Arginine-free diet suppresses nitric oxide production in wounds

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To determine the effects of dietary arginine on nitric oxide production during wound healing, we fed male Sprague-Dawley rats a low nitrate diet containing 0% or 3% L-arginine. Nitrate from daily urine samples was reduced to nitric oxide and measured with a chemiluminescence analyzer to indirectly assess nitric oxide synthesis. Once baseline nitrate levels were established, rats received circular, full thickness dermal wounds. The results demonstrate that dietary arginine is not necessary for the sudden increase in nitrate output immediately after wounding, although removal of arginine appears to limit the maximum amount of nitric oxide produced. Arginine-deficient rats had significantly lower ($P < 0.001$) urinary nitrate levels than the corresponding 3%-arginine group over the entire experiment. Despite lower nitrate output in the arginine-free animals, the early response to wounding was similar in both the arginine-free and 3%-arginine groups, as nitrate output doubled within 48 hours of wounding (+112% and +104%, respectively). However, endogenous arginine was not sufficient to maintain increased nitrate output, which returned to baseline levels in arginine-free animals within 11 days after wounding, while the nitrate output of the 3%-arginine group remained significantly elevated (+30–60%, $P < 0.001$) through day 30 and wound closure. In addition, animals on the arginine-free diet increased nitrate output 14-fold in response to lipopolysaccharide challenge. These results suggest that removing dietary arginine does not prevent the cytotoxic activity of macrophages during the inflammatory response, but suppresses nitric oxide synthesis during later tissue repair. This may help explain previous evidence that supplemental arginine improves wound healing, although video image analysis of wounds was inconclusive. (J. Nutr. Biochem. **4**:588–593, 1993.)

Keywords: arginine; nitrate; nitric oxide; video image analysis; wound healing

Introduction

L-arginine plays a pivotal role in several physiologic pathways. It is an essential amino acid for normal tissue growth during animal development and wound repair,[1–3] a major factor in the synthesis of collagen and other proteins,[1–5] and helps restore nitrogen balance after wounding.[6] In addition, arginine is the precursor of nitric oxide (NO), which is involved in diverse, biologically important processes such as wound healing, host defense, cardiovascular regulation, signal transduction, and neurotransmission.[7–10] L-arginine is converted by the enzyme nitric oxide synthase into L-citrulline and NO. Oxidation of NO produces unstable intermediates (such as N_2O_3 and N_2O_4) and subsequently the stable products nitrite (NO_2^-) and nitrate (NO_3^-).[11] Many cells involved in the wound healing process have shown NO synthase activity, including macrophages,[11,12] neutrophils,[13] fibroblasts,[7] keratinocytes,[14] endothelial cells,[15] lymphocytes,[3] and vascular smooth muscle cells.[16] The exact functions of NO in tissue repair have not been established, although it seems likely that a major role of NO is that of a cytotoxic or cytostatic agent released by macrophages and other phagocytic cells during the early inflammatory phase.[17–19] Macrophages secrete a variety of factors that induce angiogenesis, chemotaxis, and fibroblast proliferation,[20] and NO release from these and other wound resident cells may also be important in regulating granulation tissue formation and revascularization.[14]

Previous metabolic studies have established that NO_3^- is biosynthesized in rats[21] and humans,[22] and that L-arginine is a source of NO_3^- in humans.[23] Earlier work

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has shown that urinary NO_2^- is negligible in wounded or infected rats,[24,25] and that urinary NO_3^- is an accurate indirect measure of NO production. Our previous results showed that urinary NO_3^- levels rose sharply upon wounding and remained significantly elevated over the course of tissue repair in excisionally wounded rats fed a diet supplemented with 2% arginine.[24] Furthermore, urinary NO_3^- output remained elevated for 13–18 days following external wound closure. It is known that tissue remodeling and the coinciding biochemical activity in wounds continues well after external wound closure is complete.[26] Consequently, urinary NO_3^- output may reflect NO-dependent processes during all phases of wound repair. Profiles of NO_3^- output may also indicate the extent to which the healing process is completed.

