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KINETICS OF NITROGEN OXIDE PRODUCTION FOLLOWING EXPERIMENTAL THERMAL INJURY IN RATS

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Nitric oxide is biosynthesized from the amino acid L-arginine by the enzyme nitric oxide synthase. Nitric oxide is a vasodilator, a neurotransmitter, and may moduiate immune function. The experiments presented here were performed to determine whether the synthesis of nitric oxide is increased following experimental burn injury in rats. After a 30% total body surface area burn in 300-g Lewis rats, the urinary output of nitrate, a stable metabolite of nitric oxide, was significantly increased for 8 days postburn compared with that in sham-burned control rats. The origin of the urinary nitrate from L-arginine was demonstrated by administering the stable isotope $^{15}N_2$-guanido-arginine to burned and sham-burned rats and observing an immediate enrichment of ^{15}N in nitrate. The amount of administered ^{15}N recovered as $^{15}NO_3$ was <1% of the administered arginine isotope in both the burned and unburned rats; the recovery of the isotope increased tenfold over baseline recovery in burned rats. The arginine analog N-monomethyl-arginine, an inhibitor of the enzyme nitric oxide synthase, blocked the postburn rise in urinary NO_3 output in burned rats, but did not completely inhibit the output of NO_3 in burn wound-infected rats. Experimental burn injury in rats results in an increase in L-arginine-dependent nitric oxide production and urinary nitrate output.

BURN INJURY is associated with a hypermetabolic response; the degree of hypermetabolism that follows burn injury is related to burn size.[1] The hemodynamic component of the hypermetabolic response is characterized by an elevation of cardiac output and a depression of peripheral vascular resistance. Although the precise mechanisms that induce postburn hypermetabolism are not fully understood, catecholamines are considered to be one of the principal mediators of postburn hypermetabolism.[2] When burn injury is complicated by the development of a burn wound infection, there is often an associated increase in the magnitude of the hypermetabolic response.[3]

Nitric oxide (NO), biosynthesized in mammalian species by the enzyme nitric oxide synthase (NOS, EC 1.14.14.39) from the amino acid L-arginine and molecular oxygen, is a vasodilator, a neurotransmitter, and may also modulate immune function.[4-7] Nitric oxide is rapidly oxidized in vivo to the nitrogen oxides, nitrite (NO_2), and nitrate (NO_3), which are the stable metabolic end products of nitric oxide. The major route of elimination of these end products is by urinary excretion.[8] Experimental studies in animals have demonstrated an increase in both arginine-dependent NO synthesis and the urinary excretion of NO_2/NO_3 following the administration of endotoxin.[9] Also, increases in plasma levels of NO_2/NO_3 have been observed in humans with sepsis, and in patients with advanced malignancies following the administration of interleukin-2.[10,11] Blockade of NO synthesis, by stereospecific inhibitors of the enzyme NOS, has been studied in multiple species of experimental animals and results in an acute increase in blood pressure and peripheral vascular resistance, suggesting that the synthesis and release of NO may regulate, in part, both blood pressure and vascular tone.[12,13]

Nitric oxide is also produced by macrophages, and the production of NO by these cells is stimulated by mitogens (concanavalin-A), endotoxin, and cytokines (tumor necrosis factor, interleukin-2, interferon-γ).[14] Production of NO by macrophages appears to play a role in the microbiostasis of intracellular pathogens, tumor cell cytostasis, and the macrophage response to alloantigens.[15,16] The production of NO by macrophages in vitro can be blocked by stereospecific inhibitors of NOS; this blockade can be reversed by L-arginine.

It is possible that NO may be responsible, in part, for some of the hemodynamic and metabolic changes commonly observed following injury or infection. The studies

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reported here were performed to determine if arginine-dependent NO synthesis and the subsequent urinary excretion of NO_3, a stable metabolic end product of NO formation, were increased in a standard animal model of burn injury. Urinary NO_3 was measured in burned and sham-burned rats, and the conversion of L-arginine to NO_3 was quantified, using stable isotopic techniques, in burned and sham-burned rats. The conversion of arginine to an alternate metabolite, urea, was also measured in these rats by stable isotopic techniques and compared with the production of nitrogen oxides from L-arginine. Finally, the effect of N-monomethyl-arginine (NMMA), an inhibitor of the enzyme NOS, on the urinary excretion of NO_3 was studied in burned and burn wound-infected rats.

