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Nitroso-Redox Status and Vascular Function in Marginal and Severe Ascorbate Deficiency

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Abstract

Marginal vitamin C (ascorbic acid) deficiency is a prevalent yet underappreciated risk factor for cardiovascular disease. Along with glutathione, ascorbate plays important roles in antioxidant defense and redox signaling. Production of nitric oxide (NO) and reactive oxygen species and their interaction, giving rise to nitroso and nitrosyl product formation, are key components of the redox regulation/signaling network. Numerous *in vitro* studies have demonstrated that these systems are interconnected via multiple chemical transformation reactions, but little is known about their dynamics and significance *in vivo*. ***Aims:*** We sought to investigate the time-course of changes in NO/redox status and vascular function during ascorbate depletion in rats unable to synthesize vitamin C. ***Results:*** We here show that both redox and protein nitros(yl)ation status in blood and vital organs vary dynamically during development of ascorbate deficiency. Prolonged marginal ascorbate deficiency is associated with cell/tissue-specific perturbations in ascorbate and glutathione redox and NO status. Scurvy develops earlier in marginally deficient compared to adequately supplemented animals, with blunted compensatory NO production and a dissociation of biochemistry from clinical symptomology in the former. Paradoxically, aortic endothelial reactivity is enhanced rather than impaired, irrespective of ascorbate status. ***Innovation/Conclusion:*** Enhanced NO production and protein nitros(yl)ation are integral responses to the redox stress of acute ascorbate deprivation. The elevated cardiovascular risk in marginal ascorbate deficiency is likely to be associated with perturbations of NO/redox-sensitive signaling nodes unrelated to the regulation of vascular tone. This new model may have merit for the future study of redox-sensitive events in marginal ascorbate deficiency. *Antioxid. Redox Signal.* 17, 937–950.

Introduction

Ascorbic acid (vitamin C) is the most potent water-soluble antioxidant in biological fluids and is widely distributed across cells/tissues (36). At physiological pH, it exists largely in the form of its monovalent anion, ascorbate. In addition to its role as antioxidant, it is a cofactor of prolyl hydroxylases and involved in several other cellular functions. Most mammals can synthesize ascorbate from glucose in a pathway involving the enzyme L-gulonolactone oxidase (for a review on ascorbate biosynthesis, recycling and degradation, see Ref. 26). Humans, along with primates, guinea pigs, and bats, lack a functional enzyme and therefore depend on an adequate dietary supply with this vitamin. Current recommended dietary allowances are based on the absence of obvious signs of deficiency, but this may be insufficient for optimal health (12). Prolonged inadequate supply eventually leads to depletion of bodily pools and development of scurvy, an almost forgotten disease historically known to affect sailors on long voyages.

Innovation

This is the first study that investigated the time-course of changes in NO/redox status in blood and tissues during acute and marginal ascorbate deficiency *in vivo*. Our results indicate that NO is a key regulator of cellular redox homeostasis whose production and metabolism is intimately linked to local ascorbate status. The system is characterized by a compensatory upregulation of NO production and protein nitros(yl)ation as a response to the redox stress caused by ascorbate deprivation, with a compromised reactivity following prolonged marginal deficiency.

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In spite of occasional reports of its occurrence in recent years (33, 49), scurvy has become rare in industrialized societies. In contrast, marginal ascorbate deficiency, a condition caused by suboptimal vitamin C intake, is much more prevalent. Estimates suggest that >20% of the UK population and 30%–40% of US adults are affected by subclinical ascorbate deficiency (20, 35, 42, 51). Because this condition is neither associated with overt clinical symptoms nor routinely assessed, its health implications have long been underappreciated. However, low blood ascorbate levels are a strong predictor of cardiovascular disease and all-cause mortality (10), and recent observations have linked ascorbate deficiency to increased risk for cardiovascular (13, 14), respiratory (8), and metabolic disease (6, 39), warranting a re-evaluation of its physiological significance.

Several diseases linked to ascorbate deficiency are known to be associated with endothelial dysfunction, an impaired vascular reactivity due to reduced formation and/or availability of nitric oxide (NO). Endothelial dysfunction may represent the vascular manifestation of systemic events affecting NO-related processes. NO is a pleiotropic cell signaling and effector molecule that controls vital bodily functions across all organ systems (32). NO is also an important antioxidant (50) and a key regulator of mitochondrial function and redox signaling (40). Ascorbate has been shown to reverse endothelial dysfunction, but the underlying mechanism(s) of these effects remain unclear (30). The main mechanisms proposed include stimulation of nitric oxide synthase (NOS) activity by modulation of cofactor requirements, sparing of intracellular thiols, and release of NO from circulating/tissue storage forms. Irrespective of mechanism, these results are inconsistent with the reported lack of endothelial dysfunction in ODS (Osteogenic, Disorder Shionogi) rats (45), a strain derived from the Wistar rat that carries a missense mutation of L-gulonolactone oxidase, rendering these animals incapable of synthesizing ascorbate (23, 31). Interestingly, $Gulo^{-/-}$ mutant mice, a genetically engineered mouse lacking the same enzyme, present with aortic wall damage on withdrawal of ascorbate supplementation (27), consistent with the association between low ascorbate levels and cardiovascular disease.

The notion that a perturbation of NO-dependent regulatory redox circuits, coined the "nitroso-redox status" (16), may contribute to cardiovascular disease has received considerable attention lately. Together with glutathione, ascorbate represents the "heart of the redox hub" (11), and both antioxidants are linked to NO/nitrosation status via a multitude of reactions. Studies in the 1970s identified ascorbate as a nitrosation inhibitor (25), but more recent studies revealed that it can also release NO from nitrosated proteins (21). Ascorbate also reacts with nitrite (7) and plasma S-nitrosothiols (38) to produce NO, and this reactivity is exploited for S-nitrosothiol detection using the "biotin switch" assay (18). With a few exceptions (2, 22), many of these reactions have been studied using *in vitro* systems, and our knowledge about their significance *in vivo* is limited. Moreover, reaction dynamics tend to be complex, rendering network interrogation by a select few time points problematic. Reactions may also differ between organs/compartments, depending on the expression of transporters, local concentrations, and recycling mechanisms. Thus, investigations in relevant *in vivo* models, ideally with comprehensive assessment of multiple organ systems, are required to put those reactions into context.

Much of the insight into the role of ascorbate in endothelial dysfunction has been gained using pharmacological doses; yet, the chemistry of such high ascorbate concentrations can differ substantially from that prevailing at physiological levels (5). A complementary approach to studying the role of ascorbate in physiology uses inhibition of its production. A standardized technique for inducing chronic latent ascorbate deficiency has been developed in guinea pigs (14). However, the majority of published research has been carried out in rats and mice, hampering direct comparison of results due to possible species differences. In the present study we used ODS rats, in combination with bioanalytical and functional techniques, to assess the nitroso-redox status in blood and tissue and investigate mechanisms of acute and chronic ascorbate depletion. Using this integrated approach, we demonstrate a complex pattern of compartmentalized responses that suggest that NO upregulation is a crucial response to redox stress. This response is blunted following prolonged marginal ascorbate deficiency and associated with perturbations in tissue NO/redox tone. Surprisingly, these changes do not impair the regulation of vascular tone.