Arginine has also been shown to influence wound healing by acting as a regulator of secretory products of lymphocytes.[3,27–31] However, the connection between the different modes of arginine action on wound healing is ambiguous. The present study attempts to further clarify the relationship between dietary L-arginine and NO production during wound healing.

Methods and materials

Materials

L-arginine (free base) (99%) and *E. coli* lipopolysaccharide (LPS) were purchased from Sigma Chemical Company (St. Louis, MO USA). Potassium nitrate (99.999%) was purchased from Aldrich Chemical Company (Milwaukee, WI USA). The custom arginine-free modified AIN-76 low nitrate diet and the modified AIN-76 low nitrate diet (0.7% wt/wt arginine) were purchased from ICN Biochemicals (Cleveland, OH USA). The composition of the arginine-free diet is listed in *Table 1*. Vanadium(III) chloride (99%) was purchased from Johnson Matthey/Alfa Products (Ward Hill, MA USA). Water for solution preparation and rate consumption was purified with a Milli-Q cartridge filtration system (Millipore Corporation, Bedford, MA USA). Nalgene metabolic cages and all other reagent grade materials were purchased from Fisher Scientific (Pittsburgh, PA USA).

Table 1 Composition of the arginine-free, amino acid-defined diet*

Component	(g/kg)
Cornstarch	400.0
Sucrose	250.0
Corn oil	50.0
Alphacell Non nutritive bulk	50.0
AIN-76 Mineral mixture	35.0
AIN-76 Vitamin mixture	10.0
Choline bitartrate	2.0
BHT	0.01
Menadione sodium bisulfite	(0.5 mg)
Alanine	5.4
Aspartic acid	13.0
Cystine	0.8
Glycine	5.0
Glutamic acid	42.0
Histidine (free base)	5.2
Isoleucine	12.0
Leucine	18.0
Lysine(HCl)	15.0
Methionine	8.0
Phenylalanine	9.0
Proline	22.0
Serine	11.0
Threonine	9.0
Tryptophan	2.2
Tyrosine	10.5
Valine	11.9

*Diet was obtained from ICN Biochemical, Cleveland, OH USA.

Experimental method

All procedures used for animal experimentation were approved by the University of Akron Animal Care Committee. Male Sprague-Dawley rats (325–360 g) were purchased from Zivic Miller Co. (Zelienople, PA USA). The animal storage facility provided alternating 12-hour periods of light and dark with constant humidity (50–60%) and temperature (21–25° C). Rats were acclimatized for 7 days in clean bedded cages, given distilled, deionized water ad libitum, and fed a custom low NO_3^- diet containing 0.7% (wt/wt) arginine. Animals were transferred to metabolic cages and randomly assigned to one of two groups. The first group ($-$ Arg, $n = 4$) received a powdered, low-NO_3^- arginine-free diet. The second group ($+$ Arg, $n = 5$) received the identical diet supplemented with 3% (wt/wt) arginine. Both groups were provided distilled, deionized water ad libitum.

Urine was collected at 24-hour intervals for 18 days prior to wounding to establish baseline urinary NO_3^- output levels. Bacterial growth was inhibited by adding approximately 5 mL of 3 mol/L HCl to each urine collection vial, which maintained the urine at or below pH 1. Urine was collected at 24-hour intervals throughout the wound healing period. Samples were either used immediately or kept frozen until analyzed.

On the day of wounding, urine was collected and rats were anesthetized with Nembutal (40 mg/kg i.p.). The dorsal side of each rat was shaved, swabbed with a sterile, isopropanol-soaked pad, and a 2.7-cm diameter inked stencil was applied at the level of the fifth thoracic vertebrae. Using sterile instruments and aseptic technique, a circular, full thickness wound was made by removing the dermis and panniculus carnosus, resulting in wounds with an average area of 6.15 cm^2. The rats were also injected with gentamicin (4.4 mg/kg i.m.) while under anesthesia. After surgery and wound imaging, each rat was placed on an isothermal pad and monitored closely until it recovered from anesthesia, then returned to its metabolic cage.

In a second experiment, six rats were fed the $-$Arg diet for 10 days prior to wounding. Urinary NO_3^- was monitored and rats received circular, full thickness wounds as before. On day 44 post-wounding, urine samples were collected and each rat was injected (i.p.) with 1.0 mg/kg *E. coli* LPS.