MATERIALS AND METHODS

Animal Model

Lewis rats (Harlan-Sprague-Dawley Inc., Indianapolis, Ind), weighing approximately 300 g each, were utilized in these experiments. Animals were housed in individual plastic metabolic cages that allowed for the measurement of food and water intake and for the quantitative collection of urine. The animals were allowed free access to a defined, nitrate and nitrite-free amino acid diet that contained 12.1 g/kg L-arginine (Teklad Premier, TD 86529, Madison, Wis). Nitrite-free and nitrate-free, distilled, deionized water was continuously available to each animal. A 30% total body surface area (TBSA) full-thickness dorsal scald burn was produced in anesthetized (pentobarbital, 30 mg/kg intraperitoneal) rats by immersion in 100°C water for 10 seconds, using the procedure of Walker and Mason.[17] Sham-burned (control) animals underwent identical anesthetic and handling procedures, but were immersed in room-temperature water. No fluid resuscitation was given to these animals. Burn wound-infected animals underwent dorsal burn wound inoculation with 10^6 organisms of *Pseudomonas aeruginosa* (strain 1244) immediately after burn injury.[18] Urine was collected daily from each animal. Isopropyl alcohol (0.5 mL) was added daily to each urine collection container before the daily collection as a preservative. Urine samples were centrifuged at 300*g* for 10 minutes and the supernatant frozen at −80°C before analysis. At the conclusion of each experiment, animals were killed by an intraperitoneal injection of pentobarbital (100 mg/kg). These experimental studies were reviewed and approved by the United States Army Institute of Surgical Research animal use committee, and the studies conformed to guidelines established by the National Institutes of Health.

Biochemical Procedures

Urinary NO_3 was determined by reaction with the Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) following reduction of NO_3 to NO_2 by copper-coated cadmium, as previously described.[19] This method measures both NO_2 and NO_3 present in the urine. Preliminary studies utilizing urine from both burned and unburned rats demonstrated that urinary NO_2 (measured before the reaction with cadmium) was uniformly <1% of the total nitrogen oxides (NO_2 + NO_3) present in urine and was frequently undetectable. Therefore the urinary nitrogen oxide values reported in these experiments are equivalent to the urinary NO_3 levels. Urinary urea was determined on an automated analyzer by the urease method.

Gas-Chromatographic-Mass Spectrometry Procedures

The atoms percent excess (APE) of urinary $^{15}N_2$-urea and $^{15}NO_3$ was measured by gas chromatography-mass spectrometry (GC-MS) using electron impact (urea) and chemical ionization (NO_3) techniques, as previously described.[20,21] For $^{15}N_2$-urea, the trimethylsilyl derivative was used and the mass/charge (m/z) ratios of 191 (M+2) and 189 (M) were measured. For urinary $^{15}NO_3$ the nitrobenzene derivative was used and m/z ratios of 124 (M+1) and 123 (M) were measured. All isotopic enrichments were corrected for the measured natural enrichment of each molecule. In the stable isotopic experiment the percentage of administered isotope recovered in the end products, urea or nitrate, was calculated, as previously described, on a daily basis.[9]

Experimental Design

Experiment I. Twenty-eight rats were divided into burn and sham-burn groups (n=14/group). Urine was collected from each animal for 24 hours preburn to establish baseline NO_3 output, and then daily for 8 days postburn. The urine was analyzed for NO_3 and the daily nitrate output for each animal (μmol NO_3/rat/day) was calculated from the urinary concentration of NO_3 and the daily urine output for each animal.

Experiment II (Stable Isotopic Studies). The stable isotope $^{15}N_2$-guanido-arginine (99%, Cambridge Isotope Laboratories, Woburn, Mass) was utilized in these experiments. The possible metabolic pathways for arginine and the flow of the guanido ^{15}N are shown in Figure 1. Nitric oxide (and the stable metabolites NO_2 and NO_3) biosynthesized from this isotope will contain ^{15}N, since the guanido-nitrogen of arginine is the only known substrate for endogenous NO synthesis. Urea, derived from the arginine isotope by the action of the enzyme arginase, will contain both guanido-nitrogen atoms, or two ^{15}N atoms/urea molecule. Measured $^{15}N_2$-urea (M+2) will be derived directly from the arginine isotope, since it is extremely unlikely, considering the large size of the exchangeable nitrogen pool, that any recycled ^{15}N will be incorporated simultaneously into both guanido positions of endogenously synthesized arginine.