Results

Changes in NO/redox status in response to acute ascorbate depletion in optimally supplemented animals

To study the effects of acute ascorbate depletion on blood and tissue NO/redox status in animals adequately supplied with ascorbate before, ODS rats were placed on a diet lower in vitamin C and regular tap water and monitored over the subsequent 2 months. Blood and tissues were harvested just before switching from full supplementation to depletion ($t=0$), at the end of weeks 1 and 2, and on appearance of overt signs of scurvy (weeks 6–7). As shown in Figure 1a, a rapid ascorbate decline was apparent in most compartments within the first 2 weeks. This was accompanied by changes in total glutathione in which each data point for every organ rose above baseline at weeks 1 and 2, peaking at week 2 (Fig. 1c). A similar increase was observed in the GSH/GSSG ratio during that time (Fig. 1d). Although the glutathione data ($n=2$ for each tissue) may be insufficient to assess the significance of the changes in individual organs, the consistency in direction of the change in all compartments allows us to state that significant global increases in glutathione content and redox status took place within the first 2 weeks. These changes were accompanied by a dramatic yet transient improvement in ascorbate redox (AA/DHA ratio) at week 1 (Fig. 1b). This increase in AA/DHA ratio was mirrored by increases in the concentrations of nitrite (Fig. 1e), nitrate (Fig. 1f), and total nitroso (RXNO) and nitrosyl (NO-heme) products (Figs. 1g and 1h) in several compartments. By week 7, total ascorbate and AA/DHA ratios were significantly reduced in most compartments (Figs. 1a and 1b), while total glutathione and GSH/GSSG ratios returned to normal levels. Conversely, NO-related products (in particular, nitrite/nitrate in blood and tissue nitroso/nitrosyl species) showed dramatic elevations in most compartments investigated (Figs. 1e–1h). These results suggest a compensatory increase in tissue antioxidant status in response to progressively decreasing ascorbate levels, although this does not appear to be sustainable for longer periods. NO production may be upregulated, as indicated by the massive increase in NO-related metabolites, to substitute for

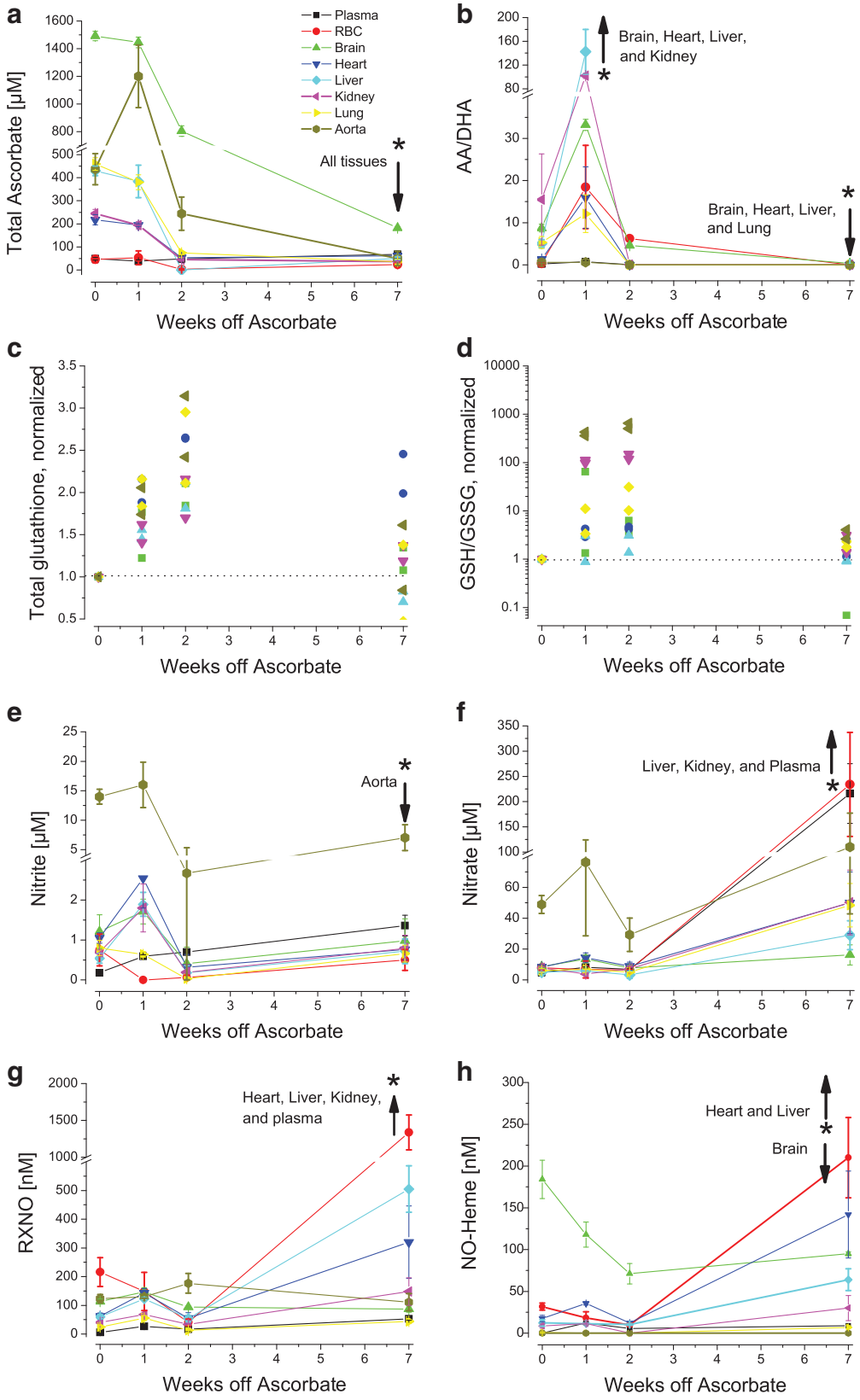

FIG. 1. Time dependence of the effects of acute ascorbate depletion on NO/redox status in blood and tissues of ODS rats. *Panels* depict steady-state concentrations and corresponding redox status of **(a)** total ascorbate and **(b)** ratio of reduced (AA) over oxidized ascorbate (DHA); **(c)** total glutathione, and **(d)** ratio of reduced (GSH) over oxidized glutathione (GSSG); and concentrations of **(e)** nitrite, **(f)** nitrate, **(g)** total nitrosation (RXNO), and **(h)** heme nitrosylation products (NO-heme) immediately before (time = 0), and 1, 2, and 7 weeks after start of ascorbate depletion. Means ± SEM of $n = 3$–5 animals/time point for ascorbate and NO measurements, and means of $n = 2$ animals/time point—normalized to control values—for glutathione determinations, with * denoting significance at $p < 0.05$ for the compartments indicated. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

the lower availability of classical antioxidants at later stages of ascorbate deficiency. These data also demonstrate that responses are not uniform across organs but highly compartmentalized.

NO/redox status during development of marginal ascorbate deficiency

A subset of ODS rats was kept on a standard rodent chow containing ascorbate in amounts just sufficient to prevent the development of visible signs of scurvy (group 2). Direct comparison of controls on full supplementation to animals with a suboptimal ascorbate supply revealed a significant decline in ascorbate across most compartments (Fig. 2). After stabilization of ascorbate levels (around day 28), a similar and often more reduced redox state was maintained compared to the beginning (Fig. 2d), suggesting the involvement of compensatory mechanisms to maintain tissue redox status. In contrast to the acute depletion study, NO-related metabolites were maintained within a rather tight concentrations range, with no further increases over time. In fact, several compartments exhibited significant decreases during this time (Figs. 2g and 2h).

Optimal time window for study of marginal ascorbate deficiency

Since none of the animals within group 2 revealed any scorbutic signs within the first 6 weeks, we extended the observation period to see what might happen over the following months while animals were kept marginally ascorbate deficient. Animals were sacrificed at regular intervals for blood/tissue analysis. No further ascorbate loss or major changes in NO metabolites were observed in the majority of animals (data not shown). However, 25% of rats (5 out of 20) kept under these conditions spontaneously developed scurvy between months 5 and 7, narrowing the window of opportunity for future studies of marginal ascorbate deficiency using this model to about 6 months. No sentinels optimally supplemented with ascorbate developed clinical symptoms at any point of the study.

NO/redox status on acute ascorbate depletion following prolonged marginal deficiency

After 7 months of asymptomatic marginal ascorbate deficiency, a subset of animals were subjected to complete ascorbate deprivation (group 3) in order to compare the biochemical changes associated with scurvy development after prolonged marginal deficiency to those of acute depletion in adequately supplemented animals (group 1). We hypothesized that animals maintained under suboptimal conditions (group 2) would develop scurvy faster than those optimally supplemented with ascorbate. As in the acute study, animals were sacrificed after 1 and 2 weeks, and following the appearance of overt signs of scurvy.

The most obvious difference between animals of groups 1 and 3 relates to the time required to develop scurvy. While in optimally supplemented animals it took up to 7 weeks to reach a scorbutic score that justified sacrifice, this period was reduced to only 4 weeks in marginally deficient animals. Indeed, 90% of the animals showed first signs of scurvy already after 1 week. In week 2 about half of the animals developed severe symptoms, necessitating sacrifice of the entire group between weeks 3 and 4 to avoid unnecessary suffering (Fig. 3). While animals in the acute study showed a gradual depletion in ascorbate over approximately 3 weeks, tissue ascorbate levels in marginally deficient rats now exhibited changes (compare Fig. 1a with Fig. 4a). As seen before, plasma continued to experience a significant increase in total ascorbate. Except for the brain, absolute levels of ascorbate in marginally deficient animals at the beginning of the depletion experiment were comparable to those in animals of group 1 after 2–3 weeks of acute ascorbate deprivation. Yet, signs of scurvy became apparent almost immediately in the former (compared to $\sim$2 more weeks to develop mild to severe symptoms in optimally supplemented rats), indicative of a dissociation between tissue biochemistry and clinical symptomology.