Video image analysis

Immediately following wounding and every 2–3 days thereafter, each wound was videotaped using a video camera (Nikon VN-3000 with a macro-focusing 6× power zoom lens) and VHS tape (Fuji A/V Master Super XG). Unanesthetized rats were manually restrained and a self-adhesive circular label (1.9-cm diameter) was placed adjacent to the wound.[32] This served as an external standard during analysis of the video image and enabled the lens-to-wound distance to vary. The camera lens was positioned perpendicular to the wound site, with the wound and external standard in the same horizontal plane, and the lens was focused to give the largest possible image.

Digital computer analysis of wound images was accomplished by playing the camera output signal to a Spectrum NTSC+ frame grabber board (Redlake Corporation, Morgan Hill, CA USA) installed in a Gateway 2000 386/16SX computer (Gateway 2000, Inc., North Sioux City, SD, USA). Using Accuware image analysis software (Automated Visual Inspection, Santa Clara, CA USA), several optimal images showing the wound and external standard were consecutively captured and displayed on a Samsung CSA7571 multiscanning 17 inch RGB monitor (Samsung Information Systems America, Inc., San Jose, CA, USA). The perimeter of the wound and external standard were traced with a mouse, and the pixel areas and perimeters of each image were computed and then averaged. Relative wound areas and perimeters were obtained by using the ratio of wound to external standard, giving measurements that were independent of camera-to-wound distance. Each relative wound area was expressed as a fraction of the original and plotted versus time to determine wound healing progress. The Gilman parameter, a measure of the linear advance of the wound edge,[33] was also calculated using relative area and perimeter measurements referenced to day zero. A paired two-tailed Student's *t* test was used to assess significant differences in wound healing between treatment groups.

Nitrate analysis

All urine samples were assayed for NO_3^- using a Monitor Labs Model 8440 Nitrogen Oxides Analyzer (Lear-Siegler Corporation, Englewood, CO USA) and a modification of the method described by Braman and Hendrix.[34] A custom impinger was filled with 40–50 ml of a reducing solution of VCl_3 (approximately 0.3 mol/L) and HCl (2 mol/L). The reducing solution was heated to 90–100° C and degassed with helium set at a flow rate of 125 mL/min. Urine samples were injected into the reducing solution through a teflon-lined septum, and the VCl_3 reduced any NO_3^- present to NO. The helium flow carried newly generated NO through a second impinger filled with 1 mol/L NaOH to remove any acidic gases. The flow rate of the analyzer vacuum pump (i.e., the sample inlet flow) was set at 150 mL/min with a micrometering valve. A "T" between the analyzer inlet and the impingers provided an open system that maintained a steady input flow rate and avoided the problem of matching analyzer inlet and helium flow rates. The NO entering the analyzer and the subsequent chemiluminescent reaction (between O_3 generated by the analyzer and NO) determined the amount of NO per sample. Known concentrations of KNO_3 were also injected and used as external standards to determine the μmol/d NO_3 output per animal. The output signal was captured by an HP 3392A integrating recorded (Hewlett Packard Co., Avondale, PA USA). Duplicate injections of all urine samples were run and the average values used as the daily NO_3^- output for each animal. An unpaired two-tailed Student's *t* test was used to assess significant differences in urinary NO_3^- concentration before and after wounding and between treatment groups.

Results

Eighteen days before wounding, the average weights of the −Arg and +Arg diet groups were 343 g and 338 g, respectively. On the day of wounding, rats fed the −Arg diet had an average weight gain of 11 g, while the +Arg group gained an average of 83.4 g. The inhibition of weight gain due to lack of dietary arginine is consistent with previous studies.[1] There were no differences in daily food intake between the two treatment groups, and our previous work showed no correlation between animal weight and NO_3^- output (data not shown).