In this experiment, ten rats were divided into two equal groups, burn and sham burned. The diet in this experiment was altered: arginine was removed from the diet and replaced isonitrogenously with alanine (Teklad TD 91230). This dietary manipulation was performed to enhance the plasma enrichment of arginine by the isotope by eliminating the dilution of isotope with dietary arginine. Previous studies by others have demonstrated that elimination of dietary arginine had no effect on the nitric oxide response to infection in an animal model.[22] Before burn or sham burn, the rats had free access to the arginine-free diet and to isotope-free distilled water for 2 days. The drinking water was then changed to a solution containing 5.7 mmol/L $^{15}N_2$-guanido-arginine; animals were allowed free access to diet and to isotope containing water for the remainder of the experiment. In contrast to other amino acids, arginine is not involved in transamination reactions during intestinal uptake and transport; orally administered arginine will be absorbed into the bloodstream intact. Baseline urine samples were collected while the animals were receiving isotope for an additional 3 days before burn or sham burn and urine samples were collected for 3 days postburn. Urine samples were analyzed for NO_3 urea, and the enrichment of $^{15}NO_3$ and $^{15}N_2$-urea. The daily intake of ^{15}N was calculated from the water intake and the concentration of isotope in the drinking water. The urinary content of ^{15}N isotope, in NO_3 (1 ^{15}N/molecule) or urea (2 ^{15}N/

Metabolic Fate of Arginine

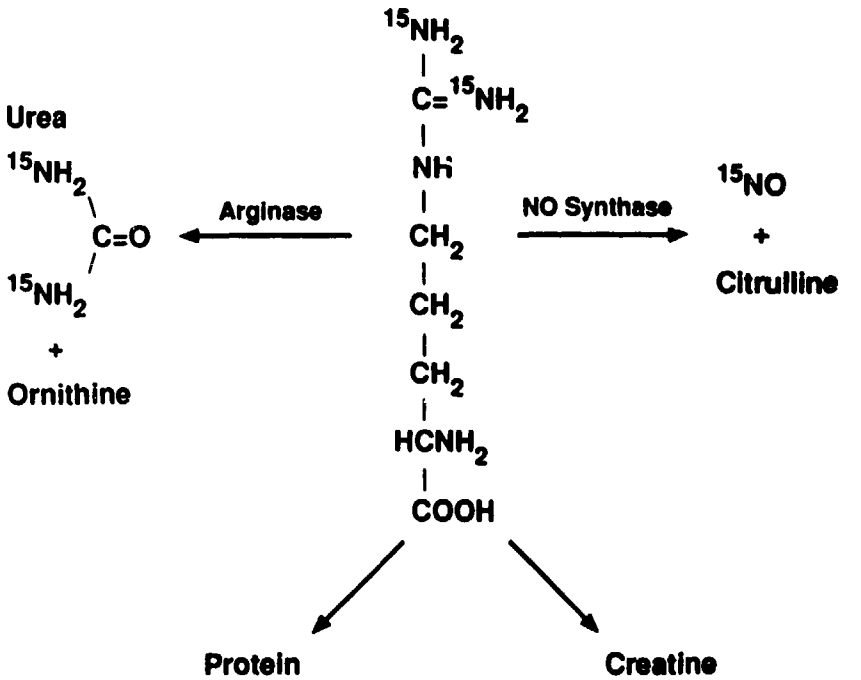

Figure 1. Possible metabolic pathways for L-arginine. The flow of ^{15}N-guanido-nitrogen is illustrated.

molecule), was calculated from the product of the quantity of each metabolite in the urine and the APE for $^{15}NO_3$ or $^{15}N_2$-urea.

Experiment III. Eight rats were divided into two equal groups: burned and burn wound-infected. The NOS inhibitor N-monomethyl-arginine (NMMA) was added to the animals' drinking water at a concentration of 50 mmol/L at the start of the experiment. Urine was collected daily for 4 days preinjury and for 5 days postinjury, and was analyzed for NO_3. The route of administration and the concentration of NMMA were chosen because they had been previously demonstrated to block the NO response to infection in a murine model.[22] The 30% TBSA burn injury that was utilized in the previous experiments produces a nonlethal injury in rats and is associated with a modest increase in metabolic rate. The burn wound infection model that was added in this experiment results in a progressive, lethal injury that is characterized by invasion of bacteria into viable tissue below and adjacent to the burn wound, bacteremia with the deposition of bacteria in distant organs, and a larger increase in metabolic rate than that observed in burned rats.[18,23] The combination of burn injury and infection may be a more potent stimulus to NO production than burn injury alone. This possibility prompted the inclusion of this group in the experiment.

Statistical Analysis

Differences between two means were analyzed by Student's t test and a p value of less than 0.05 was utilized to denote statistical significance.

RESULTS

The baseline urinary output of NO_3 in normal rats was typically 1–3 μmol/rat/day. This is similar to the NO_3 production reported by Wagner in similar sized normal rats (2–3 μmol/rat/day) but less than that reported by Leaf (9.4±3 μmol/rat/day in 280–300-g rats).[8,9] The baseline output of NO_3 will depend, in part, on the amount of NO_2 or NO_3 present in the diet. The diets used in these experiments were assayed for NO_2 and NO_3 by a commercial reference laboratory (Lancaster Laboratories, Lancaster, Penn); neither NO_2 nor NO_3 were detectable in the diets. The urinary NO_3 values from experiment I are illustrated in Figure 2. Following burn injury there was an immediate increase in urinary NO_3 excretion that peaked at approximately ten times the baseline value on postburn day 2. No increase in NO_3 was noted in sham-burned animals. Levels of urinary NO_3 output declined in burned animals after postburn day 2, but remained significantly elevated in burned animals compared with sham-burn control animals, on each of the eight postburn study days.