Several other differences in outcome between these two groups are worth mentioning. The transient increase in ascorbate redox status seen in the first week of the acute study was not observed in most tissues after prolonged marginal deficiency (Fig. 4b). Moreover, nitrite levels experienced a drop rather than an increase in the first week of total ascorbate deprivation, followed by a rebound in nitrite and nitrate in the second week, to again drop near death. These changes are almost the mirror image of what was observed in the acute study, suggesting tissues are struggling to adapt to the new situation, perhaps due to differences in NO system operation. Differences do not only relate to the time-course of biochemical changes but also to response magnitudes, consistent with a lower regulatory reserve. At the time of sacrifice of optimally supplemented animals, the NO system seems to be systemically upregulated, resulting in a massive rebound in NO metabolite concentrations (43-fold increase in steady-state levels of nitrite + nitrate (NOx) in blood) (Figs. 5a and 5b); in marginally deficient animals at the same point, this increase was not nearly as spectacular (6-fold increase for NOx in blood), although starting levels were comparable in most tissues (Figs. 5c and 5d). This observation is consistent with a specific impairment of the nitroso-redox system in marginal ascorbate deficiency which translates into a compromised response to further redox stress. Of note, the aorta differed from this general pattern of responses, showing a marked up-regulation of nitrosation near the study end (Fig. 5d), although this difference did not reach statistical significance. NO-heme levels in blood and tissues showed only minor variations (Fig. 4e), suggesting changes in overall NO availability remained moderate. This contrasts with the results obtained on acute ascorbate depletion in optimally supplemented animals, where production (indicated by NOx formation) and availability of NO (indicated by NO-heme levels in tissues and erythrocytes) were markedly upregulated (Figs. 1e, 1f, and 1h).

NO/redox status on ascorbate repletion

To test the reversibility of alterations in nitroso-redox status at advanced stages of deficiency, a subset of animals kept for 7 months marginally ascorbate deficient and subjected to full ascorbate deprivation thereafter (scorbutic score $\geq$14) were repleted with vitamin C via their drinking water and followed for another 3 weeks. Most ($\sim$ 70%) animals recovered within days of repletion, starting to gain weight with rapid behavioral improvements and gradual disappearance of scurvy

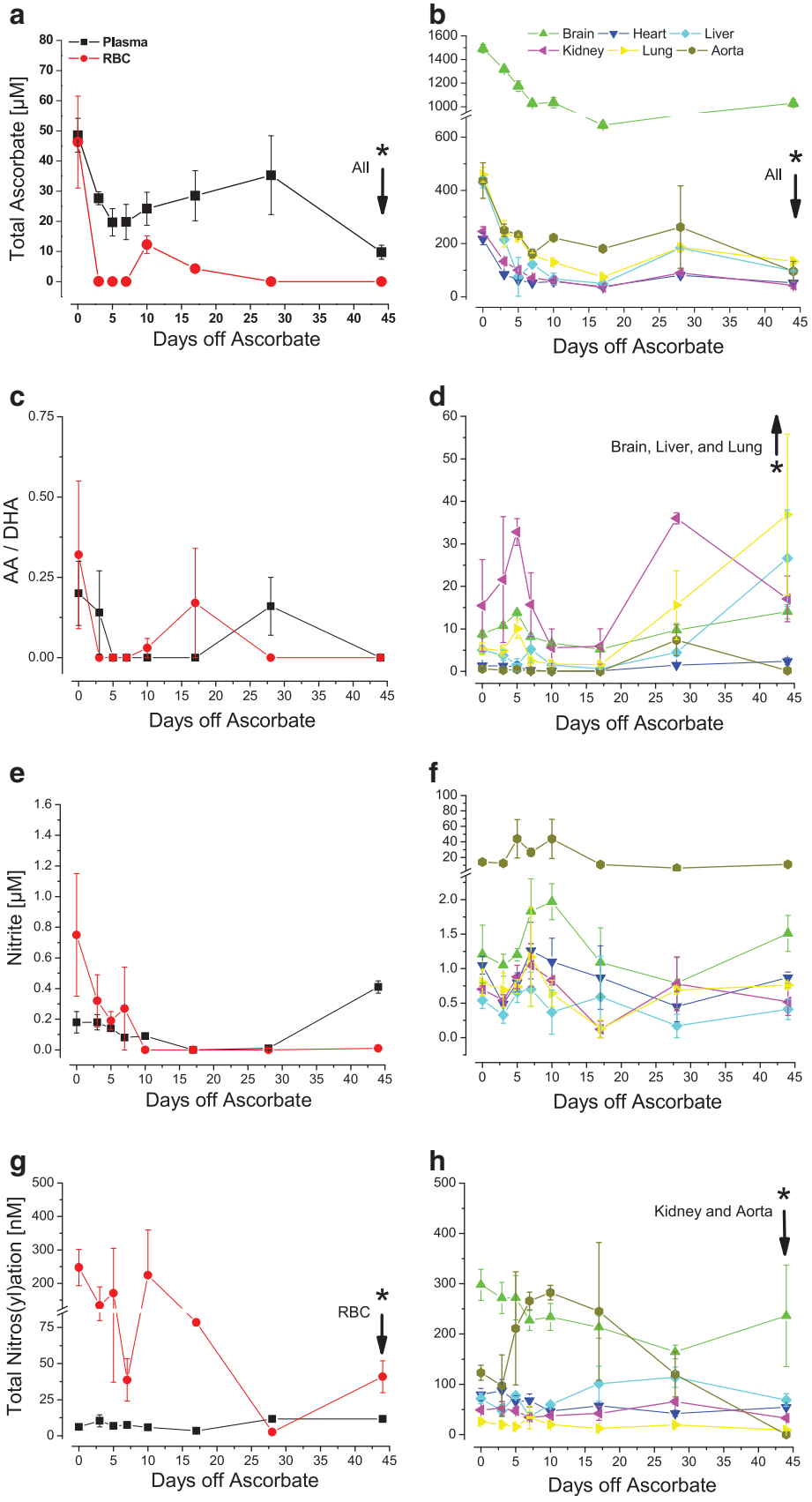

FIG. 2. Development of marginal ascorbate deficiency and effects on NO/redox status in blood and tissues of ODS rats. Time course of changes in **(a, b)** total ascorbate content; **(c, d)** ascorbate redox status, expressed as ratio of reduced (AA) over oxidized (DHA); **(e, f)** nitrite concentration; and **(g, h)** concentration of total nitros(yl)ation products (nitrosothiols + nitrosamines + NO-heme species). *Left panels* depict changes in blood, *right panels* those in tissues. Means ± SEM of n = 3–5 animals/time point; *$p < 0.05$. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

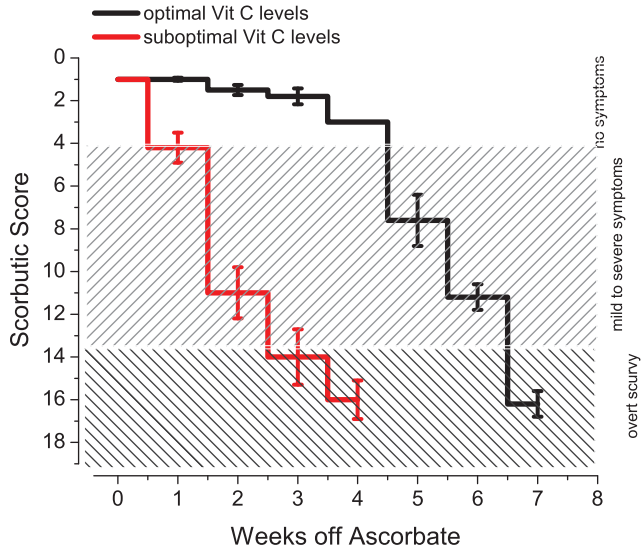

FIG. 3. Scurvy development following acute ascorbate depletion in ODS rats with previously optimal vitamin C supply compared to animals after prolonged marginal deficiency. Minimum score: 1 (no symptoms), maximum score: 19 (overt scurvy). Animals were sacrificed at scores between 14 and 17 for ethical reasons, dependent on severity of the symptoms (see Methods for details). Each time point represents the average ± SEM of individual animal scores calculated weekly ($n = 4$–20). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

symptoms. As depicted in Figure 6a, tissues fully recovered to their pre-depleted ascorbate levels. This was accompanied by a strikingly enhanced AA/DHA redox ratio in all tissues except the aorta (Fig. 6b), perhaps a result of improved recycling, and a normalization of NOx levels in all compartments (Fig. 6c). The latter suggests the increase in NO production in scorbutic animals was due to an upregulation of constitutive NOS activity rather than expression of inducible NOS (which might be expected to further increase on ascorbate repletion).