Urinary NO_3^- profiles for the −Arg (n = 4) and +Arg (n = 5) diet groups are shown in *Figure 1*. Day zero (0) is the day of wounding, and each data point represents the mean daily urinary NO_3^- output for each group. The rats fed the −Arg diet had significantly lower ($P < 0.001$) urinary NO_3^- levels than the corresponding +Arg output over the entire experiment. The mean (n = 18 days) pre-wound urinary NO_3^- output was 3.81 ± 0.72 versus 5.25 ± 0.97 μmol/d NO_3^- for the −Arg and +Arg groups, respectively. In the early post-wound phase of healing (n = 10, days 1–10), the mean NO_3^- output was 8.08 ± 0.96 and 10.69 ± 1.03 μmol/d NO_3^- for the −Arg and +Arg groups, respectively. The mean urinary NO_3^- output in the later wound healing phase (n = 12, days 11–22) was 4.27 ± 0.97 versus 8.40 ± 1.00 μmol/d NO_3^- for the −Arg and +Arg groups, respectively. Despite lower absolute NO_3^- output in the arginine-free animals, both the −Arg and +Arg groups responded to wounding by doubling their NO_3^- output within 48 hours (+112% and +104%, respectively, $P < 0.001$). The major difference between the urinary NO_3^- profiles of the two groups was evident in the later stages of wound repair. By day 11, the mean urinary NO_3^- output in the −Arg group had returned to pre-wound baseline levels (P = 0.143). However, the +Arg group maintained an elevated NO_3^- output through day 30 and wound closure. Urinary NO_3^- production was 60% higher than baseline between days 11–22 (n = 12, $P < 0.001$), and 30% higher between days 25–30 (n = 6, P = 0.003). *Figure 2* shows the wound closure profiles of the −Arg and +Arg groups. Based on both percent of initial wound area and the Gilman parameter (both relative to day zero wound measurements), the wounds of the

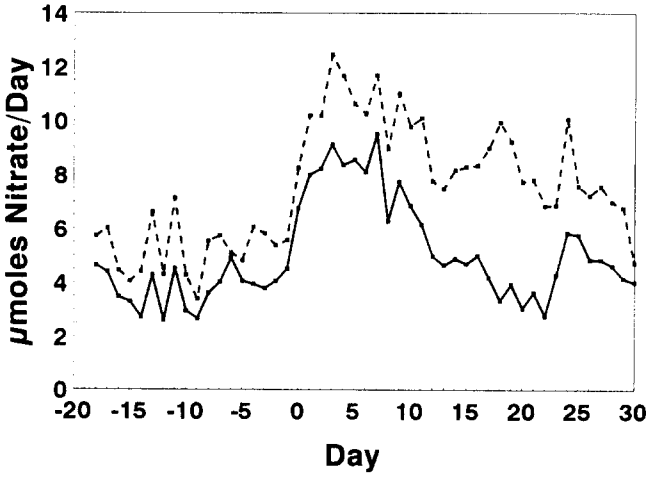

Figure 1 Urinary NO_3^- profiles for wounded rats fed an arginine-free diet (solid line) or a 3%-arginine diet (dashed line). Each rat received a full-thickness circular dermal wound on day zero. Urinary NO_3^- data for each day represent the mean of assays from four (−Arg) and five (+ Arg) rats, respectively.

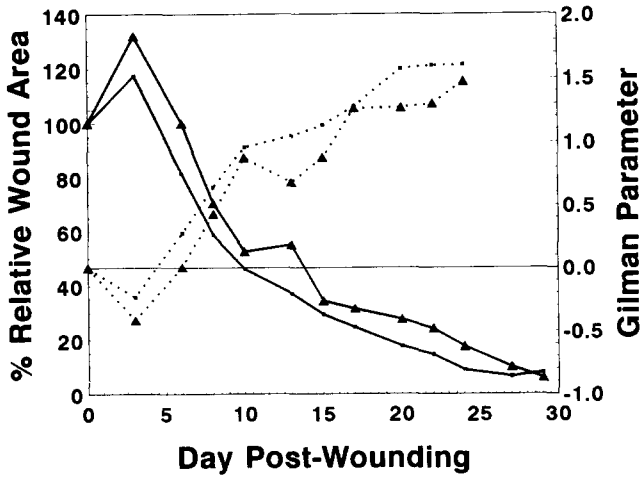

Figure 2 Wound closure profiles for wounded rats fed an arginine-free diet (■) or a 3%-arginine diet (▲). Each rat was wounded on day zero. Solid lines represent percent area closure relative to day zero, and dashed lines represent the linear advance of the wound edge relative to day zero (Gilman parameter). The wound area data were obtained by video image analysis. Each wound area data point represents the mean of measurements from four (−Arg) and five (+ Arg) rats, respectively.

