fluorescent product slowly exits the cells and can be quantified in the medium with a fluorescence microtiter plate reader. As shown in Fig. 5A, after a short delay, nitrite caused a time-dependent increase in fluorescence that became apparent at nitrite concentrations of 200 μM or greater. A representative experiment is provided, since the fluorescence varied as much as two-fold from different experiments with nitrite. However, the relative effects of a given nitrite concentration were consistent across experiments. A plot of the slope of the linear portion of the time course of the data from Fig. 5A is compared to the effects of loading the cells with ascorbate using 0.5 mM DHA in Fig. 5B. Ascorbate-loaded cells generated very little fluorescence in response to nitrite. The concentration-dependence of the effect of intracellular ascorbate is shown in Fig. 6. It can be seen that DHA loading concentrations as low as 10 μM caused appreciable decreases in nitrite-induced fluorescence. The slopes of the linear portions of the time courses are plotted in the inset, and followed a mono-exponential decay. The inhibitory effect of ascorbate was half-maximal at a DHA loading concentration of about 50 μM.

The possibility that the effect of ascorbate was due to extracellular ascorbate was investigated with the experiment shown in Fig. 7. Ascorbate oxidase was added to the cells after loading with DHA to remove any ascorbate released from the cells during the incubation with nitrite. Removal of extracellular ascorbate decreased the inhibition of fluorescence development by DHA by 25–40% in 3 experiments, indicating that most effects of ascorbate are intracellular.

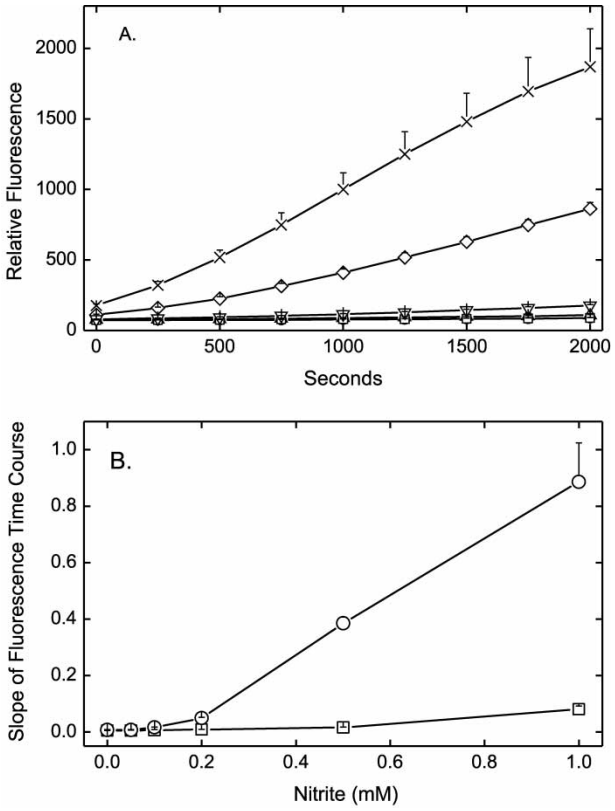

FIGURE 5 Effects of nitrite on DAF-FM fluorescence in EA.hy926 cells. Panel A: Rinsed EA.hy926 cells were loaded with 5 μM DAF-FM diacetate as described under Experimental Procedures. The cells were rinsed three times in KRH, and the cells were incubated in 37°C in KRH that contained 5 mM D-glucose and the following concentrations of nitrite: circles, 0 nitrite; squares, 50 μM nitrite; triangles, 100 μM nitrite; inverted triangles, 200 μM nitrite, diamonds, 500 μM nitrite, and crosses, 1μM nitrite. The plate was loaded into the microtiter plate reader and fluorescence was measured at the times shown as described in "Experimental Procedures" section. Panel B: Incubations were the same as in Panel A, except for the absence (circles) or presence (squares) of 0.5 μM DHA during loading with DAF-FM diacetate. The slopes of the time course values after 500 s at each nitrite concentration are plotted. Results in both panels are shown as averages from four separate wells (± SD) from an experiment representative of 4 performed.