Changes in vascular function in response to ascorbate alterations

As alluded to earlier, the aorta is the only compartment besides the brain that follows a trend for biochemical changes distinct from that in other tissues. This observation, along with puzzling reports about a lack of endothelial dysfunction in these animals (45), prompted us to investigate possible changes in vascular function in response to variations in ascorbate levels. Figure 7a depicts an original tracing of the protocol used for assessment of endothelium-dependent and independent vascular responses (see Methods for details), with quantitative data depicted below. Control experiments confirmed the lack of endothelial dysfunction in ODS rats optimally supplemented with ascorbate (Fig. 7b). In fact, endothelial vascular reactivity of ODS rats was superior to that of age-matched Wistar rats (pD_2: 8.2 ± 0.02 versus 6.94 ± 0.01 in ODS and Wistar rats, respectively). In contrast, vasorelaxant responses to the endothelium-independent NO-donor, DEA/NO were comparable between both strains (Fig. 7c), indicating a specific enhancement of vascular endothelial reactivity. Unexpectedly, marginally ascorbate deficient animals retained their improved vascular endothelial reactivity and revealed no sign of alteration in endothelium-independent relaxation to NO. Both potency and maximal relaxation to Ach were greatly improved in ODS rats rendered marginally deficient in ascorbate compared to age-matched Wistar rats (pD_2: 8.13 ± 0.04 vs. 6.94 ± 0.01 in ODS and Wistar rats, respectively; maximal relaxation: 89 ± 1% in ODS, 80 ± 0.8% in Wistar controls; Figs. 7b and 7c). Remarkably, vascular function remained unimpaired even after induction of scurvy following prolonged marginal deficiency. While calculated pD_2 values (a measure of vasorelaxant potency) for Ach were nearly identical in all ODS treatment groups, maximal relaxation responses to Ach were significantly impaired in overt scurvy (71 ± 3%; $p = 0.0004$ vs. optimally supplemented ODS controls, $p < 0.0001$ vs. marginally ascorbate deficient rats), possibly secondary to enhanced oxidative stress. In conclusion, ODS rats show improved NO-dependent endothelial reactivity compared to control animals corresponding to their genetic background (Wistar rats), and this is independent of their ascorbate status. The lack of difference in NO-dependent but endothelium-independent vasorelaxation clearly demonstrates that this improved vascular reactivity is not secondary to enhanced downstream processes mediating vasorelaxation, but the result of increased eNOS activity. This assumption is further corroborated by higher levels of basal NO release in ODS compared to Wistar rats, as evidenced by the difference in maximal contractile tone following NOS inhibition (Fig. 7c, inset).

Discussion

The key findings of the current study are that i) ascorbate deficiency is associated with characteristic changes in NO production and metabolism, accompanied by a shift towards a more reduced redox state; ii) the effects of acute ascorbate depletion on cellular redox/nitros(yl)ation status differ between well-supplemented and marginally deficient animals, with faster scurvy development in the latter; iii) changes in NO/redox status are complex and highly compartmentalized; iv) NO responses are blunted and dissociated from phenotypical changes in marginal ascorbate deficiency; and v) vascular endothelial dilatation remains largely unaffected by ascorbate deficiency. To the best of our knowledge, ours is the first study to comprehensively assess the dynamics and define the role of ascorbate in modulating cellular redox status and blood and tissue nitros(yl)ation status *in vivo*. We believe this new rodent model has merit for the future study of redox signaling mechanisms related to ascorbate deficiency.

Marginal ascorbate deficiency is a growing problem in most contemporary societies due to lifestyle-related changes in dietary habits with a lower than desirable intake of fruits and vegetables (further compounded by physical inactivity and psychosocial stress). Marginal ascorbate deficiency is associated with fatigue, increased prevalence for chronic diseases, and a higher risk of developing scurvy (19). The latter is consistent with our finding that scurvy developed faster in marginally deficient rats. That chronic latent ascorbate deficiency is associated with changes distinct from those of acutely scorbutic animals is not new (41), but we are the first to report that those differences are associated with specific alteration in NO production and metabolism. These results may be relevant to subclinical micronutrient deficiency

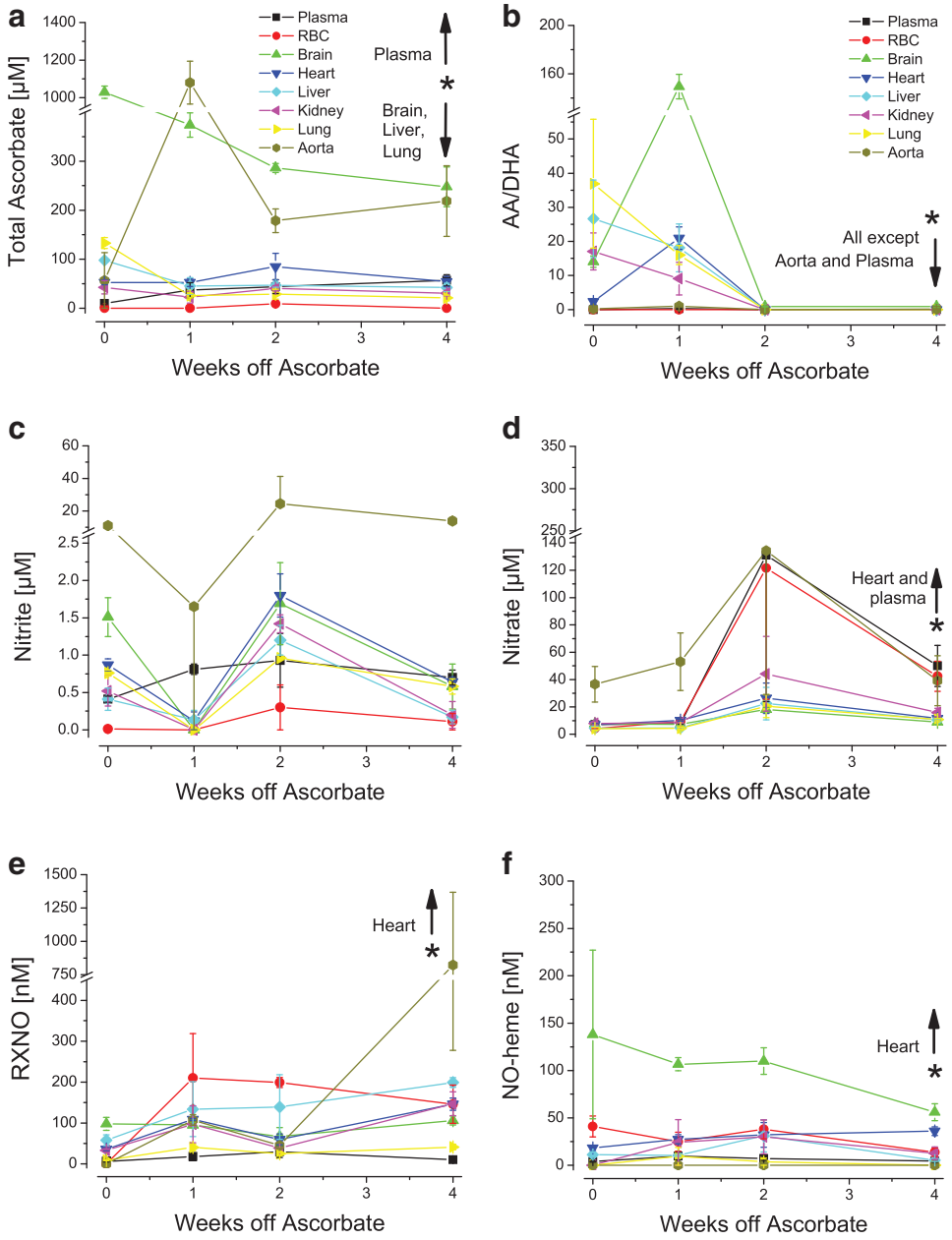

FIG. 4. Time dependence of the effects of acute ascorbate depletion on NO/redox status in blood and tissues of ODS rats subjected to prolonged marginal ascorbate deficiency. Animals were subjected to acute ascorbate depletion after 7 months of suboptimal supply with ascorbate (marginal deficiency); after 1, 2, and 4 weeks blood and tissues were analyzed for changes in **(a)** total ascorbate content, **(b)** reduced (AA) over oxidized ascorbate (DHA) ratio; and concentrations of **(c)** nitrite, **(d)** nitrate, **(e)** total nitrosation products (RXNO, nitrosothiols + nitrosamines), and **(f)** total nitrosyl (NO-heme) products. Means ± SEM of n = 3–5 animals/time point; $*p < 0.05$. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