The results of experiment II are listed in Tables 1 and 2. Baseline (preburn) output of NO_3 was similar in both the burn and sham-burn groups, and was also similar to the baseline values reported in experiment I. Following burn injury, urinary NO_3 output rose to 20.18 μmol/rat/day on postburn day 3. No elevation of NO_3 output was noted in sham-burned rats. Following the introduction of the arginine isotope into the drinking water on preburn day 3 there was an immediate enrichment of ^{15}N in NO_3. The oral route of intake of the isotope did result in some variability in isotope dose, since rats tended to drink more water after injury. The isotope intake ranged from

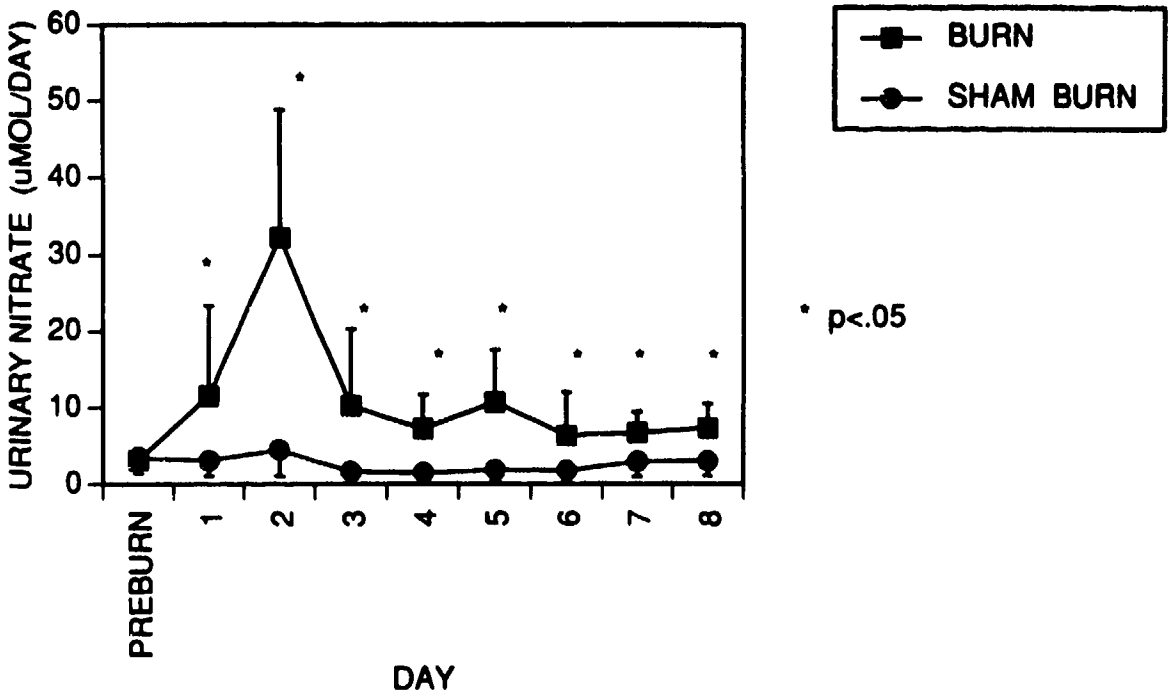

Figure 2. Urinary nitrate values for rats in experiment I (n=14/group).

Table 1
Data from stable isotope studies in burned and sham-burned rats (Mean ± SD)

