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On the Dynamics of Nitrite, Nitrate, and Other Biomarkers of Nitric Oxide Production in Inflammatory Bowel Disease

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Running title: NO metabolites in colitis

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ABSTRACT

Nitrite and nitrate are frequently used surrogate markers of nitric oxide (NO) production. Using rat models of acute and chronic DSS-induced colitis we examined the applicability of these and other NO-related metabolites, in tissues and blood, for the characterization of inflammatory bowel disease. Global NO dynamics were assessed by simultaneous quantification of nitrite, nitrate, nitroso and nitrosyl species over time in multiple compartments. NO metabolite levels were compared to a composite disease activity index (DAI) and contrasted with measurements of platelet aggregability, ascorbate redox status, and the effects of 5-aminosalicylic acid (5-ASA). Nitroso products in the colon and in other organs responded in a manner consistent with the DAI. In contrast, nitrite and nitrate, in both intra- and extravascular compartments, exhibited variations that were not always in step with the DAI. Extravascular nitrite, in particular, demonstrated significant temporal instabilities, ranging from systemic drops to marked increases. The latter was particularly evident after cessation of the inflammatory stimulus and accompanied by profound ascorbate oxidation. Treatment with 5-ASA effectively reversed these fluctuations and the associated oxidative and nitrosative stress. Platelet activation was enhanced in both the acute and chronic model. Our results offer a first glimpse into the systemic nature of DSS-induced inflammation and reveal a greater complexity of NO metabolism than previously envisioned, with a clear dissociation of nitrite from other markers of NO production. The remarkable effectiveness of 5-ASA to abrogate the observed pattern of nitrite instability suggests a hitherto unrecognized role of this molecule in either development or resolution of inflammation. Its possible link to tissue oxygen consumption and the hypoxia that tends to accompany the inflammatory process warrants further investigation.

Keywords: Inflammation, ulcerative colitis, inflammatory bowel disease, nitric oxide, nitrite, nitrosation, ascorbate, redox signaling, 5-aminosalicylate, hypoxia

Introduction

Nitric oxide (NO) is a free radical capable of reacting with a variety of molecules in biological fluids and tissues. These interactions produce not only the oxidation products, nitrite and nitrate, but also lead to formation of nitrosyl (NO-heme) species and modification of thiols and amines to produce S- and N-nitroso products[1]. The level of these NO-related substances, in fluids and in tissues, is widely assumed to reflect the activity of NO-synthases, including the inducible form of NO-synthase (iNOS) which is known to be expressed at high levels during inflammation[2], such as in inflammatory bowel disease (IBD)[3]. The link between iNOS expression and inflammation serves as a rationale for considering increased levels of NO-related products as evidence of inflammatory conditions. Nitrite and nitrate have been used extensively as markers of inflammation, largely because of their ease of determination using a variety of assays,[4] including the widely available Griess reaction that can measure the combined concentrations of both anions (NOx) in body fluids [5].

The use of NO-related products as markers of inflammation presupposes that only iNOS activity affects their concentrations in the compartment of interest. However, it is well known that nitrite and nitrate from the diet can also affect NOx levels assessed in fluids[6] and in tissues[7]. There is mounting evidence that nitrite is not as biologically inert as previously assumed, i.e. that it is a biochemically and pharmacologically active molecule[7-12] and that its levels are maintained within a tight concentration range (200-500 nM) across many mammalian species[13], suggesting some level of regulation. Further evidence for some form of nitrite homeostasis in plasma is gleaned from experiments recently conducted in rats sustained on a low NO_x diet. These experiments revealed an unusual compartmentalization of this anion, showing complete depletion in the extravascular compartment with essentially unaltered levels in plasma[7]. If the level of nitrite in plasma was indeed regulated, then the question arises as to whether the systemic concentration of this NO product is able to increase at all during a local inflammatory event. In addition to assessing the merit of NO products as markers of inflammation, we were interested in understanding how increased NO production localized to an inflammatory site, such as the colon in colitis, can affect NO status in distal tissues. This interest is grounded on evidence from clinical studies of colon inflammation showing that this disease can trigger several extra-intestinal problems that appear to be associated with a decreased bio-availability of NO, including enhanced platelet aggregation[14-17] and thromboembolic events[18-21].

The present study sought to investigate the dynamics of a wide spectrum of NO markers, including

nitrite, nitrate, nitroso and nitrosyl products, in the circulation and in tissues throughout the body of an exemplary rodent species, following the induction of acute and chronic colitis. Both forms of colitis are characterized by mucosal inflammation of the large bowel, leading to an increased NO production secondary to iNOS upregulation[22-30]. A state of acute and chronic colitis that is clinically and histologically reminiscent of human ulcerative colitis (UC) can be induced in rodents by administration of dextran sulfate sodium (DSS)[31]. In the present study, the *in vivo* assessment of NO-products was complemented by measurements of ascorbate redox status and platelet aggregability; in addition, the effects of the therapeutic drug 5-aminosalicyclic acid (5-ASA) were investigated. Our results demonstrate that colitis elicits significant NO-related reactions that are complex and at times counter-intuitive, suggesting they may not be suited as markers of disease activity until their behavior is better understood. Additionally, our results raise the prospect that nitrite plays a hitherto unrecognized role in either development or resolution of IBD and that fluctuations in nitrite concentration are intimately linked to tissue oxygen consumption and the hypoxia that tends to accompany the inflammatory process.

Methods

Materials: All chemicals were from Sigma Chemicals Co. (St. Louis, MO) unless otherwise stated.

Induction of Colitis: Male Wistar rats (Harlan, Indianapolis; 200-400g; housed 3/cage) were kept on a normal 12/12 light/dark cycle, with access to food (2018 rodent diet, Harlan Teklad) and water *ad libitum*. A minimum of 7 days was allowed for acclimatization to the institutional housing environment before experimental use. Rats were administered 5% DSS (MP Biomedicals) dissolved in tap water for 7 days (day 0-6, *ad libitum*) to induce acute colitis, followed by 4 days of tap water without DSS to assess recovery. Chronic colitis was induced by three cycles of DSS treatment, where one cycle included 7 days 5% DSS followed by 7 days water, and measurements were performed on the final day of 3 complete cycles (i.e. one week after cessation of the last DSS administration). A group of animals that received tap water only served as time-matched controls. A minimum of 3 animals/group was used for each time point in the acute study, whereas 5 (control) and 6 (DSS) animals/group were used in the chronic study. All protocols described were carried out in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NRC 1996) and were approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine.

Clinical Assessment of Colitis: The extent of colitis was assessed using a composite disease activity

index (DAI)[32, 33] determined by scoring changes in weight, hemoccult positivity (as determined by a guaiac paper test (ColoScreen; Helena Laboratories Inc., Beaumont, TX, USA) and stool consistency. The scores (0-4) for weight change, the presence of blood in the stools and stool consistency were combined and the final score divided by three where 0 represents no symptoms and 4 represents maximum symptoms. Following sacrifice colon samples were assessed for myeloperoxidase (MPO) activity and histopathological signs of disease progression.

Myeloperoxidase Assay: Colon samples were rinsed with cold phosphate buffered saline, blotted dry and immediately frozen in liquid nitrogen. Following storage at -80°C, tissues were thawed, weighed and assessed for MPO activity using the *o*-dianisidine method as described.[34, 35]

Histology: Colon cross sections were fixed with 10% neutral-buffered formalin and embedded in paraffin. Full-thickness sections were stained with hematoxylin/eosin and examined by a blinded pathologist for evidence of pathological changes and neutrophil infiltration.

NO-Related Biochemistry: To provide a comprehensive assessment of NO biochemistry during development of colon inflammation the NO-related metabolites, nitrite and nitrate as well as S- and N-nitroso species and nitrosylated heme species were measured in blood (plasma and red blood cells), brain, heart, liver, kidney, colon, aorta, and inferior vena cava (IVC). In addition, cellular redox status was assessed in the same compartments by measurement of the redox couple ascorbate/ dehydroascorbate, and platelet activity was analyzed in whole blood as detailed underneath. Analyses were performed on day 0 (control); days 1, 3, 5 and 7 of DSS administration, and on days 9 and 11 (i.e. 2 and 4 days after cessation of DSS administration) to assess recovery in the acute model. In the chronic model, measurements were taken following three cycles of DSS treatment as described above.

Experimental Protocol: Heparinized (0.07 Units/g i.p.) rats were anesthetized with diethylether (2 min) and killed by cervical 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 PBS (50 mM, pH 7.4) supplemented with N-ethylmaleimide (NEM)/ethylenediamine tetraacetic acid (EDTA) (10/2.5 mM) at a rate of 10 ml/min. The superior vena cava was cut directly after the aortic cannulation to provide an exit port for blood and buffer. After 2 min of perfusion, the brain was removed, followed by the heart. Liver, kidneys, and colon were individually cannulated and perfused through the portal vein, renal artery, and mesenteric artery respectively, and excised 2–3 min apart. The colon was removed from the cecum to the pelvic floor and prepared for histology and

assessment of MPO activity. Thoracic aorta and IVC were harvested last. This order of extraction was based on the relative metabolic rates of the organs and was strictly adhered to throughout the study to minimize tissue hypoxia. Excised tissues were blotted dry on filter paper, weighed, cut into small pieces, and homogenized immediately in ice-cold NEM/EDTA-containing perfusion buffer (5:1 v/w) by using either a Teflon-glass Potter-Elvehjem or a Wheaton glass-glass homogenizer immersed in an ice bath. Tissue homogenates were kept on ice in the dark and analyzed within 1–4 min of preparation.

Quantification of NO Metabolites: Nitroso species (i.e. the sum of S- and N-nitroso compounds) were quantified by a technique that uses reductive denitrosation followed by chemiluminescence detection of NO in the gas phase as described in detail elsewhere.[36] NO-heme species were quantified by direct injection of cell or tissue homogenates into an oxidizing solution comprised of 0.05 M ferricyanide in PBS.[1] Total amounts of NO adducts were calculated by comparison of peak areas of the samples to those of freshly prepared standards. The oxidative decomposition products of NO, nitrite and nitrate, were measured in aliquots of tissue homogenates and blood after methanol precipitation of proteins (1:1 vol/vol) using a dedicated HPLC system (ENO-20; EiCom, Kyoto) as described.[1]

NO-related responses are reported either as concentrations or fold-changes relative to the average basal levels. Most of the basal levels measured in our current study (see Tab.1) were similar to those reported in earlier studies, although some differences were noted which might be attributed to opposed light cycle conditions (animals used in the present study were kept on a normal rather than a reversed 12h/12h light/dark cycle), strain (different local supplier) or weight differences (final animal weights differed between the acute and chronic study). With the concentration of NO-heme products being noticeably lower under these conditions, no attempt was made to analyze the time course of these species. In view of our current lack of knowledge about the identity and significance of individual proteins undergoing NO-dependent post-translational modification during inflammation, in some cases we opted to collapse the data obtained for S- and N-nitroso compounds into a single value.

Redox Status: The redox status of plasma and tissues was assessed by quantification of the concentrations of the reduced and oxidized forms of ascorbate using the dinitrophenylhydrazine assay and expressed as ascorbate/dehydroascorbate ratio. Samples were treated as described by Carr et al.[37] and read at 524 nm using a 96-well plate reader. An additional blank was run in which 2,4-dinitrophenylhydrazine was not added until after the addition of sulfuric acid to account for tissue-specific background coloration of samples.[38] Total ascorbate concentrations in tissues were determined by the difference in readings of the 2,6-dichlorophenol-indophenol (DCIP)-treated and control samples

by comparison with a standard curve of ascorbic acid.