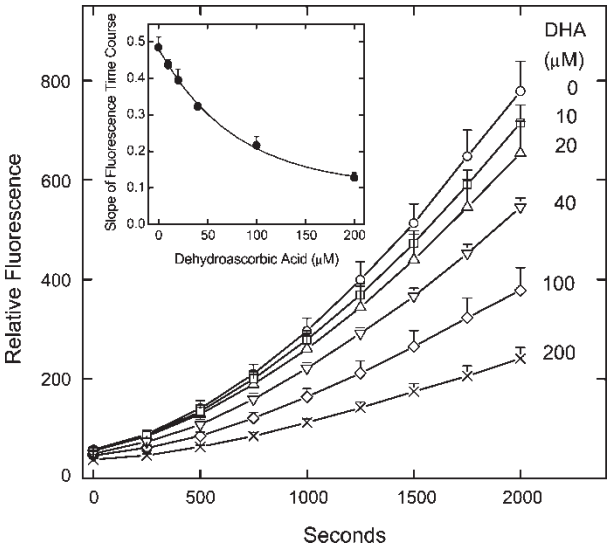

FIGURE 6 Inhibition of nitrosation by intracellular ascorbate. EA.hy926 cells were loaded with DAF-FM as described under Experimental Procedures, except that DHA was included at the indicated concentration. After rinsing three times with 0.2 ml of KRH to remove extracellular material, the cells were treated with 0.5 mM nitrite in KRH containing 5 mM D-glucose during measurement of fluorescence in the microtiter plate reader at 37°C. Time-dependent changes in fluorescence from an experiment typical of 4 performed are plotted in the main graph as mean ± SD from four wells. In the inset, the slopes of the time-course curves after 500 s were determined, and plotted as a function of the initial DHA concentration, fit to a mono-exponential decay.

To determine whether nitrosation reactions due to nitrite affect cellular processes, the activity of G3PDH was measured. It has been shown that G3PDH is inhibited by NO donors in endothelial cells.[28,32] Increasing amounts of nitrite also inhibited G3PDH activity in a concentration-dependent manner, with a half-maximal effect at about 2 mM nitrite (Fig. 8). This method was of similar sensitivity in detecting nitrosation as was measurement of increases in DAF-FM fluorescence (Fig. 5). Cells that had been loaded with ascorbate by pre-treatment with DHA showed significantly less inhibition across the range of nitrite concentrations. These results using a biologic assay confirm those observed with DAF-FM.

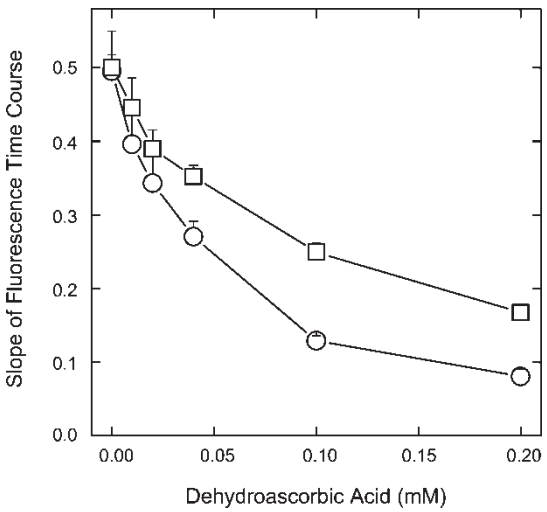

FIGURE 7 Intra- versus extracellular ascorbate effects on nitrite-induced nitrosation. EA.hy926 cells were loaded with DAF-FM as described under Experimental Procedures, except that DHA was included at the indicated concentration. After 30 min, the cells were rinsed three times with 0.2 ml of KRH to remove extracellular material.This was followed by addition of KRH containing 5 mM D-glucose, 0.5 mM nitrite, without (circles) or with 0.25 units/ml ascorbate oxidase. Changes in fluorescence were then measured in the microtiter plate reader at 37°C. Time-dependent changes in fluorescence from an experiment typical of 3 performed are plotted in the main graph as mean ± SD from four wells.