−Arg group healed slightly faster than those of the +Arg group.

The urinary NO_3^- and wound closure profiles of the −Arg group (n = 6) treated with LPS are shown in *Figure 3*. Urinary NO_3^- output and wound healing rates were similar to the −Arg group in the first experiment. The mean urinary NO_3^- output was 5.41 ± 1.38 μmol/d NO_3^- for the pre-wound phase (n = 10 days), 8.09 ± 0.77 μmol/d NO_3^- for the early wound healing phase (n = 10, days 1–10) and 5.82 ± 0.55 μmol/d NO_3^- for the late wound healing phase (n = 33, days 11–44). The relative increase in NO_3^- after wounding was lower than that of the first −Arg group (+50% versus +112%), but the return to baseline levels by day 11 was clearly evident. After receiving LPS, urinary NO_3^- output increased over 14-fold to a mean value of 84.4 μmol/d NO_3^- on day 45 before returning to baseline levels by day 47.

Discussion

The results of these experiments demonstrate that dietary arginine is not necessary for the sudden increase in NO synthesis during the inflammatory phase of wound repair, although removal of exogenous arginine does appear to limit the maximum amount of NO produced. The initial spike of urinary NO_3^- is most likely due to NO release by activated macrophages because they are among the first cells present in the wound and are major regulators of repair events.[26] Studies have shown that activated macrophages produce large amounts of NO over extended periods in vitro,[35] and high levels of activated macrophages are found in wounds throughout all phases of healing.[36,37] Recent experimental evidence indicates that release of NO, a reactive free radical, is responsible for the nonspecific cytostatic/cytotoxic functions of many immunostimulated cells, possibly by reacting with key cellular enzymes that contain heme or other Fe compounds.[18,19,38,39]

Despite lower absolute output in the arginine-free animals, the early response to wounding essentially doubled the relative urinary NO_3^- in both the −Arg and +Arg groups. The second −Arg group responded to LPS challenge with a 14-fold NO_3^- increase. This response corresponds with those previously published for animals fed diets containing arginine,[22,24] and implies that the −Arg diet did not compromise the wounded animals' ability to respond to LPS challenge. Together, these facts suggest that the cytotoxic functions of macrophages are not impaired by a lack of dietary arginine.

Recent studies have shown that neutrophils can also release NO.[13] Neutrophils are the first leukocyte to appear in the wound space and phagocytize bacteria along with macrophages. These cells may also contribute to the early rise in NO_3^- after wounding. However, continued activation of neutrophils and other inflammatory cells inhibits wound healing.[26] Furthermore, NO can inhibit cerebellum-derived NO synthase in a negative feedback manner,[40] and this may also be the case in the wound environment. Thus, once the inflammatory phase subsides. NO may play a different role during the later stages of wound healing.

As granulation tissue forms and matrix remodeling commences, the activity of fibroblasts, endothelial cells, keratinocytes, and other NO-producing cells increases. New tissue also requires an increased oxygen supply, and NO functioning as a vasodilator may be crucial to the revascularization process. Thus, during the later wound healing stages, NO may play a part in stimulating other cells for different functions, resulting in increased urinary NO_3^- output. The hypoxic nature of wounds[41] may help increase the half-life of NO and thus prolong