Day	^{15}N-Nitrogen Intake (μmol 15N/Day)	Urinary Nitrate (μmol/Day)	^{15}N-Nitrate (Atoms Percent Excess)	Recovery of ^{15}N in Nitrate (%)
Burn Group				
Preburn				
5	—	2.83 ± 1.45	—	—
4	—	1.69 ± 0.59	—	—
3	285 ± 69	1.95 ± 0.82	3.21 ± 1.5	0.021 ± 0.014
2	217 ± 48	2.78 ± 1.67	4.29 ± 0.92	0.051 ± 0.026
1	191 ± 51	1.07 ± 0.36	4.47 ± 2.34	0.024 ± 0.018
Postburn				
0	182 ± 48	0.70 ± 0.09	3.48 ± 0.99	0.015 ± 0.011
1	552 ± 178	6.20 ± 3.20*	2.82 ± 0.64	0.038 ± 0.025
2	376 ± 95	17.17 ± 7.47*	3.61 ± 1.13	0.175 ± 0.085*
3	458 ± 90	20.18 ± 11.68*	4.28 ± 1.22	0.167 ± 0.059*
Sham-burn Group				
Preburn				
5	—	2.16 ± 1.82	—	—
4	—	2.00 ± 1.15	—	—
3	319 ± 95	3.05 ± 2.61	3.80 ± 1.91	0.041 ± 0.053
2	182 ± 102	1.23 ± 0.54	3.90 ± 1.65	0.03 ± 0.02
1	251 ± 86	2.10 ± 1.94	2.61 ± 1.33	0.02 ± 0.015
Postburn				
0	205 ± 31	1.33 ± 1.43	3.06 ± 1.12	0.023 ± 0.032
1	152 ± 65	1.19 ± 0.58	2.81 ± 0.72	0.023 ± 0.013
2	266 ± 66	1.63 ± 0.63	3.67 ± 0.18	0.023 ± 0.009
3	163 ± 69	1.02 ± 2.63	2.44 ± 0.31	0.019 ± 0.015

* $P < 0.05$ vs. sham-burn group.

152 to 552 μmol of ^{15}N/day. The intake of arginine in this experiment was approximately one tenth to one third of that ingested by normal rats receiving the arginine-replete diet. Previous studies have demonstrated that isotope doses of $^{15}N_2$-guanido-arginine of up to 170 mg/kg in normal rats had no effect on urinary nitrate output.[8] It is unlikely that the variability in the quantity of isotopic arginine administered in these experiments had any influence on the NO_3 output in these animals. The percentage of administered isotope recovered in NO_3

Table 2
Urea kinetics in burned rats (Mean ± SD)

Postburn Day	$^{15}N_2$-Urea (Atoms Percent Excess)	^{15}N (μmol/day)	Recovery of ^{15}N as Urea (%)
1	1.13 ± 0.70	115 ± 78	39 ± 21
2	1.46 ± 0.75	164 ± 104	81 ± 41
3	1.08 ± 0.46	103 ± 38	45 ± 17

ranged from 0.019% to 0.051% in unburned rats and increased to 0.175% and 0.167% in burned rats on postburn days 2 and 3. The limited availability of the $^{15}N_2$-guanido-arginine isotope at the time these experiments were performed precluded studies beyond postburn day 3.

The atoms percent excess (APE) of urinary $^{15}N_2$-urea was less than the sensitivity of the GC-MS (0.5 APE) in unburned (both groups preburn) and sham-burned rats. The APE of $^{15}N_2$-urea and percentage of ^{15}N isotope recovered in $^{15}N_2$-urea in the burned animals are listed in Table 2. On postburn day 1 the recovery was 39% and it increased to 81% on postburn day 2, declining to 45% on postburn day 3. Urinary urea output (mmol/day) in the postburn period was postburn day 1, 9.50±1.95; postburn day 2, 10.30±2.27; and postburn day 3, 9.81±1.54.

The NO_3 output of the animals in experiment III is shown in Figure 3. Addition of NMMA to the animals' drinking water resulted in a decrease in the baseline (preburn) output of NO_3 to <1 μmol/rat/day. The NMMA completely blocked the urinary NO_3 response to burn injury. In the burn wound-infected rats there was a steady increase in the urinary output of NO_3 beginning on postburn day 2 and continuing until the end of the experiment on postburn day 5.

DISCUSSION

The origin, in mammalian species, of NO from the guanido-nitrogen of arginine and molecular oxygen with the production of citrulline as a byproduct catalyzed by the enzyme NOS is well established.[6] The enzyme NOS has at least four isoforms.[24,25] Several of the isoforms are constitutive, regulated by calcium/calmodulin, and are found in the brain, peripheral nerves, and in vascular endothelial cells. Other isoforms of NOS are inducible, calmodulin-independent, require flavins as co-factors, and have been isolated primarily from macrophages, although these inducible isoforms also appear to be present in other tissues, including Kupffer's cells, hepatocytes, and chondrocytes.[26-29] All isoforms require tetrahydrobiopterin and NADPH as co-factors.[30]

In the central nervous system NO functions as a neurotransmitter, the action of which is mediated by activation of soluble guanylate cyclase and the subsequent production of intracellular cGMP.[31] In the peripheral nervous system NO mediates non-adrenergic, non-cholinergic neurotransmission in the gastrointestinal and genitourinary tracts.[32-34] In both the gastrointestinal and genitourinary tracts, NO mediates smooth muscle relaxation; absence of NO-producing nerve fibers has been implicated in the development of infantile hypertrophic pyloric stenosis. Nitric oxide synthase immunoreactivity has been demonstrated in the brain (cerebral