5-ASA Administration: To investigate the effects of 5-ASA on the development of acute colitis a further set of animals received 5-ASA at a dose level of 50mg/kg/day with the drinking water, starting from day 5 of DSS treatment throughout the duration of the study period to mimic a therapeutically relevant scenario. NO-related metabolites, ascorbate/dehydroascorbate and platelet activity in blood and tissue were analyzed as described above on days 7, 9 and 11 (i.e. after 2, 4 and 6 days of 5-ASA administration).

Platelet Aggregation: Platelet aggregability was assessed in freshly obtained whole blood, sampled from the inferior vena cava of DSS-treated and control rats and diluted 1:1 with PBS, using a Whole Blood Aggregometer (Chronolog Model 590D). Amplitude and slope of the resulting changes in impedance following addition of either collagen (2 µM) or adenosine diphosphate (5 µM) were evaluated using the Aggrolink software (Chronolog).

Statistical Analysis: Data from the acute study were analyzed with a one-way ANOVA followed by Dunnett's post hoc test. Comparisons between two groups, as in the chronic study were analyzed using an unpaired *t* test. All analyses were performed using GraphPad Prism (version 4, GraphPad Software, San Diego). All values are reported as means ± SEM. Statistical significance was set at $p < 0.05$. Values reported as fold-increases were calculated from the averages at a given time-point to corresponding time-matched controls, and their respective SEM through propagation of errors.

Results

Disease Assessment: The development of colitis was accompanied by reduced body weight gain until day 3 of DSS administration and an average weight loss thereafter, leading to body weights varying from -1% (day 5) and -9% (day 7) on DSS to -6% and -10% on days 2 and 4 off DSS compared to the weight at day 0. In agreement with published data [39-44], the DAI increased gradually over the time of DSS administration, reaching a maximum of 3.11 ± 0.05 on the last day of DSS administration (day 7), and subsequently decreased to 1.55 ± 0.17 at the 4th day after DSS cessation. The full DAI time course is shown in Fig.1. The induction and extent of colitis was further confirmed by blinded histological assessment, which showed inflammatory injury in the colon of DSS-treated animals including crypt damage, mucosal ulceration, loss of goblet cells, and submucosal edema. Significant leukocyte

recruitment to the colon was observed at day 7 of DSS administration, with MPO activity remaining increased on day 11 ($p<0.05$).

Colonic Nitrosative Stress and Systemic NO Metabolite Changes: Consistent with previous findings[1], the concentrations of nitroso and nitrosyl species present in blood and tissues at baseline (day 0) were found to be in the range of 10-100 pmoles/g wet tissue weight, with distinctly different ratios in different compartments. The major nitroso compounds present in plasma and in the colon were mercury-stable species (presumably RNNOs), whereas those in venous tissue were almost exclusively of the RSNO type (data not shown). In all other compartments a mixture of S- and N-nitroso products, along with nitrosyl heme species prevailed. A complex picture of changes in NO-related metabolites was apparent, both during administration and cessation of DSS (Fig.2). In the colon, DSS-induced inflammation was accompanied by a transient decrease in total nitros(yl)ation products (i.e. the sum of all S/N-nitroso and heme nitrosyl species), followed by an increase with peak concentrations on day 7 ($p<0.01$), coinciding with the peak in DAI, and a return to control levels by day 11 (Fig.1). These alterations were accompanied by dynamic changes in the ratio of individual colon nitroso and nitrosyl products with a shift from amine to thiol nitrosation products and the appearance of nitrosyl heme species on day 7 of DSS administration, indicative of a significant change in local NO production and metabolism. Overall, shifts in metabolite composition and concentration were organ-specific and most dramatic on days 1, 5 and 7 of DSS application, suggesting the involvement of multiple competing pathways affecting local NO metabolite profiles. Importantly, these changes were evident also in tissues other than the colon, revealing the systemic nature of DSS-induced inflammation.

Plasma and Colon Nitrite and Nitrate versus DAI: Plasma nitrite and nitrate exhibited a time course that paralleled the DAI up to and including day 2 following cessation of DSS (see Fig. 3A). By day 4 after DSS cessation, however, both anions experienced a resurgence in concentration, in sharp contrast to the DAI which continued to drop. The time course for changes in nitrite and nitrate concentrations in the colon is shown in Fig. 3B and contrasted in the same figure with the kinetics of the DAI. While nitrate exhibits a relatively small reduction during the course of the disease, nitrite experiences two surges, including a 2.5 fold-increase 3 days after DSS administration and a 2.2 fold-increase after its cessation. A considerable decline in colon nitrite is observed in the intervening time, particularly at day 2 after DSS cessation when levels of the anion dropped from a basal level of 1.4 μM to below our detection limit of about 0.01 μM. Thus, NOx species in the colon experience changes very distinct from those seen in plasma.

***Systemic Changes in Nitrite, Nitrate, Redox and Tissue Nitrosation Status*:** Nitrite concentrations in all organs studied exhibited changes remarkably similar to one another and to those observed in the colon (see Fig.4 for absolute changes at select time points and Fig.5 for fractional changes over the entire time course). These included (1) a general decline in nitrite, bottoming at levels below our detection limit at day 2 following DSS cessation, superimposed with (2) a spike at day 3 into the DSS treatment, and (3) a profound surge by day 4 after DSS cessation. In the course of these changes, the redox couple ascorbate/dehydroascorbate (Asc/DHA) remained steady except at day 4 after cessation of DSS administration, when a statistically significant redox change occurred, characterized by a decrease in ascorbate ($p<0.05$) and an increase in the corresponding oxidation product, DHA ($p<0.05$) (Fig. 5A). Increases in nitrite concentrations after DSS cessation correlated well ($R^2=0.89$, data not shown) with decreases in the logarithm of the AA/DHA ratio, strongly suggesting the existence of a redox-related link between the changes in ascorbate and nitrite. In contrast to the many-fold changes exhibited by nitrite and AA/DHA (Fig. 5A), and total ascorbate (not shown), nitrate remained largely unchanged throughout the acute phases of the disease (Fig. 5B). In all organs studied, tissue nitrosation levels (i.e., the concentrations of S- and N-nitroso products combined) exhibited remarkably similar changes in response to DSS administration (see Fig. 5C). Those trends included a moderate decline during the first 5 days of DSS administration, followed by a sudden rise on the last day of the treatment. The sudden rises in tissue nitrosation levels at day 7 are in stark contrast to the nitrite downward trends observed during that period in the same organs.

Platelet Aggregation during Acute Colitis: Irrespective of the test stimulus applied, platelet aggregability was found to be elevated both during acute DSS administration (Fig. 6) and following cessation (not shown), although the differences to untreated controls did not reach statistical significance. Given that a certain percentage of UC cases is associated with a hypercoagulable state we reasoned that the changes observed in the acute model may be indicative of a stage that precedes this state and may become more pronounced only in the chronic setting. To investigate this possibility a more chronic form of colitis was induced in a separate set of animals and the above measurements repeated.

Chronic Colitis Model: The chronic model of colitis yielded a DAI of 1.3 ± 0.1 compared to time-matched controls ($p<0.01$). Changes in absolute concentration of NO metabolites in blood and tissues are shown in Figure 7, and fractional changes from control levels are summarized for NO-metabolites and AA/DHA ratios in Table 2. As observed during DSS administration in the acute model, plasma

nitrite levels remained significantly elevated in the chronic model (mean fractional change of 2.26 over 11 days for the acute model versus 2.25 for the chronic model). In contrast to the changes in tissue nitrite concentrations observed during acute DSS administration, which dropped to levels below our detection limit, those levels remained either unchanged or increased in the chronic model. Tissue nitrosation also exhibited a general tendency toward systemic increases that was particularly evident in targets of the cardiovascular system (exemplified by effect on heart and aorta; see Fig.7 and Tab.2), whereas changes in cellular redox status and nitrate showed no clear systemic trends. While plasma nitrate in particular remained almost unchanged in the chronic model, platelet aggregation (Fig. 6) was elevated to levels comparable to those observed in the acute model, with differences reaching statistical significance when compared to age and time-matched controls.

5-ASA Treatment: Histological assessment of the colon in those animals that received 5-ASA between day 5 of DSS administration through to day 4 after removal from DSS revealed no measurable reduction in colon damage. However, 5-ASA treatment was accompanied by a reduction in DAI compared to animals receiving DSS alone (2.70 ± 0.07, day 7; 1.22 ± 0.40, day 11). The difference in DAI was not due to reduced weight loss, which was comparable between the 5-ASA treatment group and the DSS-treated control group that received only water in the recovery phase (-7% and -11% at days 9 and 11, respectively). Similarly, no changes in MPO activity were observed compared to animals treated with DSS alone. Given the short time the animals received 5-ASA treatment on top of DSS these results are perhaps not surprising. Unexpectedly, however, 5-ASA had a dramatic effect on NO-related products and redox status. Co-administration of 5-ASA together with DSS completely abolished the increased formation of nitrosated species to levels virtually indistinguishable from those of control animals (data not shown). Moreover, 5-ASA treatment largely prevented the drop in tissue nitrite concentrations at the height of DSS-induced inflammation and fully prevented the systemic increase in nitrite and oxidative stress observed in all compartments on the fourth day of recovery following DSS administration (Fig. 8). In addition to the abrogation of oxidative stress following DSS cessation a trend towards a more reduced status became apparent in several compartments with 5-ASA administration, in particular in the brain. The significance of this finding remains unclear at present. In contrast, blood nitrate levels remained unchanged with 5-ASA treatment compared to DSS administration alone (data not shown).

Discussion

The present study provides a first comprehensive assessment of the global dynamics of a series of NO-related metabolites during development, manifestation and remittance of DSS-induced colon inflammation in a rodent model in vivo. Our results reveal an unexpected level of complexity concerning the fate of NO and are at odds with the contemporary (and often indiscriminate) use of NO-related metabolites as universal markers of enhanced NOS activity which typically accompanies inflammatory events. These findings are of relevance not only for the monitoring of disease progression and for biomarker research, but may have important implications for the role of NO and nitrite in inflammation in general. The appreciation that iNOS induction during inflammation can be sometimes beneficial and on other occasions detrimental,[3, 45] depending in part on whether the enzyme is induced in blood cells or tissues,[46] does little to answer the question as to whether NO is protective or deleterious.[47, 48] This has created a situation, characterized as "NO paradox", that not only continues to confuse clinical and basic investigators but represents a significant impediment to research progress in this area. By addressing this situation from an analytical angle, our study contributes to a better understanding of the chemical biology of NO and its reaction products in the setting of gastrointestinal inflammation, an important prerequisite for defining novel treatment strategies aimed at modulating NO bioavailability. The demonstration that 5-ASA leads to almost complete normalization of the NO metabolite profile and oxidative stress-induced perturbations in tissues, even when administered well after colon inflammation is established, further suggests that the systems-based experimental paradigm used in the present investigation may hold promise for future mechanistic studies and drug discovery efforts.