in humans, particularly in the pediatric population and the elderly where unspecific ascorbate deficiency symptoms are easily mistaken for something else. Moreover, while imminently treatable once recognized, the amelioration of symptoms may not always lead to full restoration of health. Our data suggest that an adequate NO production combined with a change in NO metabolism are key elements in the counter-regulatory response to acute and chronic ascorbate deficiency, but there is a paucity of information on what other redox-sensitive processes might be affected in addition. Moreover, our results suggest a dissociation of tissue biochemistry and clinical symptomology at a stage of ongoing redox perturbation the long-term consequences of which remain unexplored. The relationship between low ascorbate levels and all-cause mortality (10) suggests some of these changes are crucial for survival. The observation that endothelial vasoreactivity remains largely unaffected by ascorbate depletion suggest that regulation of vascular tone (and thus convective oxygen and nutrient delivery) are unlikely to be involved. An alternative possibility is that endothelial function is so vital that it is maintained irrespective of the level of ascorbate supply. This interpretation would be consistent with the finding that NO metabolite levels are highest in overt illness.

Glutathione and NO as first-line of defense against acute ascorbate depletion

In the acute study group, animals fully deprived of ascorbate developed scurvy in the same fashion as originally described (31), with hindlimb disorders appearing after 3 weeks, body weight gain arrest at week 4, and death ensuing after 7–8 weeks. These symptoms were accompanied by specific changes in NO/redox status. Ascorbate depletion was associated with a compensatory increase in total glutathione

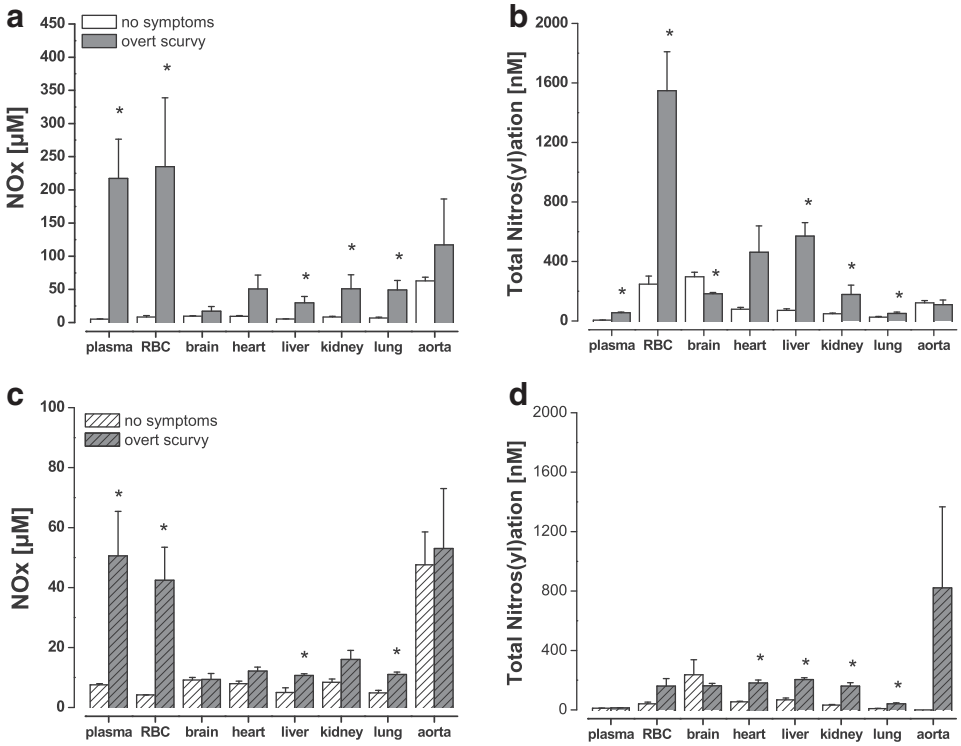

FIG. 5. Comparison of magnitudes in NO-related responses between symptom-free and overtly scorbutic animals in adequately supplemented (a, b) and marginally deficient ODS rats (c, d). *Bar charts* depicting steady-state concentrations in blood and tissues of **(a)** NOx (nitrite + nitrate) and **(b)** total nitros(yl)ation products (nitrosothiols + nitrosamines + NO-heme species) in animals during acute induction of scurvy (no symptoms = day 0; overt scurvy = 6–7 weeks after full ascorbate depletion); **(c)** NOx and **(d)** total nitros(yl)ation products in animals with marginal ascorbate deficiency and subsequent induction of scurvy (no symptoms = day 0; overt scurvy = 3–4 weeks of full ascorbate depletion). Means ± SEM of $n = 3–5$ animals/group); $^*p < 0.05$.

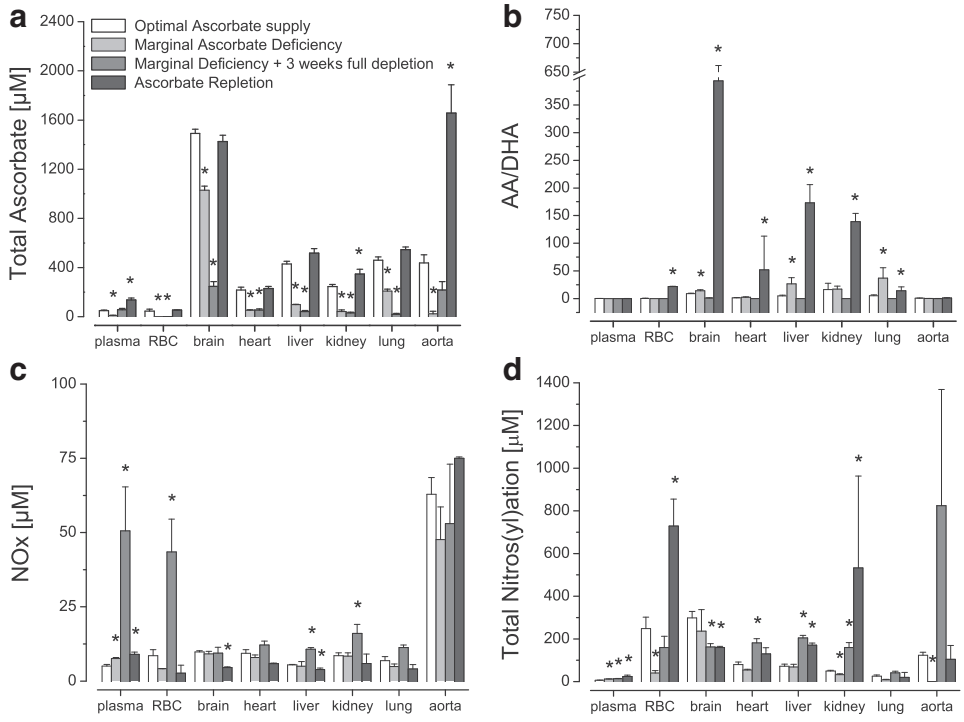

FIG. 6. Effects of manipulations in ascorbate supply on nitroso-redox status in blood and tissues of ODS rats. *Bar charts* depicting steady-state concentrations of **(a)** total ascorbate, **(b)** reduced (AA) over oxidized ascorbate (DHA) ratio, **(c)** NOx (nitrite + nitrate), and **(d)** total nitros(yl)ation products (nitrosothiols + nitrosamines + NO-heme species). *Open bars*: ODS rats supplemented with 1 mg/mL ascorbic acid in the drinking water. *Light gray bars:* ODS rats with marginal ascorbate deficiency (symptom-free). *Gray bars:* ODS rats with marginal ascorbate deficiency completely deprived of ascorbate for 4 weeks (average scorbutic score: 14). *Dark gray bars:* ODS rats with marginal ascorbate deficiency, completely deprived of ascorbate for 4 weeks and then replenished with 1 mg/mL ascorbate with the drinking water for another 3 weeks (at which time average scorbutic scores returned to values within healthy limits). Means ± SEM of n = 3–5 animals/group; $^*p < 0.05$.