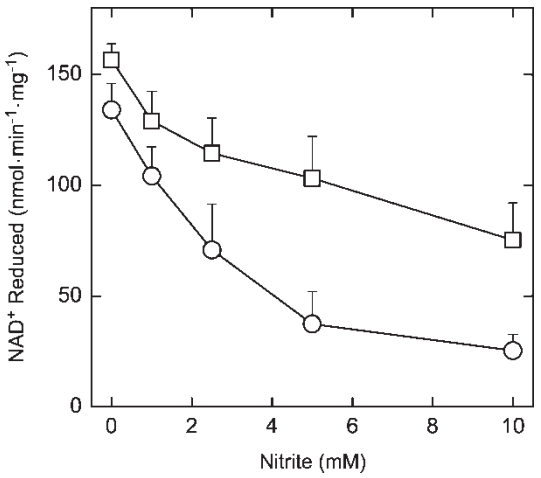

FIGURE 8 Nitrite effects on nitrosation measured by inhibition of glyceraldehyde 3-phosphate dehydrogenase. Cells were incubated for 15 min at 37°C in KRH containing 5 mM D-glucose and either no DHA (circles) or 0.5 mM (squares). After rinsing, the cells were incubated under the same conditions for 60 min with the indicated concentration of nitrate before measurement of glyceraldehyde 3-phosphate dehydrogenase activity. Results from 5 experiments and shown as mean ± SE from 5 separate experiments. The curves for control and DHA-treated cells were different at the 0.05 level.

DISCUSSION

As one of the end products of aerobic metabolism of NO, nitrite is generated in significant amounts in the vascular bed, and much of this likely derives from endothelial cells. Nitrite in the bloodstream is taken up by erythrocytes by the $Na-P_i$ cotransport system, and probably by diffusion of a small amount of nitrous acid across the cell membrane.[19] Although EA.hy926 endothelial cells in culture contain 50–70 μM intracellular nitrite (Figs. 1 and 2), they take up millimolar concentrations at rates comparable to those observed in erythrocytes.[19] These concentrations are higher than the cells are likely to encounter *in vivo*. During nitrite uptake, intracellular nitrate concentrations are ten-fold higher than those of nitrite (Fig. 2), indicating that the actual rate of nitrite uptake is likely to be considerably greater than estimated from changes in intracellular nitrite concentrations. Although the mechanism of nitrite uptake by EA.hy926 cells was not explored in detail in the present work, nitrite uptake was not inhibited by 25 μM DIDS, so its uptake does not occur on the anion transporter protein (AE-1).[33]