NO Metabolites as Markers of Inflammation – Prospects and Limitations

While elevated NOS activity has been found in experimental rodent models of colon inflammation as well as in patients with Ulcerative Colitis and Crohn's Disease, the relationship between this activity and the severity of bowel inflammation remains ill defined.[49, 50] This may be due, in part, to the fact that most individuals from whom colon biopsy samples are obtained typically receive either corticosteroids or other anti-inflammatory medications all of which have the potential to interfere with NOS expression and activity. Information in the literature about iNOS expression in DSS colitis (in particular changes over time) is sparse, sometimes conflicting, and differs between species and strains. We opted not to determine message or protein expression for endothelial or inducible NOS in our present study. We reasoned that such measurements may be of little help in interpreting the changes in NO metabolite

patterns observed without concomitant information about true NOS activity (as opposed to a potential activity as measured by citrulline or oxyHb assay, which is typically determined in the presence of optimal cofactor and substrate supply), reactive oxygen species production and NO/nitrite consumption processes. While we could speculate about oxidative stress levels taking into account the ascorbate redox status measured, NO/nitrite consumption is likely to change dynamically as inflammation progresses or animals recover. Thus, all these processes have the potential to affect NO availability and metabolic profile even if NO production progressively increased or remained constant. Other complications include the multitude of reactions NO and its metabolites can undergo in a biological environment, the chemical instability of some of the reaction products and analytical challenges related to their quantification at trace level in biological samples. Sampling frequency and logistical challenges related to sample processing further limit the mechanistic insight one can gain from an analysis of clinical specimen in this particular area of research, emphasizing the need to address issues of greater complexity in animal models of disease such as the one employed in the present study.

Another formidable confounding factor is dietary intake. Rodent diets are known to contain varying amounts of NOx which, if not controlled for, may contribute to poor comparability between studies. Since stress and/or sickness are typically accompanied by reduced food intake progression of disease may translate into changes in bodily NOx supply and their concentration in tissues. Since accurate quantification of food consumption is only possible with individually housed animals (which itself is a stressor that may impact on the inflammatory process) weight gain is often used as a proxy for food intake. In agreement with other investigations a variable degree of weight loss was observed in DSS-treated animals in the present study. However, with regard to its relevance to NO metabolite concentration this data has to be interpreted with caution as loss in body weight may be due to changes in metabolism, extracellular fluid volume depletion and other factors unrelated to food intake.

Irrespective of these limitations, both nitrite and nitrate seem to track the DAI in our acute colitis model, with the exception of the sudden surge in nitrite/nitrate observed four days after DSS cessation (discussed below). In the chronic model, however, nitrate levels remained essentially unaltered (fractional change of 1.27 ± 0.19) while nitrite levels more than doubled (2.25 ± 0.31). The finding that nitrate increases significantly only in the acute model of inflammation suggests that plasma nitrite may be a better overall marker of inflammatory events. Consequently, any plasma assay that combines nitrite with nitrate into one single marker of NO synthesis (i.e. NOx assays) will not provide a more sensitive disease index since only nitrite appears to be changing consistently during inflammation. In fact, since

plasma nitrite concentrations are usually an order of magnitude lower than those of nitrate, any changes in nitrite may be overwhelmed by sampling errors in nitrate. Thus, our results suggest that plasma nitrite by itself may be better suited as a marker of inflammatory activity than assays that combine nitrite and nitrate values. However, caution is recommended in associating the time-course of this product too closely to the DAI, given our observation that its levels rebounded even as the DAI subsided (Fig 3A).

A surprising finding from our acute model is the extent of NO responses (Figs. 2-5) that occurs in the extravascular compartment. Our results clearly indicate that the levels of nitrite and nitroso products, but not nitrate, are significantly altered synchronously in all organs including the colon. Additionally, the data obtained in the chronic model (Figs. 7 and 8, Tab.2) indicate that the colon exhibits no particular pattern of NO-metabolites that would differentiate it significantly from other organs. Thus, given the similarity in changes in NO-metabolite profiles between the colon (where supposedly inflammation is confined to) and other organs, it appears that these species may not serve as markers suitable for confirmation or localization of inflammation in tissues, neither in the acute nor in the chronic stage.

Compartmentalization of Nitrite Between Intra- and Extravascular Space

Another surprising finding from our study is the degree of compartmentalization of nitrite between intra- and extravascular space. For instance, a salient feature in the nitrite signals from the colon and from other organs (Fig 5A) is the presence of two surges, at days 3 after initiation of DSS treatment and at day 4 following its withdrawal. The first nitrite surge which is prominent in the colon but also present in other tissues, may originate from the induction of iNOS in macrophages and other cells in those tissues. Interestingly, there is no evidence that this added nitrite diffuses into the intravascular compartment, which is surprising considering the relatively large volume of extravascular spaces that experience increases in nitrite. Further evidence of compartmentalization is seen in the dramatic loss in extravascular nitrite observed in the intervening time between the two surges. This loss occurs while levels of nitrite in the intravascular compartment continue to increase above basal values. Further evidence for nitrite compartmentalization can be gleaned from our 5-ASA data, which demonstrates that therapeutic administration of this drug selectively affects nitrite in the extravascular compartment.

The degree of compartmentalization between the extra- and intravascular compartments is surprising, given the established ability of this anion to cross cell membranes through ion channels and possibly also via passive transport in the form of nitrous acid (HNO_2)[51]. This level of compartmentalization is

reminiscent of those from another recent study[7]. In that particular study, animals sustained on a low NOx diet exhibited a systemic loss of nitrite from all tissues except the circulation where levels remained unchanged compared to those of animals on a normal diet. Thus, the low NOx diet also did not lead to a loss of tissue nitroso products when nitrite was lost. The similarities between the findings of that study with those reported here might be part of a common reaction channel yet to be characterized that is activated under certain physiological stresses. In the following section we elaborate further on the nature of this response and the difficulty in reconciling it with our current understanding of NO biochemistry in inflammation.

A Need to Re-Examine the Chemical Biology of NO and Nitrite in Inflammation

Most NO-metabolites found in vivo, including nitrite, nitrate, and nitrosation products are thought to arise primarily from the NO autoxidation pathway, in which NO and O_2 react to form NO_2, from which either N_2O_4 or N_2O_3 are formed:

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$2\ NO_2 \rightarrow N_2O_4 \quad (2)$$

$$NO + NO_2 \rightarrow N_2O_3 \quad (3)$$

These higher N-oxides can subsequently lead to the formation of NOx and nitroso products according to the following reactions:

$$N_2O_4 + RXH \rightarrow RXNO + NO_3^- + H^+ \quad (4)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (5)$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (6)$$

$$N_2O_3 + RXH \rightarrow RXNO + NO_2^- + H^+ \quad (7)$$

Thus, if inflammatory responses were associated with an increase in NO synthesis while the availability of other reactants (O_2, H_2O, RXH) was kept essentially constant, one would expect that levels of nitrite, nitrate, and nitroso species all would rise simultaneously in inflamed tissues to eventually end up in the circulation. However, this is not what we observe. Instead, nitrite levels in tissues drop even as

nitrosation in that compartment rises, while no appreciable changes in nitrate concentration are apparent. Furthermore, our data suggest that these products behave differently in the circulation and are not in equilibrium with their counterparts in tissues.

The departure from the chemistry outlined in reactions (1-7) might be attributed to a shift in NO-biochemistry driven by increased levels of a third reactant, namely superoxide (O_2^-). This oxygen-derived radical anion, which is not merely a by-product of aerobic respiration but fulfills important signaling functions[52], is produced at much higher levels during inflammatory responses. Superoxide reacts very rapidly with NO to form peroxynitrite:

$$O_2^- + NO \rightarrow ONOO^- \qquad (8)$$

Scavenging of NO by this reaction would reduce its bioavailability, leading to lower levels of nitrite from reactions (5-7). While a decrease in NO bioavailability might account for the lower levels of nitrite in tissues and the increased platelet aggregability in the circulation, much of the product peroxynitrite decays readily to form nitrate,

$$ONOO^- \rightarrow NO_3^- \qquad (9)$$

leading to what we should have registered as increased levels of nitrate in tissues. As clearly seen from Fig. 3B for the colon, and echoed in the data for other organs (Fig. 5B), nitrate either remains unchanged or even decreases somewhat during this period. This scenario also fails to account for the seemingly contradictory rise in tissue nitrosation (Fig. 5C) when NO bioavailability is presumably diminished. The significant rise in nitrite in the circulation observed at days 5 and 7 (Fig. 3A), at a time when all tissues supposedly produce much less of it (Figs. 4 and 5), also cannot be rationalized as a consequence of superoxide production.

The increased production of nitroso products at the peak of the disease activity invites consideration of a third scenario involving a recently proposed mechanism for thiol nitrosation via a radical mechanism[53]. In this mechanism, NO_2 produced via reaction 1 reacts directly with thiols to produce a thiyl radical, RS· via the following reaction:

$$NO_2 + RSH \rightarrow RS\cdot + NO_2^- + H^+ \qquad (10)$$

Once formed, the RS· radical can react very rapidly with NO to produce the nitroso product RSNO via a radical-radical reaction:

$$NO\cdot + RS\cdot \rightarrow + RSNO \qquad (11)$$

If inflammation somehow produces conditions that favor reactions (10-11) over reactions (2-7), the net result would be a chemical shift that produces less nitrate in favor of nitroso (and nitration) products and nitrite. The increased nitrite, however, is not consistent with the observed disappearance of this anion from tissues, including the colon.

The difficulty in reconciling current models of NO biochemistry with the observed dynamics of NO products in our model of acute colitis incites the question of whether additional mechanisms are intervening to modulate nitrite, nitrate, or nitroso products. For instance, several mechanisms have been proposed in the literature that could reduce nitrite to NO in vivo and generate nitroso products through the nitrosative chemistry described above. Mechanisms include interaction of nitrite with the mitochondrial electron transport chain, haemoglobin and myoglobin, xanthine oxidase, cytochrome P_{450}, and simple protonation to form nitrous acid[10]. In addition, nitrite may be consumed in reactions with peroxidases/H_2O_2, which are known to be present at high levels in inflamed tissues (and documented by MPO measurements in colon tissue in the present study), leading to the formation of nitrotyrosine, nitrated fatty acids and other nitration products[54, 55] which would go largely undetected in our assay systems. While these may readily account for the diminishing levels of nitrite we observe, they could only account for the increased nitrosation if we assumed the increased NO fluxes from nitrite reduction enhanced the nitrosative chemistry described above. However, that same nitrosative chemistry would inevitably yield increased levels of nitrate, a trend we do not observe. To complicate matter further, the action of peroxidase/H_2O_2 on nitrite gives rise to NO_2 to either form S-nitrosothiols via reactions (10-11) or nitrite via reactions (3, 5). Furthermore, the increased platelet aggregability we measured at the peak of the disease activity (i.e., at day 7 of DSS administration) would also argue against increased systemic NO bioavailability during the course of the disease, as NO is well known to inhibit platelet activation.

A possible way to resolve the apparent conflict between the formation of nitroso products without increased NO or other NO products, such as nitrate, is to consider an alternative mechanism that produces nitrosation from nitrite without the intermediacy of NO or nitrosative species such as N_2O_3. The existence of such a mechanism was indeed demonstrated in a recent study of the biochemical response of rat tissues to nitrite, both in vivo and in vitro[7]. This study found that tissue nitrosation was directly correlated with nitrite levels. A unique feature of this process, which involves a cooperative action between thiols and hemes, is its use of nitrite as a substrate to produce nitrosation products

without the intermediacy of NO. If the same mechanism was responsible for the nitrosation observed in the present study, our results would suggest that this may be part of an enzymatic process that is significantly enhanced in inflammation.