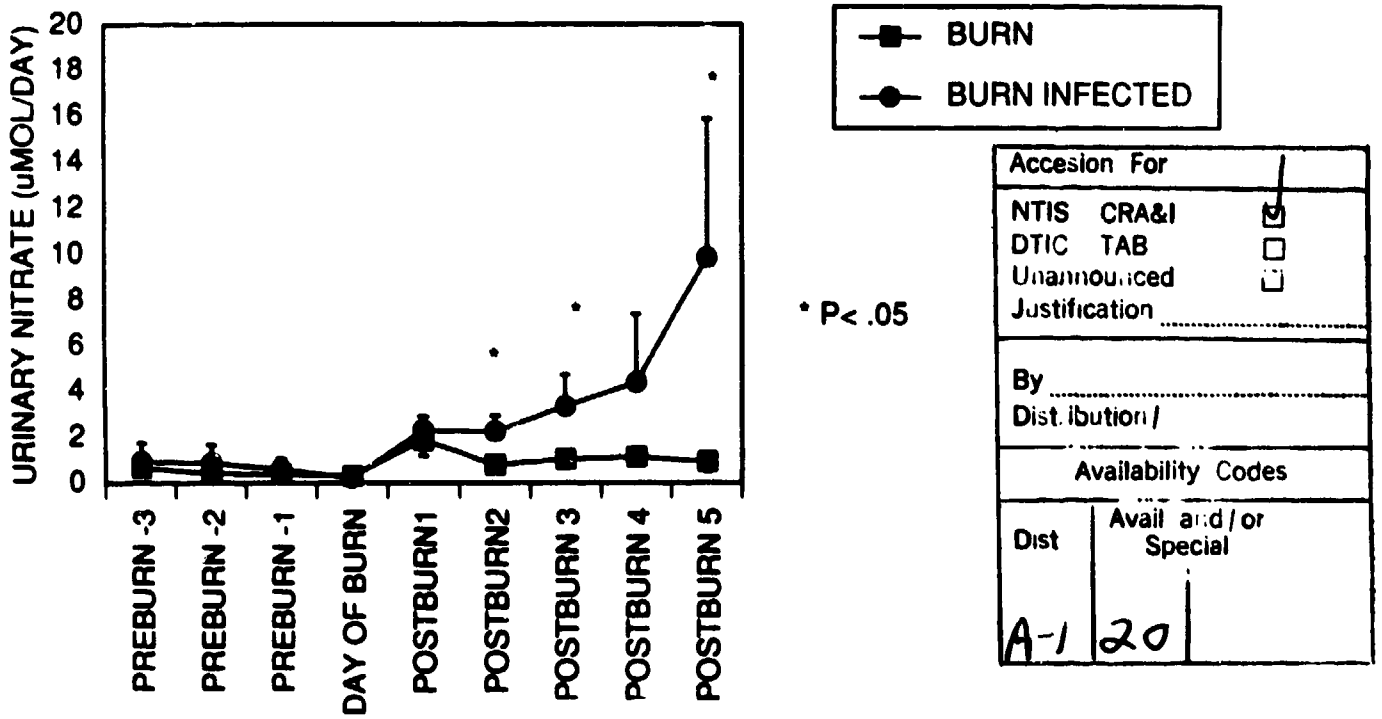

Figure 3. Urinary nitrate values for rats in Experiment III, receiving the nitric oxide synthase inhibitor NMMA.

cortex and cerebellum) and in the neurons of the myenteric plexus of the gastrointestinal tract.[35]

Vascular endothelial cells contain a constitutive form of the enzyme NOS and produce NO from L-arginine. Nitric oxide, like other nitrates, is a vasodilator; endogenous production of NO by vascular endothelial cells appears to regulate, in part, blood pressure and vascular tone. The acute administration of stereospecific, competitive inhibitors of the enzyme NOS, such as NMMA or L-nitro-arginine, to experimental animals results in an increase in vascular tone and blood pressure.[36-38] This increase in vascular tone and blood pressure can be reversed by the administration of L-arginine. Chronic blockade (2 months) of NOS in rats results in systemic hypertension, reduction in glomerular filtration, proteinuria, and histopathologic evidence of glomerulosclerosis.[39] Nitric oxide synthase inhibitors have been utilized to elevate blood pressure in animals with endotoxin shock and inhaled NO has been utilized to treat experimental pulmonary hypertension.[40,41]

The current studies document a significant increase in the arginine-dependent urinary excretion of NO_3 following burn injury in rats. Sham-burned animals did not have an increase in urinary NO_3 excretion. The increase in NO_3 excretion in burned animals peaked in the early postburn period and persisted, at lower levels, for at least 8 days postinjury. The animals in these studies had no exogenous source of NO_2 or NO_3; therefore the NO_3 recovered must have been produced endogenously. Synthesis of NO, by the enzyme NOS, with the subsequent oxidation of NO to NO_3, is the only known pathway for conversion of arginine guanido-nitrogen to NO_3. Seventy-five percent of NO_3 administered to experimental animals is recovered in the urine within 24 hours.[8] In the absence of renal dysfunction, renal excretion is the major pathway for the elimination of NO_3. Although NO was not directly measured in these experiments, because of its short biologic half-life, the urinary NO_3 content that was measured is considered to be an index of NOS activity and NO production.