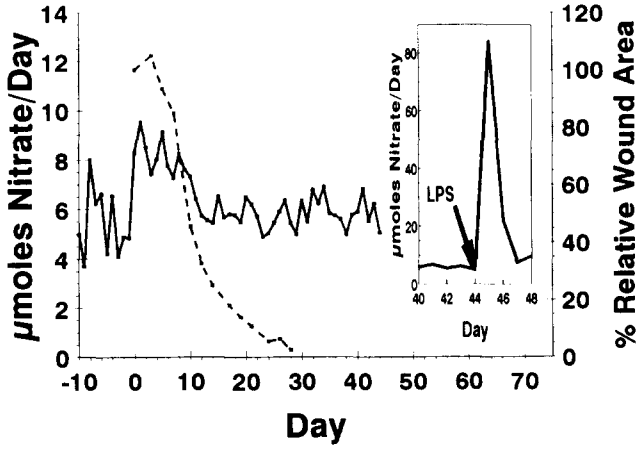

Figure 3 Urinary NO_3^- and wound closure profiles for wounded rats fed an arginine-free diet. Each rat received a full-thickness circular dermal wound at day zero. Urinary NO_3^- (solid line) and wound area (dashed line) data represent the mean of assays from six rats. The wound area data were obtained by video image analysis. Inset: Urinary NO_3^- data on the same rats following i.p. injection of 1.0 mg/kg *E. coli* LPS at 44 days post-wounding.

its effects on target cells. Although NO is involved in signal transduction in neurons and endothelial cells,[7–10,15] no evidence for this type of signaling mechanism in wounds has been shown.

While the exact functions of NO in the later stages of wound healing remain unclear, the return to baseline urinary NO_3^- levels in −Arg animals by day 11 post-wounding suggests that endogenous arginine is not sufficient to maintain increased NO levels for the duration of tissue repair. Possible explanations for this reduced NO synthesis are suggested by the results of some recent studies. In wounds deprived of dietary arginine, the majority of endogenous arginine may be used in other pathways to meet the increased demand for DNA synthesis and cell replication. Once wound inflammation subsides, prodigious quantities of new cells are needed for tissue repair. Arginine is required for the synthesis of polyamines and histones, which are necessary for cell proliferation.[5,42] An arginine-free diet lowers plasma arginine levels[43] and would reduce the amount of arginine available to the wound. The resulting competition between separate pathways for substrate arginine may reduce NO synthesis. Similarly, arginine may also be metabolized to support increased synthesis of collagen and other proteins, and this would reduce the amount of arginine available for NO synthase. Results of several tumorigenesis experiments suggest that this mechanism operates in cancerous tissues, where macrophages can both promote and inhibit tumor growth.[17,44,45] However, contradictory results have also been reported,[46] and no evidence for this mechanism has been shown in wound healing studies. Arginine has also been shown to stimulate the immune system.[3,30,46] An arginine-free diet may thus disrupt the regulation of growth factors and hormones that regulate NO production in wounds, resulting in reduced urinary NO_3^- output in later phases of wound repair. It is quite possible that several inhibitory effects are involved during healing of arginine-deficient wounds. The underlying mechanisms will require further investigation.

Our results did not demonstrate that dietary arginine enhanced wound healing. Excisional wounds in rodents heal primarily by contraction,[47] which may have masked any differences due to diet. Furthermore, the healing of uncovered wounds could have been physically disturbed by either the rats or by contact with metabolic cages, although this was not observed during the experiments. Previous research has shown that dietary arginine improves wound healing based on improved breaking strength of incisional wounds or by increased hydroxyproline and collagen synthesis in implanted wound chambers.[1–3] Several studies have shown neutral or deleterious effects from higher levels of arginine supplementation (4% or 6%) in animals recovering from burns[48] or sepsis.[49] However, other experiments show that pre-feeding rats with arginine improves survival of sepsis.[28] Given the incomplete knowledge of the interactions between different physiological processes involving arginine, it is possible that high levels of dietary arginine could actually impede wound healing.

The manipulation of the L-arginine-to-NO pathway can potentially lead to clinical applications in such diverse fields as cancer therapy, wound healing, and hypertension. However, the interrelationships between the various cellular systems that involve L-arginine and NO are not well defined. Current evidence illustrates that altering NO production to benefit one system may cause harmful effects on tissues in another.[7,50,51] A better understanding of these relationships is required before L-arginine or NO therapy is optimized. Our results indicate that removing dietary arginine does not prevent the antimicrobial activity of macrophages during the inflammatory response, but suppresses NO levels during later tissue repair. This may help explain previous evidence that supplemental arginine improves wound healing. Experiments to evaluate the effects of different variables on NO synthesis in wounds are in progress.

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