Links Between Nitrite, Ascorbate Status and the Pathology of Gut Inflammation

Many of the findings discussed above point to an unusual behavior of nitrite during the course of the acute inflammatory process, i.e. one that extends beyond a passive role as an inert end-product of NO formation. One of those findings relates to the extraordinary surge in the levels of this anion that take place on the fourth day after withdrawal of DSS, i.e. at a time when iNOS expression is supposedly no longer massively up-regulated. Importantly, this surge is observed in every organ examined, and appears to be correlated with a significant oxidative event in those organs as indicated by the drop in their AA/DHA ratio (Fig. 5A). The connection between nitrite and the redox status in those tissues is further strengthened by the relation between the drop in the log of the AA/DHA ratio and the magnitude of the nitrite surge (R^2=0.89). The response is particularly large in extra-colonic tissues such as the liver and kidney (Fig 5A), but its mechanism remains unclear at present. Dehydroascorbate formation is unlikely a consequence of direct chemical interaction between ascorbate and nitrite given that intracellular concentrations of the former exceed those of the latter by at least an order of magnitude in most tissues. Rather, it may originate from an impaired capacity of the mucosa to reduce DHA back to ascorbate, which has been observed in colonic biopsies from IBD patients[56]. Regardless of the underlying mechanism, an impairment of ascorbate-mediated antioxidative defense may render inflamed tissue more susceptible to oxidative damage. Moreover, these changes lead to an impaired availability of reduced ascorbate in tissues and may therefore affect hydroxylation processes and other reactions in which ascorbate is acting as a specific cofactor rather than an antioxidant.

Another curious connection between nitrite and acute colitis is gleaned from the results obtained during co-administration of 5-ASA with DSS. This drug is highly effective in the setting of acute inflammatory bowel disease in the clinic and has become the gold standard for the therapy of ulcerative colitis.[57] Its intestinal anti-inflammatory effects are believed to be mediated via interaction with the peroxisome proliferator-activated receptor-γ.[58] In the present study, no significant change in either weight gain or MPO activity was observed following 5-ASA administration, while intestinal bleeding and effects on stool consistency were clearly reduced. Yet, both circulating and tissue nitrite levels (Fig 8C) showed

substantial reversal toward basal levels, both at the peak of the DAI (day 7) and at day 4 after DSS withdrawal when nitrite would otherwise surge (Fig. 8A). While we cannot exclude the possibility that reduced food intake may contribute to the progressive decrease in tissue NOx levels once colitis is established, we do not believe that dietary changes can account for the dramatic surge in nitrite following cessation of DSS treatment for two reasons: 1. reduced body weight gain persisted well into the recovery phase; 2. there was no difference in weight loss after DSS cessation between the group that received only water and the one that received 5-ASA, although the surge in nitrite was completely abrogated in the latter. The prevention or reversal of the nitrite surge toward basal levels was accompanied by a notable attenuation of oxidative stress (compare Figs. 8B and 8D). It has been demonstrated that the p-amino phenol moiety of 5-ASA confers both potent antioxidant effects[59] and a reactive nitrogen oxide species scavenging ability[60] to the molecule. Both of these actions might work in concert to reduce nitrite accumulation and prevent the occurrence of oxidative and nitrosative stress. These results appear to be of particular relevance in the context of concerns that the nitrite produced during inflammatory conditions in the gut may enhance, via formation of carcinogenic N-nitrosamines, the risk for colon cancer[61, 62]. Of note, nitrite has recently been suggested to modulate T-cell function and cytokine release[63]. Given the significance of T-lymphocytes in the disease process[64] the avoidance of abrupt fluctuations in nitrite concentration may prove beneficial. Our biochemical findings with 5-ASA would seem to lend support to the contemporary interest in its broader use for chemoprevention of colorectal cancer, one of the more fearsome complications of IBD[65, 66].

Thus, parallels between the behavior of nitrite described above and known disease progression/ complications of colitis seem inescapable. Firstly, ulcerative colitis is a disease characterized by recurring cycles of acute illness and remission. Our study shows that an underlying product of the inflammatory process, nitrite, also undergoes considerable cycling throughout the disease, dipping to vanishingly low levels and suddenly rising to values many-fold above basal ones. Secondly, IBD is often accompanied by complications in distal organs, including the liver and the kidney, for reasons that are yet unclear[67]. Our findings show that those two organs are the main targets of nitrite overload and significant oxidative stress. Thirdly, our findings show that the drug 5-ASA, which is effective in the treatment of ulcerative colitis, also normalizes the levels of nitrite in the circulation and in the extravascular compartment. Together, these parallels raise the prospect that an intimate connection exists between nitrite and IBD. Whether this relationship is causal or consequential and whether nitrite plays a beneficial or detrimental role in gut inflammation remains to be determined.

Chronic gastrointestinal inflammation is accompanied by specific alterations in oxygen supply and utilization as well as increased tissue metabolism, rendering inflamed mucosal tissue hypoxic.[68] Tissue hypoxia leads to activation of the hypoxia-responsive transcription factor HIF-1α, and this response is subject to redox modulation by the combined production of NO and reactive oxygen species.[69] Whilst mitochondrial reactive oxygen species production is enhanced in hypoxia,[70] mitochondrial NO consumption is reduced,[71] with important consequences for local NO availability and redox signaling. Hypoxia is linked to inflammation via the transcription factor, NF-κB.[72] Ascorbate is a cofactor of prolyl hyroxylases, and inhibition of HIF hydroxylases has been shown to be protective in rodent models of colitis.[68] Thus, changes in cellular redox status secondary to alterations in NO and ascorbate availability may affect hypoxic signalling and key inflammatory mediators in a complex fashion via several different angles. Nitrite is known to be consumed by mammalian tissues under hypoxic conditions,[1] with NO being produced along the process.[10] The formation of hydrogen sulphide (H_2S), produced in the gut by commensal sulfate-reducing bacteria or in tissues by mammalian enzymatic processes, has been suspected to be involved in the etiology of IBD, possibly as a result of impaired detoxification.[73] Of note, H_2S formation is markedly upregulated in inflammation and known to affect NO availability.[74] It is possible, therefore, that this cross-talk between mediators of inflammatory and hypoxic processes is sufficient to explain the fluctuations in nitrite observed in the present study, and tissue NO metabolite profiling an informative tool to study this cross-talk *in vivo*. Given the effectiveness of 5-ASA to prevent and/or abolish the alterations in NO-related metabolite levels and redox status, this systems cross-talk would seem to deserve further investigation.

Conclusions

This study investigated the formation and fate of nitrite, nitrate, and nitroso products, following the induction of colitis in a rodent model of colitis *in vivo*. Our main findings are summarized as follows:

1) Plasma nitrite and nitrate correlate well with disease activity, but only in the acute case and during the onset of the inflammatory response. In the recovery phase, both plasma anions rise again to very high levels 4 days after withdrawal of the inflammatory stimulus, even when disease activity continues to fall. In the chronic stage, nitrite shows a significant increase in plasma whereas nitrate remains essentially unchanged. Our findings suggest that plasma nitrite by itself provides a more consistent indication of colitis, although rises of this indicator may not be synchronized with either

progression or resolution of inflammation.

2) In the extravascular space during acute colitis, only nitroso products correlate well with disease activity. This response, however, seems to be systemic in nature inasmuch as qualitatively identical transient rises in nitroso products are also seen in other tissues. At the same time nitrite levels in all organs, including the colon, actually drop as the disease intensifies, while nitrate remains essentially unchanged. Thus, none of these NO-related products appear to be suitable as biomarkers of inflammation in tissues.

3) Our assessment of NO metabolite profiles during inflammation reveals dynamical features of NO biochemistry that cannot be fully rationalized in terms of classical oxidative and nitrosative textbook chemistry. In particular, the dynamics of nitrite and its compartmentalization between intravascular and extravascular spaces seems to imply that this anion is under the control of yet unidentified mechanisms that are activated as part of the inflammatory response. A potential major factor in this context may be the degree of tissue hypoxia accompanying the inflammatory process, and this relationship deserves further investigation. In any case, our systems analysis of individual NO-related metabolites *in vivo* has unmasked a richer complexity of NO biochemistry as previously appreciated from analyses of NOx levels in blood alone.

4) Finally, our study brings to light the destabilizing effect that colon inflammation has on the redox state and on nitrite levels in other tissues. The level of oxidative stress appears to be particularly significant in the liver and kidneys, organs well known to suffer from complications of IBD. This destabilizing effect was effectively abolished by the therapeutic drug 5-ASA, suggesting that nitrite may play a key role in the modulation of inflammation.

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Figure legends

Figure 1.

Disease activity index (DAI) and concentration of total nitroso compounds in the colon of DSS-treated rats. Data displayed as mean ± SEM, n=3/day. DAI was significantly increased from day 3 of DSS application on and remained significantly ($p<0.01$) elevated 4 days after cessation of DSS administration (asterisks omitted for sake of clarity). Colon nitrosation was significantly (** $p<0.01$) increased on day 7 of DSS administration.

Figure 2.

Systemic changes in concentration of NO-related metabolites in blood and exemplary tissues of the rat during development of acute colon inflammation during DSS administration (days 0-7) and following cessation of DSS treatment (recovery phase; days 9-11). Nitros(yl)lation products are exemplified by S-nitrosothiols (white), N-nitrosamines (black) and nitrosyl heme species (grey), whereas nitrate (black) and nitrite (grey) represent products of oxidative NO metabolism (NOx). Data are means ± SEM; n=3/day/tissue.

Figure 3.

Inflammatory response to continuous administration of DSS over a period of 7 days (area shaded in grey). The blue and green curves contrast the time course of nitrite and nitrate, respectively, with that of the DAI, in plasma (panel A) and in the colon (panel B). Each time point represents a fold-change from basal levels, calculated from the ratio of the mean concentration (n=3) at the day indicated on the X axis compared to the basal level (n=3) assessed at day 0 (Controls). Error bars were omitted for clarity.

Figure 4.

Blood and tissue concentration of nitrite in controls (white bars, day 0), following 7 days DSS treatment (grey bars, day 7) and four days after treatment cessation (black bars, day 11). Data displayed as mean ± SEM, n=3/day/tissue. Where * represents $p<0.05$ and ** represents $p<0.01$ vs. matched control (day 0). (IVC, inferior vena cava; RBC, red blood cells).

Figure 5.

Comparisons of the changes in redox status (expressed as AA/DHA ratio; panel A) as well as in the

concentrations of nitrate (panel B) and nitroso products (panel C) with those in nitrite (solid curves) across various organs and in the circulation, in response to DSS administration over a course of 7 days followed by 4 days of recovery. Each time point represents a fold-change from basal levels, calculated from the ratio of the mean concentration (n=3) at the day indicated on the X axis compared to the basal level (n=3) assessed at day 0 (Controls). Error bars were omitted for clarity. Aortic AA/DHA levels were not determined due to limited sample size.

Figure 6.

Platelet aggregation as assessed by addition of collagen and adenosine diphosphate (ADP) to whole blood under control conditions (white bars, n=6) and following 7 days of DSS administration (grey bars, n=4), and in the chronic model of colon inflammation (white hatched bars, n=9) with time-matched controls (grey lined bars, n=4). Data displayed as mean ± SEM (* $p<0.05$, ** $p<0.01$ vs. control).

Figure 7.