The administration of the stable isotope $^{15}N_2$-guanido-arginine to rats resulted in prompt enrichment of ^{15}N in urinary NO_3, providing evidence that the guanido-nitrogen of arginine was the source of the urinary NO_3. Following burn injury there was a tenfold increase in the recovery of the administered isotope as NO_3, providing additional evidence of the arginine-dependent origin of the urinary NO_3. The stable isotopic studies also demonstrated that NO synthesis represents only a small fraction (<1%) of arginine metabolism. In contrast, following burn injury a much larger quantity of the administered arginine isotope was recovered as an alternate arginine metabolite, $^{15}N_2$-urea. The metabolism of L-arginine in vivo is complex. Arginine can be synthesized in multiple tissues and there are several routes of catabolism (see Fig. 1). The stable isotopic studies performed in these experiments allow a comparison of the relative activities of the various catabolic pathways of arginine. Because some of the $^{15}N_2$-urea synthesized from $^{15}N_2$-guanido-arginine will be hydrolyzed in the intestine and the ^{15}N recycled, the isotopic recovery calculation can only be used as an estimate of arginine catabolism. In addition, the arginine-free diet used in this experiment may have facilitated the incorporation of the isotopic arginine into urea; exogenous arginine is required for the efficient production of urea in rats.[42]

The percent recovery of administered arginine guanido-N as $^{15}NO_3$ in these experiments was similar to that reported by others. Wagner reported a recovery of approximately 0.003% of administered ^{15}N-ammonia (a precursor of one of the two guanido-N of arginine) in normal rats; the recovery of this isotope increased 25-fold following the administration of endotoxin to the rats.[9] Hibbs measured the recovery of $^{15}N_2$-gaunido-arginine as urinary $^{15}NO_3$ or ^{15}N-urea in two patients following the administration of interleukin-2 to treat advanced cancer. In these two patients, 0.7% of the isotope was recovered as NO_3 and 16% as urea.[10]

The increase in NO production associated with a burn injury that was demonstrated in these experiments could be blocked by the administration of the NOS inhibitor NMMA. The administration of NMMA to rats also resulted in a decrease in the baseline urinary output of NO_3. In burn wound-infected animals the early NO response was blunted by NMMA, but an increase in NO production was noted on postburn day 2 and continued until the end of the experiment on postburn day 5, suggesting that the stimulus to NO production induced by burn wound infection was sufficient to overcome the effects of the NOS inhibitor. Burn wound infection in this rat model has previously been demonstrated to result in a greater increase in indices of hypermetabolism, including oxygen consumption and core temperature, compared with burn injury alone.

The current studies measured the dynamics of nitrogen oxide synthesis and excretion in a standard model of burn injury in rats. It was not possible, based on these experiments, to determine the mechanism by which burn injury results in an increase in NO production. The physiologic significance (if any) of the increase in nitrogen oxide synthesis noted in these experiments was not directly studied, nor was it possible to determine the specific tissue of origin of the excess nitrogen oxides generated postburn. Other studies suggest that macrophages are quantitatively the most important producers of NO, compared with other NO-producing tissues.[43] Based on the physiologic properties of NO, as demonstrated by others in experimental studies, it is possible for us to speculate that the increase in NO synthesis observed following experimental burn injury may be responsible in part for some of the hemodynamic and metabolic changes classically observed following burn injury. Further studies that directly investigate the phys-

iologic and metabolic effects of NO production in the postburn period will be required to test this hypothesis.

In conclusion:

1. Arginine-dependent synthesis of NO, as measured by the urinary excretion of NO_3, and the incorporation of guanido-^{15}N in NO_3, is significantly increased in a standard animal model of burn injury.

2. The synthesis of NO in both normal rats and rats with burn injury represents only a small fraction (<1%) of arginine metabolism.

3. The increase in synthesis of NO following burn injury in rats can be blocked by the NOS inhibitor NMMA. In burn wound-infected rats the NO response is blunted by NMMA, but not entirely blocked at the dose of NMMA utilized in these experiments.

4. The physiologic significance of the increase in NO synthesis in this experimental burn model remains unclear. Further studies will be necessary to determine if NO is directly involved in mediating any of the hemodynamic or metabolism responses that typically follow burn injury.