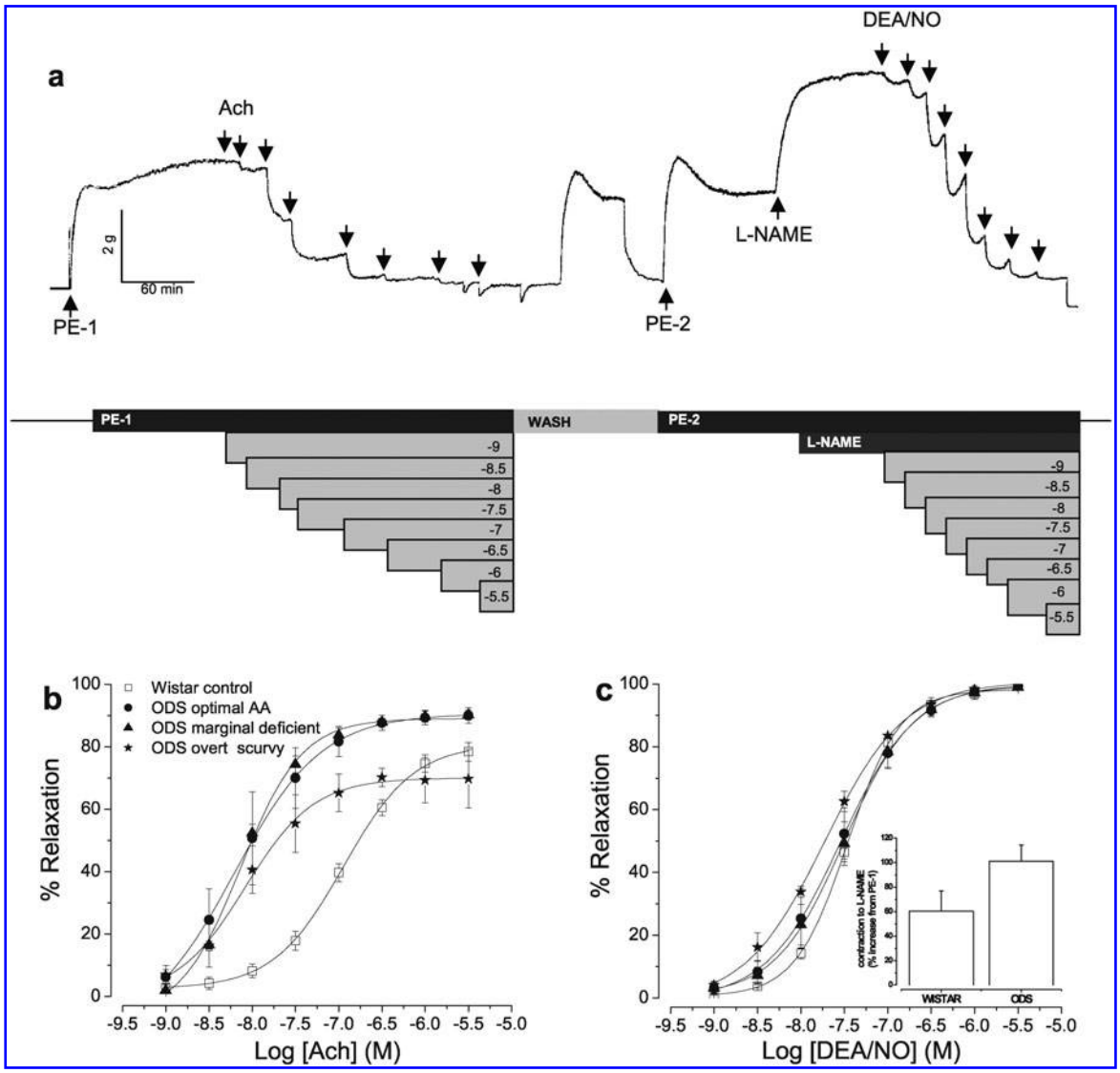

FIG. 7. ODS rats exert improved vascular endothelial reactivity compared to normal Wistar rats, irrespective of ascorbate status. (a) Representative original tracing of organ bath experiments on the relaxation to acetylcholine (Ach) and DEA/NO in endothelium-intact thoracic aortic rings of ODS rats. **(b)** Endothelium-dependent relaxation induced by increasing concentrations of Ach in phenylephrine (PE, 0.2 μM) pre-contracted vascular rings, expressed as percentage of contractile tone induced by the first PE stimulus (PE-1). **(c)** Endothelium-independent relaxation induced by the NO-donor, DEA/NO in aortas washed and re-contracted with PE (PE-2), following addition of the NOS inhibitor L-NAME (100 μM). Results are expressed as percentage of contraction induced by PE + L-NAME. *Inset:* Contraction induced by L-NAME after PE re-contraction in Wistar rats vs. ODS rats fully supplemented with ascorbic acid; results are expressed as percentage of contraction elicited by first PE stimulus. Means ± SEM of a minimum of two-paired rings from 3–6 individual animals. *Groups*: □ age matched Wistar controls, ● ODS rats supplemented with 1 mg/mL ascorbic acid in drinking water, ▲ ODS rats with marginal ascorbate deficiency, * ODS rats with marginal ascorbate deficiency subjected to acute ascorbate depletion (overt scurvy). Endothelium-dependent relaxant responses to Ach in ODS rats were significantly different from those in Wistar rats under all conditions ($p < 0.001$); maximal vasorelaxant responses in overt scurvy also differed from those in fully supplemented and marginally deficient ODS rats (see text for details).

concentration and a shift towards a more reduced redox state. These results are consistent with the counter-regulatory nature of responses described by Wang et al. (48) and the notion that ascorbate and glutathione form an inter-connected antioxidant network (28); they are also in line with the observation that ODS rats are unable to compensate the oxidative stress elicited by inhibition of glutathione production (37). It is apparent from our results that the adjustments in glutathione metabolism alone are not sufficient, as indicated by the concomitant increase in NO production during the same period. More importantly, glutathione redox changes cannot be sustained for long, and tissues become gradually more oxidized again. It appears whether the NO system compensates for the ascorbate loss, in particular as the deficiency becomes more severe. This interpretation is in agreement with the observation that ODS rats develop alternative antioxidative defenses as ascorbate level vanish (46). Our data would seem to suggest that this alternative defense system involves the production of NO.

NO responses differ in dependence of ascorbate supplementation status

The consequences of acute ascorbate depletion differ between optimally supplemented and marginally deficient animals. Either group experiences an early upregulation of the NO system that coincides with the initial drop in ascorbate. While in marginal ascorbate deficiency levels of NO-related metabolites and ascorbate tend to stabilize thereafter, NO upregulation in optimally supplemented animals then translates into gradual increases in NO metabolites, which is most pronounced towards the study end. These findings are consistent with earlier reports that nitrate levels in plasma and urine of ascorbate-deficient ODS rats are higher than in fully supplemented animals (44). The concomitant increase in tissue nitros(yl)ation status, which is normally maintained within rather tight limits (2) and may also rise as a result of the lower availability of ascorbate to act as nitrosation scavenger, may serve to protect critical protein sulfhydryl and tryptophan groups from irreversible oxidation. The same adjustments seem to prevent animals from developing scurvy during marginal ascorbate deficiency. However, although ascorbate and NO/redox levels do not seem to deteriorate further, more subtle alterations of the NO system may render animals more susceptible to scurvy development. In fact, 25% of animals subjected to prolonged marginal ascorbate deficiency developed scurvy without further reduction in supply, limiting the complication-free period for study of marginal ascorbate deficiency using this model to 5–6 months. When marginally deficient animals were subjected to further depletion, the increases in NO metabolite levels were not as obvious as under optimal supplementation, suggesting NO-dependent responses are compromised following chronic marginal ascorbate deficiency.

Is increased NO production due to enhanced endothelial NOS activity?