Systemic changes in concentration of NO-related metabolites in blood and representative organs during chronic colon inflammation. Nitros(yl)lation products are exemplified by S-nitrosothiols (white), N-nitrosamines (black) and nitrosyl heme species (grey), whereas nitrate (black) and nitrite (grey) represent products of oxidative NO metabolism (NOx). Data are means ± SEM, n=5-6/day/tissue.

Figure 8.

Comparison of changes in nitrite concentration and ascorbate redox status in blood and tissues of DSS-treated animals in the absence (panels A and B) and presence (panels C and D) of 5-ASA. Upper panel: (A) Blood and tissue concentrations of nitrite and (B) tissue concentrations of ascorbate and dehydroascorbate (DHA; displayed as a ratio of ascorbate/DHA) in controls on day 0 (white bars), day 7 (grey bars) and day 11 (black bars). Lower panel: (C) Blood and tissue concentrations of nitrite and (D) tissue concentrations of ascorbate and dehydroascorbate (DHA; displayed as a ratio of ascorbate/DHA) on day 0 (white bars), day 7 (grey bars) and day 11 (black bars) of DSS administration with concomitant 5-ASA treatment starting on day 5. Data displayed as mean ± SEM from 3 animals/day (* $p<0.05$). IVC, inferior vena cava; RBC, red blood cells. No ascorbate/DHA measurements were performed in vascular tissue and blood due to limited sample availability.

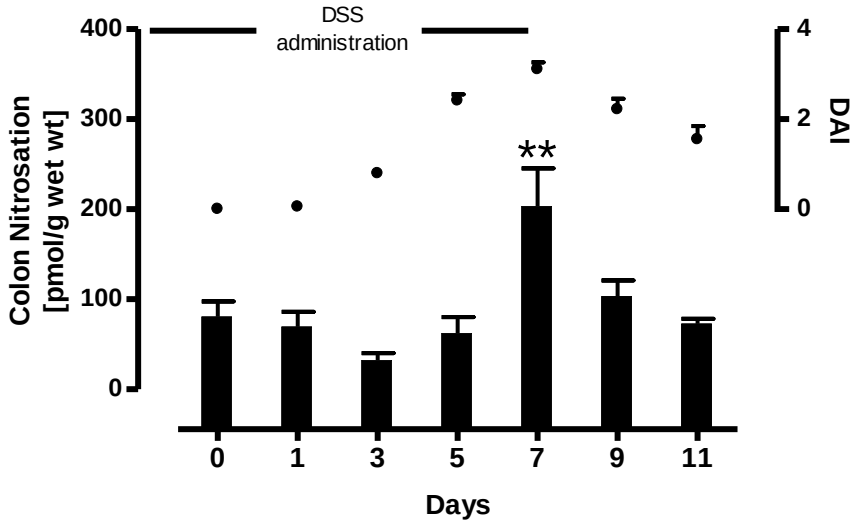
DSS
administration
Colon Nitrosation
[pmol/g wet wt]
400
300
200
100
0
**
4
2
0
DAI
0
1
3
5
7
9
11
Days

Figure 1

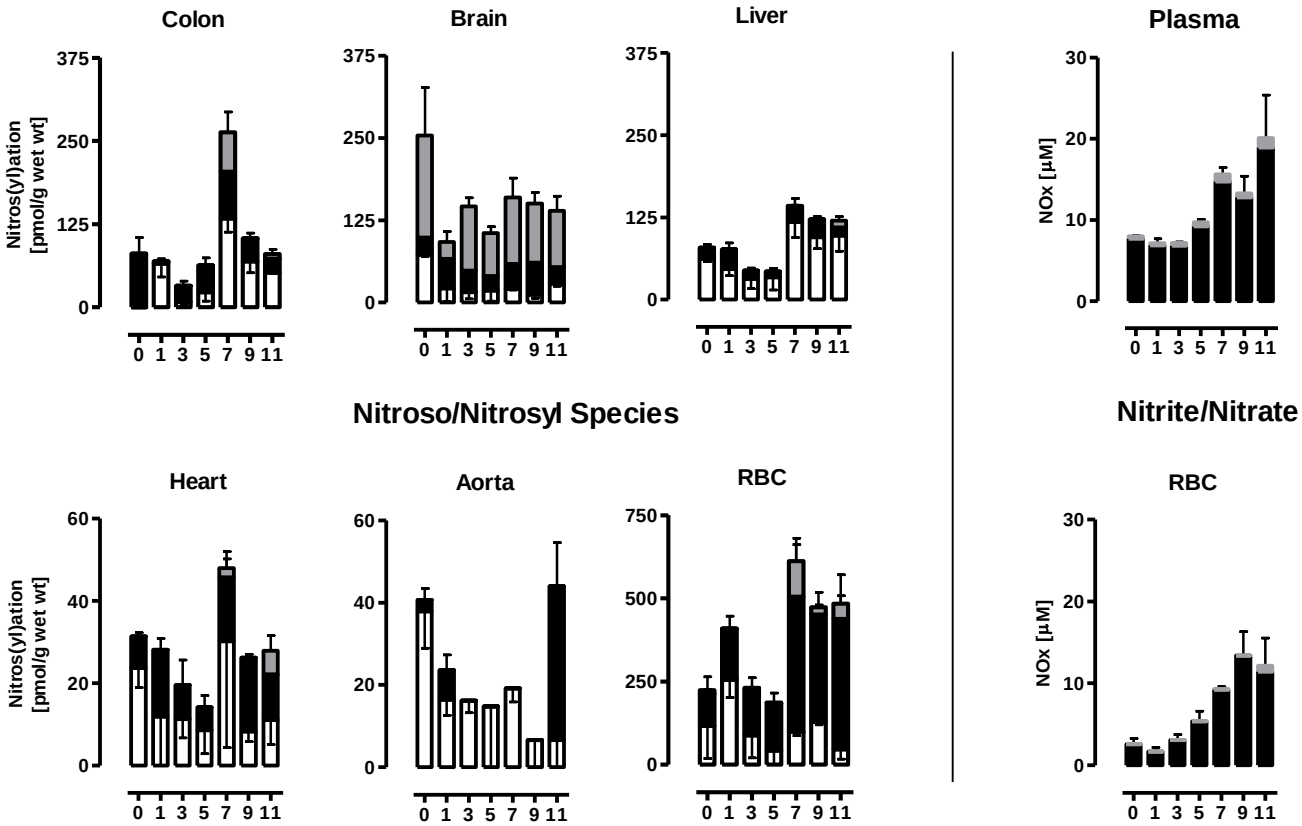
Colon
Brain
Liver
Plasma
Nitros(yl)ation [pmol/g wet wt]
NOx [µM]
Nitroso/Nitrosyl Species
Nitrite/Nitrate
Heart
Aorta
RBC
RBC
0 1 3 5 7 9 11

Figure 2

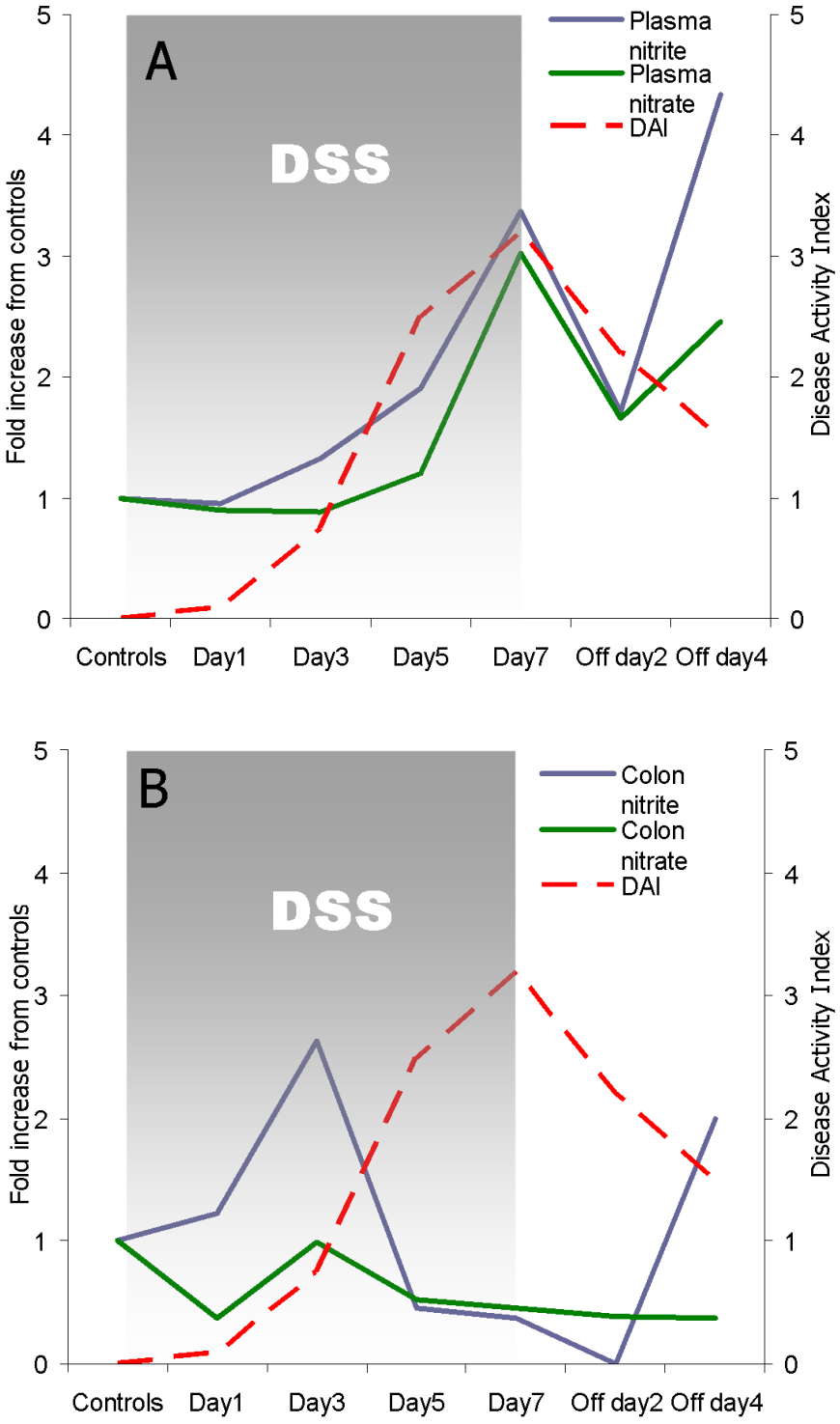
A
DSS
Plasma nitrite
Plasma nitrate
DAI
Fold increase from controls
Disease Activity Index
0
1
2
3
4
5
Controls
Day1
Day3
Day5
Day7
Off day2
Off day4
B
DSS
Colon nitrite
Colon nitrate
DAI
Fold increase from controls
Disease Activity Index
Controls
Day1
Day3
Day5
Day7
Off day2
Off day4