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DISCUSSION

Dr. Takayoshi Matsuda (Chicago, Illinois): I would like to thank the Association for the privilege of new membership and also for the privilege of discussing this paper. Doctor Becker, I congratulate you for a fine presentation. Your study has shown that nitric oxide production is increased in the postburn period in rats with 30% total body surface area burns. It has been reported by Ochoa and his colleagues from Pittsburgh that plasma nitrate levels are elevated in patients with sepsis. However, they have shown decreased plasma nitrate levels in trauma patients, including trauma patients who became clinically septic.

Could you elaborate as to why the nitrate levels are decreased in trauma patients but elevated in rats with thermal injuries?

You have also demonstrated that the inhibitor can abolish the increased production of nitrates in rats with burns, but not in rats with both burns and infection. Do you think it is because of an inadequate dosage of the inhibitor in rats with both burns and infection, or do you think there are other mechanisms for sepsis to cause an increased nitrate production?

There are no data in the present study regarding the direct cause and effect between increased nitric oxide production and postburn hypermetabolism. But let us assume that data become available in the near future. Then, what should be done? Should we block or suppress the increased nitric oxide production? It has been reported that immunologic activation of microphages induces the activity of nitric oxide synthase, thus resulting in an increased production of nitric oxide. This synthesis of nitric oxide mediates much of the antimicrobial activity of microphages against bacteria and other pathogens. In this scenario, the inhibition of nitric oxide production could be detrimental instead of therapeutic to the host.

My final question is regarding your method of statistical analysis. In your full manuscript, you stated that the differences between two means are analyzed by the Student's *t* test.

Your data contain two factors. The first factor is the injury factor; that is, animals are either burned or not burned or the animals are burned or burned with infection. The second factor is the time factor, in that the measurements were made at different time intervals using the same animal. It appears that a two-way ANOVA with repeated measures is an appropriate initial analysis to examine two-factor interaction. And, if this two-factor interaction is significant, then the data can be analyzed by one of the various post-hoc tests.

I am a little confused because in the abstract in the program, the data appear to be analyzed by the ANOVA, but there is no mention of ANOVA in the final manuscript. Did you use the ANOVA? If so, what specific post-hoc test did you use to analyze within-group data?

Dr. J. Wesley Alexander (Cincinnati, Ohio): Just one question. Did the NMMA alter mortality in the animals?

Dr. David J. Dries (Maywood, Illinois): I very much enjoyed the paper, and I have just a couple of quick questions following up on Doctor Alexander. What was the mortality of the 30% in this model? And did you see increased nitric oxide production with animals having larger injury and decreased nitric oxide production in animals having smaller injury?

Finally, what was the ambient temperature in which the animals were kept, and was there any attempt made to treat these wounds? I wonder whether, as your clinical data seem to suggest, the conditions of the management of the patient or the animal may in part ameliorate the nitric oxide response.

Dr. William K. Becker (Closing): I would like to thank each of the discussants for their comments. I will go in order. First a question about our results in the animals compared with the results published by Dr. Ochoa from the Pittsburgh group that demonstrated decreased nitrate levels in trauma patients. There are significant differences in arginine and nitric oxide metabolism between rats and humans. It has been far more difficult to document changes in nitric oxide production in humans than it has been in rats or mice, and this may indeed explain some of the differences that we've observed versus the differences observed by investigators looking at humans.

There was a question about the NMMA inhibitor in the burn-infected animals. Actually, the burn-infected animals had received more inhibitor than the burn animals alone, so I don't think that there was an insufficient amount of inhibitor in that case.

I really cannot comment on cause and effect between the burn injury and what is actually inducing the production of nitric oxide. It is interesting to speculate that perhaps there is a direct activation of microphages by the wound or some factor released by the burn wound, but we don't have a good answer as to cause and effect.

We are not advocating the clinical use of any type of nitric oxide synthase inhibitor in humans. Indeed, it may be detrimental to use this in the hypermetabolic, hyperdynamic response that burn patients exhibit, so we're not advocating the use of the inhibitor at all.

Finally, a question on the statistics. We used purely a Student's *t* test. We did not analyze over time. Two means were compared on the same day. So we did not use ANOVA.

Dr. Alexander asked questions about mortality. The burn infected animals experiment was terminated before any mortality occurred, so we have not looked at mortality in these animals.

Additional questions were, was there a difference in size of injury. We really have only looked at a 30% body surface area burn in these animals, so we really have not been able to compare size of injury versus the amount of nitrate response.

The animals were kept at room temperature. There was no wound treatment. And in the burned rats, it is almost impossible to cause a burn wound infection in any burned rat without adding a significant number of bacteria to the wound, so we really did not treat any of the wounds at all.