While a quantification of NOS isoform expression profiles in different tissues was beyond the scope of the present study, our vascular function studies suggest that the increase in NO-related products observed in response to any form of ascorbate deficiency is related to an upregulation of eNOS activity and/or altered cofactor association. This is at variance with much of the information gained from vitamin C supplementation studies. If high doses of ascorbate augment cardiac function, confer cardioprotection, and enhance endothelium-dependent vasorelaxation (3), then its depletion might be expected to cause the opposite. However, Vergely et al. (45) had reported that ODS rats reveal improved vascular reactivity compared to Wistar controls. We here confirm and extend those results to show that endothelial reactivity is enhanced regardless of ascorbate supplementation status and associated with an increase in basal and stimulated endothelial NO production without changes in sensitivity of the NO receptor, soluble guanylyl cyclase, or downstream processes of vasorelaxation. Future studies should clarify whether this is a feature of this particular rat strain or perhaps a consequence of ascorbate deficiency during embryonic development.

Is nitrite linked to ascorbate oxidation?

Following prolonged marginal deficiency, the development of overt signs of scurvy did neither seem to depend on massive further ascorbate loss from a specific bodily compartment nor did the drop in plasma ascorbate correlate with scurvy development, raising concern about the predictive power of predefined concentration thresholds for ascorbate. Whether this reflects the crucial importance of a minimal bodily pool of ascorbate and is associated with alternative pathways of ascorbate production independent of L-gulonolactone oxidase activity warrants further study.

An intriguing association between nitrite concentration changes and ascorbate oxidation status may be gleaned from a comparison of NO/redox status between tissues. These observations may be a result of direct ascorbate oxidation by nitrite (6) and accompanied by the release of NO. The increase in NO-related metabolites in conjunction with a more reduced redox state observed in the first week of the acute study was absent in the majority of tissues following marginal ascorbate deficiency. In fact, a dramatic drop in nitrite and AA/DHA ratio was apparent instead. Likewise, during the development of scurvy in marginally deficient animals, AA/DHA ratios dropped whenever nitrite levels fell. Brain and aorta were notable exceptions to this rule, perhaps due to a heightened reliance on ascorbate, which may provide an explanation for the finding that levels in these tissues never drop below 300 μM. Indeed, the aorta was the only organ that responded with an increase in ascorbate in the first week of acute depletion, demonstrating a distinct pattern of regulation in the vasculature compared to the rest of the body. These observations are reminiscent of changes in tissue nitrite in the same compartments, which follow trends opposite to all other organs (1), and may reflect a re-prioritization of tissue ascorbate pools. Whether these differences are due to tissue-specific variations in ascorbate transporter expression or perhaps their susceptibility to redox regulation is unknown.

Antioxidant rebound following ascorbate repletion

Approximately 70% of animals suffering from advanced ascorbate deficiency recovered upon repletion with vitamin C. This percentage of recovery is comparable to that seen in human studies (33). Even though NO-related metabolite levels had returned to control levels following repletion, tissue redox status tended to remain more reduced, consistent with earlier studies in guinea pigs that provided evidence for an antioxidant rebound in preparation for future oxidative challenges (28).

Conclusions and Future Directions

Our results reveal a close relationship between the ascorbate and the NO system that is characterized by a compensatory upregulation of NO production and protein nitros(yl)ation as a response to the redox stress of acute ascorbate deprivation, with a compromised reactivity following chronic marginal ascorbate deficiency. The difference in response between acute ascorbate depletion and chronic marginal deficiency is likely a consequence of adaptive changes to a prolonged inadequate supply with vitamin C, consistent with changes in nutrient handling upon prolonged alterations in intake (17). We believe that a parallel assessment of global protein nitrosation and tissue redox status across multiple organ systems *in vivo* is key to gaining a deeper understanding of redox signaling/regulation; this may not require a certain balance to be maintained for optimal functioning, as originally put forward as part of the "nitroso

redox" concept (16). The current study did not address which biological targets are affected most by the variations in NO/redox status. Scurvy is associated with compromised collagen production due to the role of ascorbate as cofactor of prolyl hydroxylase, and activity of the latter may be further inhibited by enhanced NO production; perturbation of HIF-1α hydroxylation with consecutive alterations in hypoxic signaling is another obvious possibility. Future studies are warranted to investigate the possible involvement of other redox-sensitive signaling pathways affected by prolonged marginal ascorbate deficiency.

Methods

Materials, animals, diets, and assignment to experimental groups

All chemicals were of the highest purity available and were purchased from Sigma-Aldrich (St. Louis, MO). Male ODS rats (100–120 g) were obtained from Clea, Inc. (Tokyo, Japan); male Wistar rats were purchased from Harlan (South Easton, MA). ODS rats were initially kept on a standard rodent chow (2018, Harlan Teklad, South Easton, MA) and supplemented with ascorbic acid (1 mg/mL in drinking water). Wistar rats were maintained on the same diet with tap water *ad libitum*. The ODS phenotype was confirmed by measurement of L-gulonolactone oxidase activity (24, 34) in hepatic microsomes isolated from one random animal/shipment (3 in total). After a 3-week acclimatization period, animals were randomly allocated to 3 groups (Fig. 8): 1. *Acute ascorbate depletion group*. Ascorbic acid-supplemented animals (1 mg/mL in the drinking water) were placed on an ascorbic acid-free diet (AIN93M, Harlan Teklad) with *ad libitum* access to tap water and monitored until overt signs of scurvy became apparent. 2. *Marginal ascorbate deficiency group*. Ascorbic acid-supplemented animals were taken off the supplementation by switching to regular tap water. These animals were kept on a standard diet (2018, Harlan) containing low levels of ascorbic acid (translating to an intake of 2.6 mg/day; according to supplier information, this corresponds to approximately twice the minimum level required to prevent death within the first 2 months), and monitored for 30 weeks. 3. *Marginal ascorbate deficiency group subjected to acute ascorbate depletion*. After 30 weeks of marginal ascorbate deficiency, remaining animals from group 2 were switched from their regular diet (2018) to ascorbic acid-free diet (AIN93M) and tap water for direct comparison with acute depletion in group 1. A subset of these animals starting to show signs of scurvy was repleted with ascorbic acid (1 g/L in drinking water) for 3 weeks to determine the extent of recovery from ascorbate deprivation. Rats were sacrificed at regular intervals for biochemical and functional analyses. All experiments complied with federal and state regulations in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council) and were approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine.

Scorbutic Rating Scale

In order to quantitatively assess the clinical symptoms developing during ascorbate depletion, all animals were inspected daily and rated according to a scorbutic rating scale (47). Seven symptoms were defined (hemorrhage, behavior, piloerection in the back/neck, weakness, ataxia, dysbasia, and weight changes), and scored on a scale of 0 (minimum) to 3 (maximum), except for ataxia which was scored as either 0 (absent) or 1 (present). Minimum and maximum scores possible were 1 and 19, respectively. All animals were considered almost terminal, and consequently sacrificed, when they reached a score of >16, to avoid unnecessary suffering. At predefined times, animals were sacrificed regardless of their symptom score for biochemical and vascular functional analyses.

Blood collection

Venous blood was collected from the inferior vena cava of anesthetized animals into tubes containing N-ethylmaleimide (NEM; 10 m*M*), and ethylenediaminetetraacetic acid (EDTA; 2.5 m*M*), to block thiol groups, inhibit transition metal-catalyzed transnitrosation reactions, and prevent artificial nitrosation, as well as thiolate- and ascorbate-mediated degradation of nitroso species (9, 21). Plasma and erythrocytes were obtained by centrifugation at 1600 *g* for 8 min; erythrocytes were subjected to hypotonic lysis in NEM/EDTA-containing (10/2.5 m*M*) water (1:3 v/v) immediately before analysis.