In human erythrocytes, nitrite is oxidized to nitrate by reaction with oxyhemoglobin.[9] Whereas this reaction may constitute the major "sink" for nitrite in the erythrocyte, nitrite concentrations of 2–5 mM activate the erythrocyte pentose phosphate cycle, an effect that is associated with oxidative damage to protein.[34] More recently, we provided additional evidence for nitrite-induced oxidant stress in erythrocytes with the finding that 5 mM nitrite significantly decreased ascorbic acid concentrations.[19] Although EA.hy926 cells do not contain hemoglobin, they do oxidize nitrite to nitrate (Fig. 2). Several results suggest that this oxidation is associated with increased oxidant stress in EA.hy926 cells. First, nitrite both decreased ascorbate in EA.hy926 cells, and lowered GSH in cells with decreased GSH reserve due to reduction of DHA (Fig. 3). Second, intracellular nitrite and nitrite concentrations were halved by the presence of ascorbate in the cells (Fig. 2). Finally, at loading nitrite concentrations of 0.5 mM and greater, oxidation of dihydrofluorescein to fluorescein was increased, and this was prevented by loading cells with ascorbate (Fig. 4). Peroxide-mediated oxidation of dichlorodihydrofluorescein in endothelial cells has been linked to increases in the labile intracellular iron pool and GSH depletion.[35] We have shown that chelation of intracellular iron with desferrioxamine blunted menadione-induced oxidation of dihydrofluorescein,[36] and find similar effects with nitrite in the present work (Fig. 4C). These results support the notion that intracellular free iron contributes to oxidation of dihydrofluorescein within cells. The effect of ascorbate to blunt nitrite-induced dihydrofluorescein oxidation could be due to reduction of ferric iron, or more likely to scavenging of radicals generated by nitrite. Regarding the latter, radicals might be generated when nitrite is oxidized by H_2O_2 in reactions catalyzed by cellular heme-based peroxidases.[6,37] For example, nitrite has been shown to enhance the ability of lactoperoxidase to use H_2O_2 to oxidize low molecular weight antioxidants that are present in cells, including NAD(P)H, GSH, and ascorbate.[6] This antioxidant consumption is thought to relate to generation of highly reactive nitrite metabolites, such as the nitrogen dioxide radical.[6] A role for ascorbate in scavenging toxic products of nitrite is also supported by an *in vivo* study showing that induction of ascorbate-deficiency in guinea pigs increased the deaths attributable to nitrite toxicity.[38] Nonetheless, given the relatively high nitrite concentrations required to demonstrate excess oxidant stress in endothelial cells, it is unlikely that such concentrations will be generated *in vivo*, except perhaps in areas of inflammation, inducible nitric oxide synthase can release of a large amounts of NO.

Whereas nitrite causes oxidant stress in EA.hy926 cells, it also generates a nitrosating species.[39,40] Thus, treating EA.hy926 cells with relatively high nitrite concentrations increases fluorescence due to DAF-FM. Diaminofluoresceins have been used to detect intracellular NO generation in several studies.[31,41,42] DAF-FM is loaded into cells as the diacetate derivative, where the acetate groups are removed. This traps non-fluorescent DAF-FM in the cells. Diaminofluoresceins do not react directly with NO, but with an oxidized product of NO

(probably nitrous anhydride, N_2O_3).[41,42] This reaction results in a triazole derivative that is highly fluorescent and readily detected in the cells or incubation buffer.[41–43] Since nitrite does not react directly with diaminofluoresceins,[41] it must generate N_2O_3 or some other product that reacts with DAF-FM and other derivatives.

Nitrite may also generate NO in cells either from its direct disproportionation or form its acidotic degeneration.[44] Another possibility is that xanthine oxidase[12,13] or a related enzyme reduces nitrite to NO within the cell. Whereas all these reactions are favored by low pH and low oxygen tension, there may be compartments in the cell that allow such a reaction. Ascorbate can also reduce nitrite to NO at low pH.[17] This reaction involves nitrosation of the 3-hydroxy group of ascorbate by N_2O_3 and subsequent release of NO.[45] Thus, ascorbate will compete with DAF-FM for N_2O_3, as it does with other amines,[17] and could, depending on the fate of NO generated from ascorbate, decrease the amount of fluorescent triazole product generated from DAF-FM. Similarly, ascorbate could protect G3PDH from nitrosation by scavenging N_2O_3.

The predominant effect of intracellular ascorbate in endothelial cells is likely to increase NO by enhancing the activity of endothelial nitric oxide synthase.[46,47] If excess nitrite is generated, either by the cells or due to local inflammation and inducible nitric oxide synthase activation, ascorbate will either reduce nitrite back to NO or will scavenge potentially toxic products of nitrite metabolism before they can damage the cells.

Acknowledgements

This work was supported by NIH grant DK 50435 and by the Vanderbilt Diabetes Research and Training Center (DK 20593).

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