Figure 3

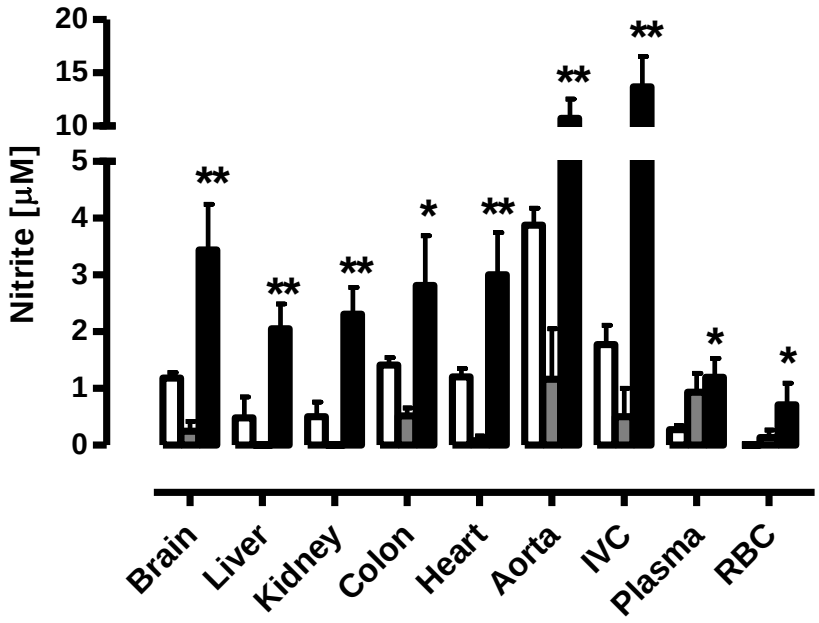
Nitrite [μM]
20
15
10
5
4
3
2
1
0
**
**
**
*
**
**
**
*
*
Brain
Liver
Kidney
Colon
Heart
Aorta
IVC
Plasma
RBC

Figure 4

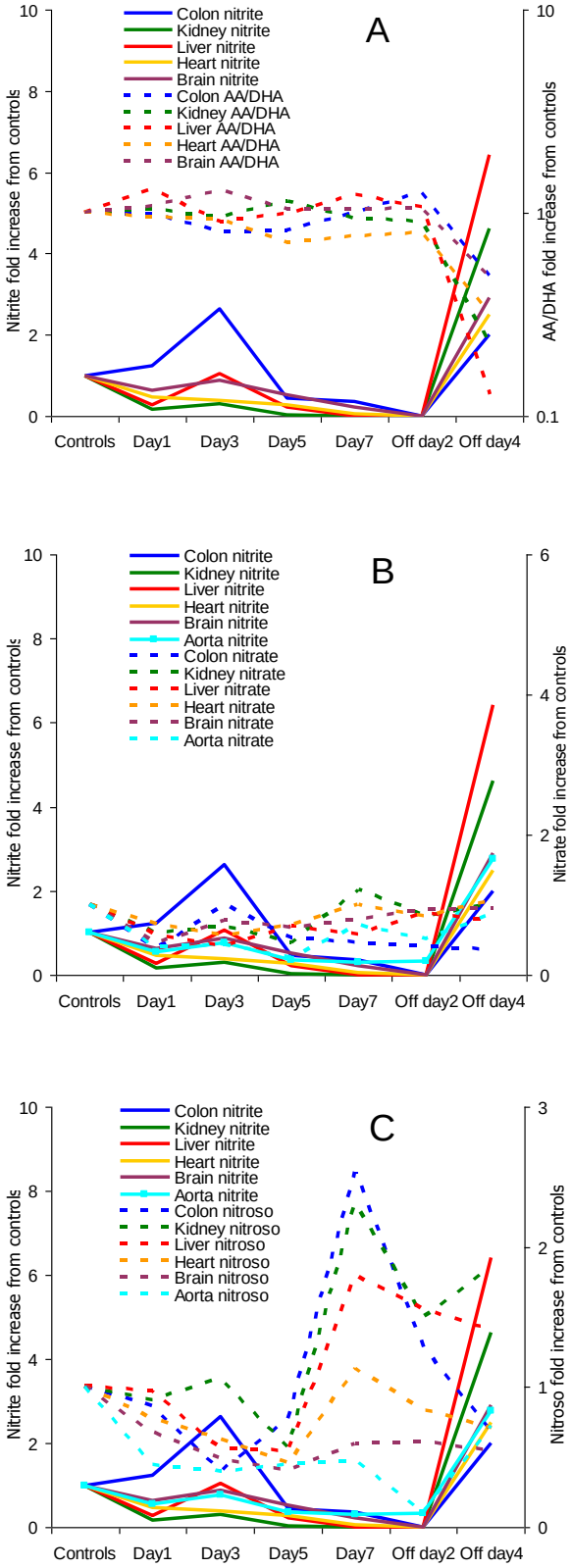
A
Colon nitrite
Kidney nitrite
Liver nitrite
Heart nitrite
Brain nitrite
Colon AA/DHA
Kidney AA/DHA
Liver AA/DHA
Heart AA/DHA
Brain AA/DHA
Nitrite fold increase from controls
AA/DHA fold increase from controls
Controls Day1 Day3 Day5 Day7 Off day2 Off day4
B
Colon nitrite
Kidney nitrite
Liver nitrite
Heart nitrite
Brain nitrite
Aorta nitrite
Colon nitrate
Kidney nitrate
Liver nitrate
Heart nitrate
Brain nitrate
Aorta nitrate
Nitrite fold increase from controls
Nitrate fold increase from controls
Controls Day1 Day3 Day5 Day7 Off day2 Off day4
C
Colon nitrite
Kidney nitrite
Liver nitrite
Heart nitrite
Brain nitrite
Aorta nitrite
Colon nitroso
Kidney nitroso
Liver nitroso
Heart nitroso
Brain nitroso
Aorta nitroso
Nitrite fold increase from controls
Nitroso fold increase from controls
Controls Day1 Day3 Day5 Day7 Off day2 Off day4

Figure 5

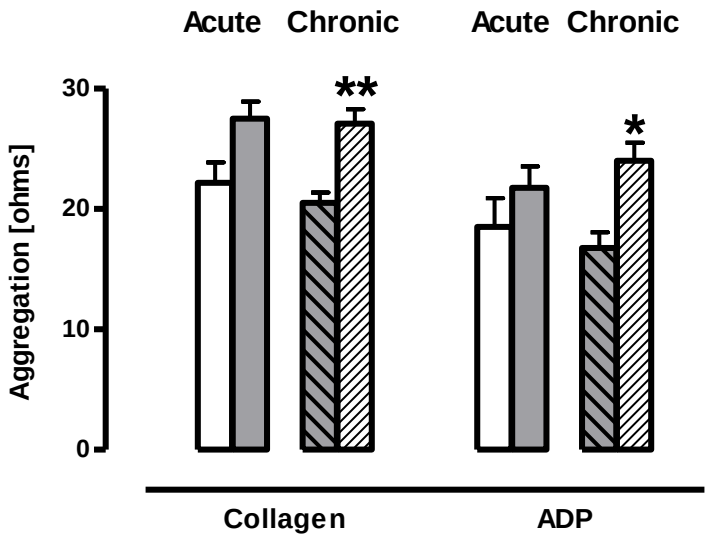
Acute
Chronic
Acute
Chronic
30
20
10
0
Aggregation [ohms]
**
*
Collagen
ADP

Figure 6

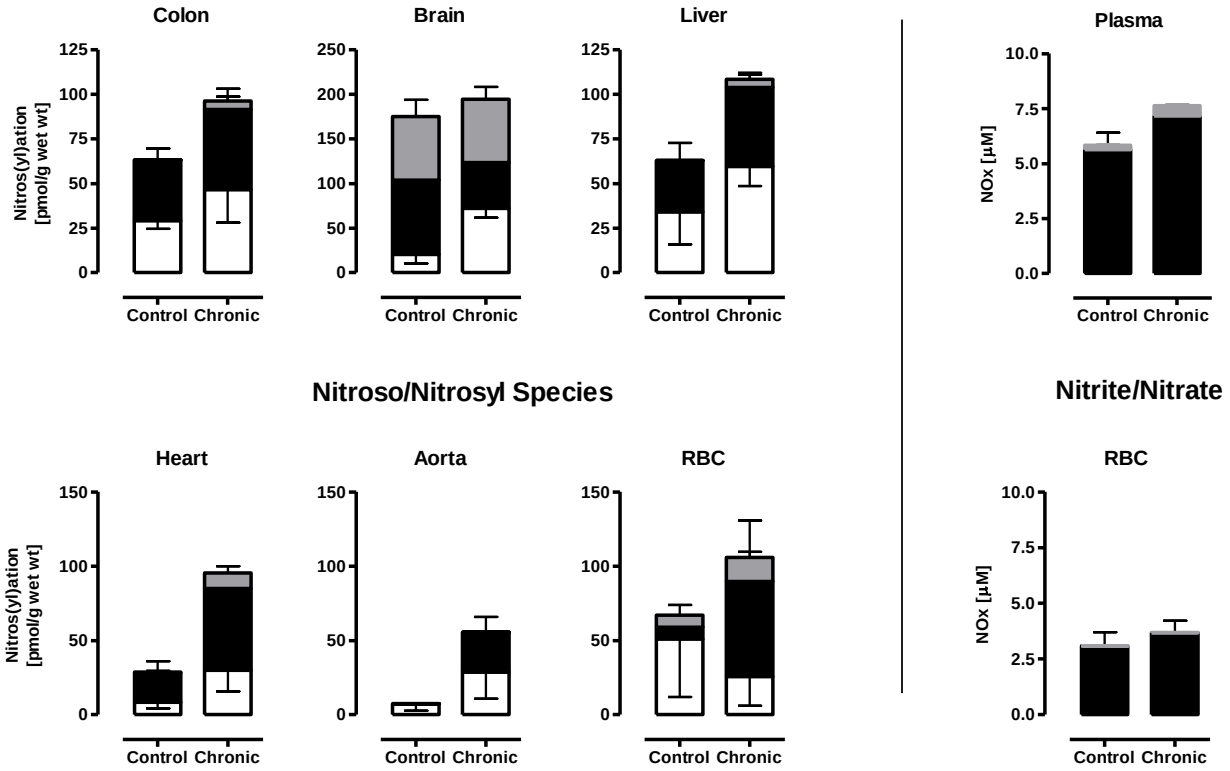
Colon
Brain
Liver
Plasma
Nitros(yl)ation [pmol/g wet wt]
NOx [µM]
Control Chronic
Nitroso/Nitrosyl Species
Nitrite/Nitrate
Heart
Aorta
RBC
RBC