Organ harvest and tissue homogenization

Heparinized (0.07 units/g body weight, i.p.) rats were anesthetized with diethylether and euthanized by cervical

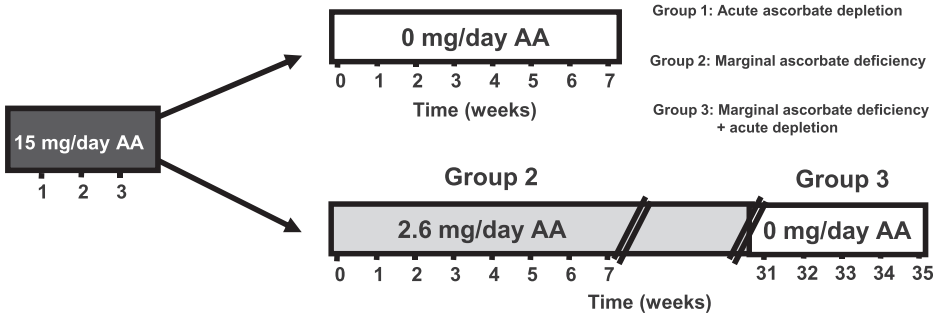

FIG. 8. Ascorbic acid supplementation and deprivation regimen, and time-line for experimental study groups. Upon arrival, ODS rats were supplemented with 15 mg ascorbic acid (AA)/day via the drinking water for 3 weeks. Thereafter, animals were randomly divided into groups 1 (no AA supplementation in water or diet) and 2 (2.6 mg AA/day supplementation in rodent chow but none in water). Group 2 was monitored for up to 30 weeks and then fully deprived of ascorbate by switching to an ascorbic acid-free diet and tap water (group 3).

dislocation. After thoracotomy, a catheter was inserted into the infrarenal part of the abdominal aorta, and organs were flushed free of blood by retrograde *in situ* perfusion with air-equilibrated, NEM/EDTA (10/2.5 m*M*) supplemented phosphate buffered saline (PBS) at a rate of 10 ml/min, immediately followed by tissue harvest and homogenization (2). For glutathione measurements, separate animals from the same treatment group/stage were used in which tissue perfusion and homogenization was carried out using EDTA-containing buffer without NEM. All steps were carried out under reduced ambient light to prevent photolytic decomposition of nitroso products.

Determination of tissue nitroso/nitrosyl and nitrite/nitrate concentrations

Tissue nitroso/nitrosyl compounds were quantified using group-specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence (9). Given that the identity of most nitroso species in the various biological compartments examined remains unknown, the concentrations of *S*- and *N*-nitroso species (nitrosothiols and nitrosamines, respectively) are reported as total nitroso species (RXNO) in the present study. NO-heme levels were determined by parallel injection of aliquots of tissue homogenates into 0.05 *M* ferricyanide in PBS (2). This method employs one-electron oxidation rather than reduction to achieve denitrosation, with the liberated NO also being quantified by gas-phase chemiluminescence. In some cases, the sum of nitroso and nitrosyl species are reported as "total nitros(yl)ation" products. Nitrate and nitrite were quantified by ion chromatography with on-line reduction of nitrate to nitrite and post-column derivatization (ENO20 Analyzer; Eicom, Kyoto, Japan).

Redox measurements (ascorbate and glutathione)

Redox status was expressed as ratio of reduced over oxidized forms of ascorbate and glutathione, respectively, with determinations carried out in either duplicate or triplicate.

Blood and tissue ascorbate content was assayed essentially as described by Carr et al. (4). Tissues were homogenized in PBS supplemented with 4 m*M* EDTA at a ratio of 1:5. After separation of plasma and RBCs, 100 μL of 50% metaphosphoric acid was added to 900 μL of plasma, RBC lysate, or tissue homogenate, followed by vortexing and centrifugation at 14,000 rpm for 10 min at 4°C. Reduced ascorbic acid (AA) in the deproteinized samples was oxidized to dehydroascorbic acid (DHA) by addition of 25 μL of 0.2% 2,6-dichlorophenolindophenol (DCIP) to 250 μL of sample. Following 1 h of incubation at room temperature, 250 μL each of 2% thiourea (in 5% metaphosphoric acid) and 2% 2,4-dinitrophenylhydrazine (DNPH) (in 12 *M* H_2SO_4) were added, and the samples further incubated for 3 h at 60°C. Reactions were stopped by addition of ice-cold 18 *M* H_2SO_4 (500 μL) to each tube, and the content transferred to 96-well plates for immediate reading at 524 nm using a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). Total ascorbate (TA) (i.e., the sum of oxidized and reduced forms of ascorbate) was calculated from these readings. Vials containing water instead of DCIP were used to determine DHA concentrations in each sample. An additional control was run in which DNPH was not added until after the addition of H_2SO_4 to account for sample-specific background coloration. TA and DHA concentration in tissues and blood were determined by the difference in readings of the DCIP/H_2O-treated samples, after correction for background, by comparison to a standard curve with authentic ascorbate. AA concentrations were determined by the difference in readings of the DCIP-treated and H_2O-treated samples.

Tissue glutathione concentrations were determined using a modified Tietze recycling assay (43). Tissues were homogenized in PBS supplemented with 4 m*M* EDTA at a ratio of 1:5. After separation of plasma and RBCs, 300 μL of 5% metaphosphoric acid were added immediately to 100 μL of plasma, RBC lysate, or tissue homogenate, followed by vortexing and centrifugation at 14,000 rpm for 10 min at 4°C. Total glutathione content was determined in the supernatant solution by the recycling assay. Briefly, reduced glutathione (GSH) is oxidized by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to yield oxidized glutathione (GSSG) with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). Then GSSG is reduced to GSH by the action of GSSG reductase and NADPH. The rate of TNB formation is followed at 412 nm using a microplate reader and is proportional to the sum of GSH and GSSG in the sample. To determine GSSG, a parallel 100 μL sample, previously derivatized with 2-vinylpiridine for 60 min, is assayed as described above, and GSH concentrations are calculated from the difference between total glutathione and 2xGSSG. Calibration curves for GSH and GSSG were run daily to calculate final blood/tissue concentrations.

Assessment of vascular function

To assess vascular reactivity, duplicate animals were sacrificed and their descending thoracic aorta (until just above the celiac trunk, excluding the aortic arch) was harvested for organ bath studies (the same tissue segment was used in the biochemical studies to allow for a direct comparison between vascular reactivity and ascorbate/NO-related metabolite status). The aorta was placed in ice-cold, oxygenated Krebs-Henseleit solution, carefully dissected free of adipose and connective tissue, and cut into 4 mm-wide rings. Vascular rings were then mounted on tungsten hooks and suspended in water-jacketed, 20 ml organ baths containing oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (pH 7.4) supplemented with indomethacin (5 μ*M*), and tissues were allowed to equilibrate for 90 min under a resting tension of 2.0 g at 37°C. During this period, the bathing medium was exchanged every 15 min. After final adjustment of the passive tension to 2.0 g, vascular segments were contracted submaximally with 0.2 μ*M* L-phenylephrine (PE). To assess NOS-related endothelial reactivity, a concentration-response curve (CRC) to acetylcholine (Ach) was performed. Following completion and extensive wash-out vessels were re-contracted with 0.2 μ*M* PE. After reaching a stable contractile tone, N^G-nitro-L-arginine methylester (L-NAME; 100 μ*M*) was added to allow assessment of basal NO vasodilator tone under the conditions of this study. The contribution of endothelial NO formation to overall vascular tone was judged by comparison of the contraction in the absence and presence of complete NOS inhibition, and the NOS-inhibitable component was expressed as percent increase from the contraction achieved in the absence of L-NAME. Thereafter, vascular smooth muscle sensitivity to NO was assessed by constructing a CRC for

the NOS-independent NO-donor, 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO). Changes in isometric tension were measured by means of force displacement transducers and documented on a six-channel recorder, and relaxant responses were expressed as a percentage of the contraction achieved with PE + L-NAME.

Statistic analyses

All graphical data is expressed as means ± SEM from *N* experiments. Statistical analysis was performed using either Origin or GraphPad Prism. Comparison between groups was achieved by one-way ANOVA with statistical significance set at $p < 0.05$. Analysis of dose-response curves were performed through nonlinear regression to a sigmoidal function with three parameters, including bottom, EC_{50}, and top.

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Author Disclosure Statement

No competing financial interests exist for any of the authors.

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Abbreviations used

AA = ascorbic acid
Ach = acetylcholine
Asc = ascorbate
CRC = concentration-response curve
DCIP = 2,6-dichlorophenolindophenol
DEA/NO = 2-(N,N-diethylamino)-diazenolate-2-oxide
DHA = dehydroascorbate (oxidized ascorbate)
DNPH = 2,4-dinitrophenylhydrazine
EDTA = ethylenediaminetetraacetic acid
GSH = reduced glutathione
GSSG = oxidized glutathione
L-NAME = N^G-nitro-L-arginine methylester
NEM = N-ethylmaleimide
NO = nitric oxide
NOS = nitric oxide synthase
ODS = Osteogenic Disorder Shionogi
PE = phenylephrine
TA = total ascorbate (reduced+oxidized)
TNB = 5-thio-2-nitrobenzoic acid

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