Figure 7

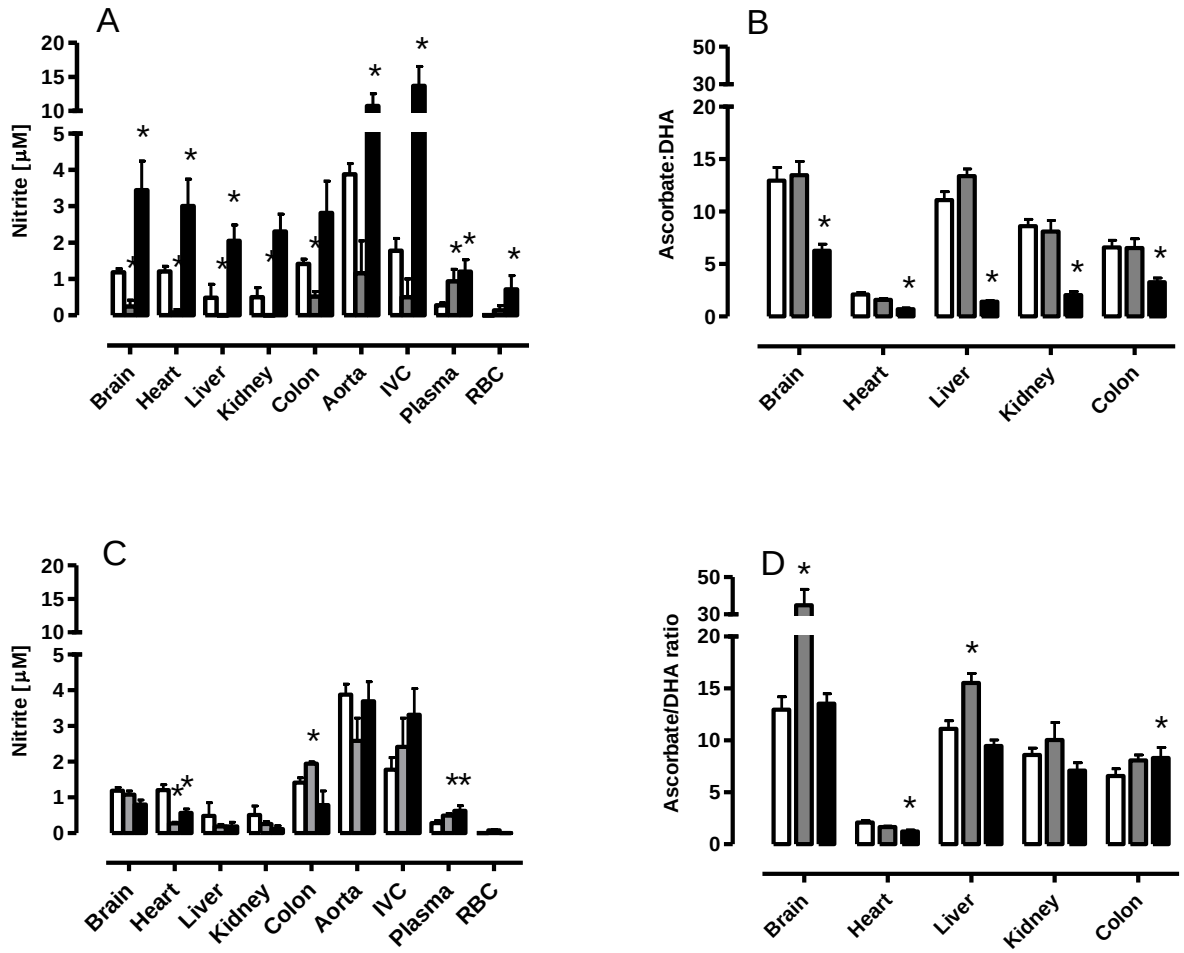
A
Nitrite [μM]
Brain
Heart
Liver
Kidney
Colon
Aorta
IVC
Plasma
RBC
B
Ascorbate:DHA
C
Nitrite [μM]
D
Ascorbate/DHA ratio

Figure 8

Table legends

Table 1.

Basal concentrations of nitrite, nitrate, nitroso, and nitrosyl species in plasma and tissues of Wistar rats enrolled in the acute (grey shaded) and chronic (unshaded) model of colitis (mean ± SEM; n=3 for acute study, time-matched to day 7 of DSS administration; n=5-6 for chronic study, corresponding to end of the treatment cycle).

Table 2.

Global changes of NO-related metabolites in plasma and representative organs during chronic colon inflammation. Fractional changes in nitrite, nitrate and total nitroso products (RXNO) and redox status (expressed as the ratio of ascorbic acid/dehydroascorbate, AA/DHA) in rats subjected to three cycles of DSS treatment compared to age- and weight-matched controls. Data displayed as mean ± SEM from n=5-6 animals. ND not determined.

	Nitrite, μM	Nitrate, μM	RSNO, nM	RNNO, nM	NO-heme, nM
Plasma	0.28 ± 0.07	7.70 ± 0.40	0.37 ± 0.37	11.83 ± 0.58	<1.0
	0.21 ± 0.01	5.63 ± 0.77	0.98 ± 0.87	13.84 ± 7.20	<1.0
Brain	1.18 ± 0.10	8.05 ± 0.53	72.47 ± 2.82	26.40 ± 5.61	154.83 ± 73.25
	0.61 ± 0.14	4.96 ± 1.03	20.56 ± 10.11	83.02 ± 31.84	71.64 ± 18.72
Heart	1.21 ± 0.14	10.66 ± 1.92	23.73 ± 4.77	7.67 ± 0.97	<1.0
	0.28 ± 0.08	3.87 ± 0.83	8.45 ± 4.17	19.66 ± 7.86	<1.0
Liver	0.32 ± 0.27	7.21 ± 1.43	61.7 ± 4.2	16.67 ± 4.87	<1.0
	0.02 ± 0.01	1.65 ± 0.25	34.04 ± 18.08	28.83 ± 9.80	<1.0
Kidney	0.50 ± 0.26	7.71 ± 0.91	24.57 ± 9.14	8.83 ± 3.34	<1.0
	0.08 ± 0.04	3.14 ± 0.65	19.8 ± 5.25	6.00 ± 2.62	<1.0
Colon	1.41 ± 0.14	23.79 ± 8.33	0.20 ± 0.05	80.57 ± 24.57	<1.0
	1.25 ± 0.22	5.31 ± 0.88	29.18 ± 4.48	33.96 ± 6.46	<1.0
Aorta	3.88 ± 0.29	24.22 ± 12.5	37.93 ± 9.02	2.77 ± 2.77	<1.0
	3.27 ± 8.3	6.31 ± 0.52	7.46 ± 4.73	<1.0	<1.0

Table 1

	Nitrite	Nitrate	Total RXNO	AA/DHA
Plasma	2.25 ± 0.31	1.27 ± 0.19	1.05 ± 0.67	ND
Brain	1.51 ± 0.42	0.77 ± 0.20	1.19 ± 0.29	0.98 ± 0.08
Heart	1.73 ± 0.57	2.03 ± 0.46	3.96 ± 1.69	0.46 ± 0.19
Liver	4.77 ± 2.99	1.14 ± 0.46	2.28 ± 0.79	0.45 ± 0.16
Kidney	1.46 ± 1.00	0.88 ± 0.24	1.44 ± 0.30	0.62 ± 0.13
Colon	1.24 ± 0.28	0.85 ± 0.20	1.44 ± 0.43	1.41± 0.27
Aorta	1.26 ± 0.35	1.14 ± 0.45	7.45 ± 5.34	ND

Table 2

References

1. Bryan, N. S.; Rassaf, T.; Maloney, R. E.; Rodriguez, C. M.; Saijo, F.; Rodriguez, J. R.; Feelisch, M. Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. *Proc Natl Acad Sci U S A*;**101**:4308-13;2004.
2. Stuehr, D. J.; Griffith, O. W. Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol*;**65**:287-346;1992.
3. Kolios, G.; Valatas, V.; Ward, S. G. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology*;**113**:427-37;2004.
4. Tsikas, D.; Gutzki, F. M.; Stichtenoth, D. O. Circulating and excretory nitrite and nitrate as indicators of nitric oxide synthesis in humans: methods of analysis. *Eur J Clin Pharmacol*;**62 Suppl 13**:51-9;2006.
5. Guevara, I.; Iwanejko, J.; Dembinska-Kiec, A.; Pankiewicz, J.; Wanat, A.; Anna, P.; Golabek, I.; Bartus, S.; Malczewska-Malec, M.; Szczudlik, A. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin Chim Acta*;**274**:177-88;1998.
6. Wang, J.; Brown, M. A.; Tam, S. H.; Chan, M. C.; Whitworth, J. A. Effects of diet on measurement of nitric oxide metabolites. *Clin Exp Pharmacol Physiol*;**24**:418-20;1997.
7. Bryan, N. S.; Fernandez, B. O.; Bauer, S. M.; Garcia-Saura, M. F.; Milsom, A. B.; Rassaf, T.; Maloney, R. E.; Bharti, A.; Rodriguez, J.; Feelisch, M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol*;**1**:290-7;2005.
8. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* **7**:156-67; 2008.
9. Butler AR, Feelisch M. Therapeutic uses of inorganic nitrite and nitrate: from the past to the future. *Circulation*. **117**:2151-9; 2008.
10. van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J, Gladwin MT. Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev*. **29**:683-741; 2009
11. Angelo, M.; Singel, D. J.; Stamler, J. S. An S-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate. *Proc Natl Acad Sci U S A*;**103**:8366-71;2006.
12. Calvert J.W., Lefer D.J. Myocardial protection by nitrite. *Cardiovasc Res.* 83(2):195-203; 2009.
13. Kleinbongard, P.; Dejam, A.; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Godecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G. A.; Bryan, N. S.; Feelisch, M.; Kelm, M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med*;**35**:790-6;2003.
14. Andoh, A.; Yoshida, T.; Yagi, Y.; Bamba, S.; Hata, K.; Tsujikawa, T.; Kitoh, K.; Sasaki, M.; Fujiyama, Y. Increased aggregation response of platelets in patients with inflammatory bowel disease. *J Gastroenterol*;**41**:47-54;2006.

15. Danese, S.; Motte Cd Cde, L.; Fiocchi, C. Platelets in inflammatory bowel disease: clinical, pathogenic, and therapeutic implications. *Am J Gastroenterol*;**99**:938-45;2004.
16. Collins, C. E.; Cahill, M. R.; Newland, A. C.; Rampton, D. S. Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology*;**106**:840-5;1994.
17. Kapsoritakis, A. N.; Koukourakis, M. I.; Sfiridaki, A.; Potamianos, S. P.; Kosmadaki, M. G.; Koutroubakis, I. E.; Kouroumalis, E. A. Mean platelet volume: a useful marker of inflammatory bowel disease activity. *Am J Gastroenterol*;**96**:776-81;2001.
18. Miehsler, W.; Reinisch, W.; Valic, E.; Osterode, W.; Tillinger, W.; Feichtenschlager, T.; Grisar, J.; Machold, K.; Scholz, S.; Vogelsang, H.; Novacek, G. Is inflammatory bowel disease an independent and disease specific risk factor for thromboembolism? *Gut*;**53**:542-8;2004.
19. Quera, R.; Shanahan, F. Thromboembolism--an important manifestation of inflammatory bowel disease. *Am J Gastroenterol*;**99**:1971-3;2004.
20. Solem, C. A.; Loftus, E. V.; Tremaine, W. J.; Sandborn, W. J. Venous thromboembolism in inflammatory bowel disease. *Am J Gastroenterol*;**99**:97-101;2004.
21. Talbot, R. W.; Heppell, J.; Dozois, R. R.; Beart, R. W., Jr. Vascular complications of inflammatory bowel disease. *Mayo Clin Proc*;**61**:140-5;1986.
22. Dijkstra, G.; Moshage, H.; van Dullemen, H. M.; de Jager-Krikken, A.; Tiebosch, A. T.; Kleibeuker, J. H.; Jansen, P. L.; van Goor, H. Expression of nitric oxide synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. *J Pathol*;**186**:416-21;1998.
23. Godkin, A. J.; De Belder, A. J.; Villa, L.; Wong, A.; Beesley, J. E.; Kane, S. P.; Martin, J. F. Expression of nitric oxide synthase in ulcerative colitis. *Eur J Clin Invest*;**26**:867-72;1996.
24. Herulf, M.; Ljung, T.; Hellstrom, P. M.; Weitzberg, E.; Lundberg, J. O. Increased luminal nitric oxide in inflammatory bowel disease as shown with a novel minimally invasive method. *Scand J Gastroenterol*;**33**:164-9;1998.
25. Ikeda, I.; Kasajima, T.; Ishiyama, S.; Shimojo, T.; Takeo, Y.; Nishikawa, T.; Kameoka, S.; Hiroe, M.; Mitsunaga, A. Distribution of inducible nitric oxide synthase in ulcerative colitis. *Am J Gastroenterol*;**92**:1339-41;1997.
26. Kimura, H.; Hokari, R.; Miura, S.; Shigematsu, T.; Hirokawa, M.; Akiba, Y.; Kurose, I.; Higuchi, H.; Fujimori, H.; Tsuzuki, Y.; Serizawa, H.; Ishii, H. Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. *Gut*;**42**:180-7;1998.
27. Lundberg, J. O.; Weitzberg, E.; Lundberg, J. M.; Alving, K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut*;**35**:1543-6;1994.
28. Rachmilewitz, D.; Stamler, J. S.; Bachwich, D.; Karmeli, F.; Ackerman, Z.; Podolsky, D. K. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut*;**36**:718-23;1995.
29. Reynolds, P. D.; Middleton, S. J.; Hansford, G. M.; Hunter, J. O. Confirmation of nitric oxide

synthesis in active ulcerative colitis by infra-red diode laser spectroscopy. *Eur J Gastroenterol Hepatol*;**9**:463-6;1997.

30. Singer, II; Kawka, D. W.; Scott, S.; Weidner, J. R.; Mumford, R. A.; Riehl, T. E.; Stenson, W. F. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology*;**111**:871-85;1996.
31. Okayasu, I.; Hatakeyama, S.; Yamada, M.; Ohkusa, T.; Inagaki, Y.; Nakaya, R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology*;**98**:694-702;1990.
32. Cooper, H. S.; Murthy, S. N.; Shah, R. S.; Sedergran, D. J. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest*;**69**:238-49;1993.
33. Onderdonk, A. B.; Bartlett, J. G. Bacteriological studies of experimental ulcerative colitis. *Am J Clin Nutr*;**32**:258-65;1979.
34. Krawisz, J. E.; Sharon, P.; Stenson, W. F. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology*;**87**:1344-50;1984.
35. Vowinkel, T.; Kalogeris, T. J.; Mori, M.; Krieglstein, C. F.; Granger, D. N. Impact of dextran sulfate sodium load on the severity of inflammation in experimental colitis. *Dig Dis Sci*;**49**:556-64;2004.
36. Feelisch, M.; Rassaf, T.; Mnaimneh, S.; Singh, N.; Bryan, N. S.; Jourd'Heuil, D.; Kelm, M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *Faseb J*;**16**:1775-85;2002.
37. Carr, R. S.; Bally, M. B.; Thomas, P.; Neff, J. M. Comparison of methods for determination of ascorbic acid in animal tissues. *Anal Chem*;**55**:1229-32;1983.
38. Terada, M.; Watanabe, Y.; Kunitomo, M.; Hayashi, E. Differential rapid analysis of ascorbic acid and ascorbic acid 2-sulfate by dinitrophenylhydrazine method. *Anal Biochem*;**84**:604-8;1978.
39. Kihara, N.; de la Fuente, S. G.; Fujino, K.; Takahashi, T.; Pappas, T. N.; Mantyh, C. R. Vanilloid receptor-1 containing primary sensory neurones mediate dextran sulphate sodium induced colitis in rats. *Gut*;**52**:713-9;2003.
40. Murthy, S.; Cooper, H. S.; Yoshitake, H.; Meyer, C.; Meyer, C. J.; Murthy, N. S. Combination therapy of pentoxifylline and TNFalpha monoclonal antibody in dextran sulphate-induced mouse colitis. *Aliment Pharmacol Ther*;**13**:251-60;1999.
41. Stucchi, A. F.; Shofer, S.; Leeman, S.; Materne, O.; Beer, E.; McClung, J.; Shebani, K.; Moore, F.; O'Brien, M.; Becker, J. M. NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats. *Am J Physiol Gastrointest Liver Physiol*;**279**:G1298-306;2000.
42. Ahn, B. O.; Ko, K. H.; Oh, T. Y.; Cho, H.; Kim, W. B.; Lee, K. J.; Cho, S. W.; Hahm, K. B. Efficacy of use of colonoscopy in dextran sulfate sodium induced ulcerative colitis in rats: the

evaluation of the effects of antioxidant by colonoscopy. *Int J Colorectal Dis*;**16**:174-81;2001.

43. Togawa, J.; Nagase, H.; Tanaka, K.; Inamori, M.; Nakajima, A.; Ueno, N.; Saito, T.; Sekihara, H. Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. *J Gastroenterol Hepatol*;**17**:1291-8;2002.
44. Osman, N.; Adawi, D.; Ahrne, S.; Jeppsson, B.; Molin, G. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of Lactobacillus and Bifidobacterium. *Dig Dis Sci*;**49**:320-7;2004.
45. Billiar, T. R.; Harbrecht, B. G. Resolving the nitric oxide paradox in acute tissue damage. *Gastroenterology*;**113**:1405-7;1997.
46. Krieglstein, C. F.; Anthoni, C.; Cerwinka, W. H.; Stokes, K. Y.; Russell, J.; Grisham, M. B.; Granger, D. N. Role of blood- and tissue-associated inducible nitric-oxide synthase in colonic inflammation. *Am J Pathol*;**170**:490-6;2007.
47. Rumi G, Tsubouchi R, Nishio H, Kato S, Mózsik G, Takeuchi K. Dual role of endogenous nitric oxide in development of dextran sodium sulfate-induced colitis in rats. *J Physiol Pharmacol.* **55**:823-36; 2004.
48. Aoi Y, Terashima S, Ogura M, Nishio H, Kato S, Takeuchi K. Roles of nitric oxide (NO) and NO synthases in healing of dextran sulfate sodium-induced rat colitis. *J Physiol Pharmacol.* **59**:315-36, 2008.
49. Kimura, H.; Miura, S.; Shigematsu, T.; Ohkubo, N.; Tsuzuki, Y.; Kurose, I.; Higuchi, H.; Akiba, Y.; Hokari, R.; Hirokawa, M.; Serizawa, H.; Ishii, H. Increased nitric oxide production and inducible nitric oxide synthase activity in colonic mucosa of patients with active ulcerative colitis and Crohn's disease. *Dig Dis Sci*;**42**:1047-54;1997.
50. Guihot, G.; Guimbaud, R.; Bertrand, V.; Narcy-Lambare, B.; Couturier, D.; Duee, P. H.; Chaussade, S.; Blachier, F. Inducible nitric oxide synthase activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease. *Amino Acids*;**18**:229-37;2000.
51. May, J. M.; Qu, Z. C.; Xia, L.; Cobb, C. E. Nitrite uptake and metabolism and oxidant stress in human erythrocytes. *Am J Physiol Cell Physiol*;**279**:C1946-54;2000.
52. Finkel, T. Signal transduction by reactive oxygen species in non-phagocytic cells. *J Leukoc Biol*;**65**:337-40;1999.
53. Jourd'heuil, D.; Jourd'heuil, F. L.; Feelisch, M. Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism. *J Biol Chem*;**278**:15720-6;2003.
54. Eiserich, J. P.; Hristova, M.; Cross, C. E.; Jones, A. D.; Freeman, B. A.; Halliwell, B.; van der Vliet, A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature*;**391**:393-7;1998.
55. Schopfer, F. J.; Baker, P. R.; Freeman, B. A. NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? *Trends Biochem Sci*;**28**:646-54;2003.

56. Buffinton, G. D.; Doe, W. F. Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic Res*;**22**:131-43;1995.
57. Nikolaus, S.; Folscn, U.; Schreiber, S. Immunopharmacology of 5-aminosalicylic acid and of glucocorticoids in the therapy of inflammatory bowel disease. *Hepatogastroenterology*;**47**:71-82;2000.
58. Rousseaux C, Lefebvre B, Dubuquoy L, Lefebvre P, Romano O, Auwerx J, Metzger D, Wahli W, Desvergne B, Naccari GC, Chavatte P, Farce A, Bulois P, Cortot A, Colombel JF, Desreumaux P. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. J Exp Med. **201**:1205-15; 2005.
59. Pearson, D. C.; Jourd'heuil, D.; Meddings, J. B. The anti-oxidant properties of 5-aminosalicylic acid. *Free Radic Biol Med*;**21**:367-73;1996.
60. Grisham, M. B.; Miles, A. M. Effects of aminosalicylates and immunosuppressive agents on nitric oxide-dependent N-nitrosation reactions. *Biochem Pharmacol;***47**:1897-902;1994.
61. Roediger, W. E.; Lawson, M. J.; Radcliffe, B. C. Nitrite from inflammatory cells--a cancer risk factor in ulcerative colitis? *Dis Colon Rectum*;**33**:1034-6;1990.
62. de Kok, T. M.; Engels, L. G.; Moonen, E. J.; Kleinjans, J. C. Inflammatory bowel disease stimulates formation of carcinogenic N-nitroso compounds. *Gut*;**54**:731;2005.
63. Garcia-Saura M.F., Fernandez B.O., McAllister B.P., Whitlock D.R., Cruikshank W.W., Feelisch M. Dermal Nitrite Application Enhances Global Nitric Oxide Availability: New Therapeutic Potential for Immunomodulation? *J Invest Dermatol*. 2009 Oct 8 [Epub ahead of print].
64. Ostanin D.V., Bao J., Koboziev I., Gray L., Robinson-Jackson S.A., Kosloski-Davidson M., Price V.H., Grisham M.B. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physio;***296**:G135-46;2009.
65. Cheng, Y.; Desreumaux, P. 5-aminosalicylic acid is an attractive candidate agent for chemoprevention of colon cancer in patients with inflammatory bowel disease. *World J Gastroenterol*;**11**:309-14;2005.
66. Velayos, F. S.; Terdiman, J. P.; Walsh, J. M. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol*;**100**:1345-53;2005.
67. Danese, S.; Semeraro, S.; Papa, A.; Roberto, I.; Scaldaferri, F.; Fedeli, G.; Gasbarrini, G.; Gasbarrini, A. Extraintestinal manifestations in inflammatory bowel disease. *World J Gastroenterol*;**11**:7227-36;2005.
68. Taylor, C.T., Colgan, S.P. Hypoxia and gastrointestinal disease. *J Mol Med;***85**:1295-1300;2007.
69. Brüne B, Zhou J. Nitric oxide and superoxide: interference with hypoxic signaling. *Cardiovasc Res.* **75**:275-82;2007.
70. Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol.;**91**:807-19; 2006.

71. Taylor CT, Moncada S. Nitric Oxide, Cytochrome C Oxidase, and the Cellular Response to Hypoxia. *Arterioscler Thromb Vasc Biol.* 2009 Aug 27. [Epub ahead of print]

72. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature*. **453**:807-11;2008.

73. Taniguchi E, Matsunami M, Kimura T, Yonezawa D, Ishiki T, Sekiguchi F, Nishikawa H, Maeda Y, Ishikura H, Kawabata A. Rhodanese, but not cystathionine-gamma-lyase, is associated with dextran sulfate sodium-evoked colitis in mice: a sign of impaired colonic sulfide detoxification? *Toxicology* **264**:96-103; 2009.

74. Whiteman M, Moore PK. Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? *J Cell Mol Med.* **13**:488-507;2009