Nitric oxide production is intensely and persistently increased in tissue by thermal injury

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NO is a major messenger molecule which regulates immunity and vascular tone, serves as a neurotransmitter and participates in wound healing. It has also been known to be increased *in vivo* by thermal injury. Urinary nitrate excretion and tissue levels of NO synthase activity were measured after a non-lethal thermal injury. Urinary nitrate excretion decreased by 90 % 24–48 h after injury, but dramatically increased by 10-fold thereafter for 30–40 days after injury and remained elevated until the wound healed. This response was inhibited by a competitive inhibitor of NO

synthase. These rates of urinary nitrate excretion suggest that NO production is dramatically affected by injury in a pattern that is distinct from that observed after lipopolysaccharide administration. On the basis of these findings, we suggest that the hyperdynamic cardiovascular and hypermetabolic responses seen to continue weeks after thermal injury could be a result of the autocrine and paracrine effects of NO generated locally within the tissues in addition to that generated by inflammatory cells.

INTRODUCTION

Thermal injury is characterized by very dramatic changes in peripheral vascular resistance, cardiac index and metabolic rate which continues for weeks or months after injury. Increased urinary nitrate excretion has been demonstrated to occur for 8 days after thermal injury [1]. The increased rates of urinary nitrate excretion probably reflect changes in arginine metabolism, because *N*^G-methyl-L-arginine (NMA) blocked this increase and the recovery of ¹⁵N in urinary nitrate increased 10-fold when [*guanidino*-¹⁵N₂]arginine was given.

In the current study, which measured urinary nitrate excretion and NO synthase activity specifically within important organs, the increases in NO production resulting from thermal injury were of a greater magnitude and a much longer duration than the lipopolysaccharide (LPS) response.

MATERIALS AND METHODS

Animal protocol

Female rats (175–200 g) were purchased from Taconic Animals Farms. All animals were fed a low-nitrate diet (AIN-76, Bioserv Inc., New York, NY, U.S.A.), given either as a solid pellet or a liquid diet (Shake and Pour) for 2 weeks. Non-lethal thermal injury was inflicted as described previously [2] using a protocol approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. Briefly, rats were anaesthetized with ether and their backs shaved with animal hair clippers. The dorsum of each rat was then placed in a water bath at 100 °C for 10 s. Each rat was immediately given saline (15 ml/kg) by intraperitoneal injection to replace intravascular volume. These rats are hereafter referred to as injured rats. Sham-operated rats, hereafter referred to as controls, were anaesthetized with ether, their backs shaved, and saline injected. All rats were individually caged for the duration of the experiment.

Chemicals

Tris/HCl, dithiothreitol, Hepes, soyabean trypsin inhibitor, leupeptin, aprotinin, antipain, phenylmethanesulphonyl fluoride, FAD, FMN, valine, ornithine, citrulline, arginine, nicotinamide, EGTA and NADPH were obtained from Sigma, St. Louis, MO, U.S.A. Sucrose and CaCl₂ were from Baxter Scientific Co., Boston, MA, U.S.A. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was obtained from B. Schirck Laboratories, Jona, Switzerland. NMA was from Calbiochem, La Jolla, CA, U.S.A. [¹⁴C]Arginine was purchased from Dupont–NEN Research Products, Boston, MA, U.S.A.

Determination of urinary nitrate

Faeces-free urine was collected from individual rats into 1 ml of 1 M NaOH, using a metabolic cage assembly (Lab Products Inc.). Total urinary nitrate was measured by an automated modified Griess procedure [3]. Total urinary creatinine was determined in the samples collected for nitrate analysis using Sigma Kit no. 555.

Determination of NO synthase activity

Rats were killed by cervical dislocation, and their tissues frozen on solid CO₂. Frozen tissues were homogenized in a Polytron in 5 vol. of buffer containing 0.32 M sucrose, 50 mM Tris/HCl (pH 7.4), 0.1 mM EGTA, 1 mM dithiothreitol, 10 µg/ml soya-bean trypsin inhibitor, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml antipain, 1 mM phenylmethanesulphonyl fluoride, 2 µM FAD, 2 µM FMN and 2 µM tetrahydrobiopterin hydrochloride. The homogenates were centrifuged at 100 000 *g* for 30 min and assayed for NO synthase activity as described previously [4] within 2 h. The 300 µl assay mixture contained 50 µl of supernatant, 200 mM Hepes (pH 7.4), 1 mM dithiothreitol, 4 µM FAD, 4 µM FMN, 50 mM valine, 1 mM ornithine, 1 mM

citrulline, 0.24 mM CaCl_2 , 1.2 mM MgCl_2 , 4 μM tetrahydrobiopterin hydrochloride, 1 mM NADPH and 100 μM arginine containing [^{14}C]arginine (labelled in the guanidino portion; 3.3 $\mu\text{Ci}/\mu\text{mol}$). Ca^{2+} -dependent NO synthase activity was determined by subtracting the radioactivity lost on addition of 1 mM EGTA from that obtained in the absence of EGTA. Ca^{2+} -independent NO synthase activity was determined by subtracting the radioactivity lost on addition of 1 mM NMA and 1 mM EGTA from that obtained in the presence of 1 mM EGTA alone. Protein content of the supernatants was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.).

Data analysis

Urinary nitrate concentrations for injured and control rats were compared by Student's *t* test at each time point. NO synthase activities for injured rats were compared with pooled control values by Student's *t* test. Unless otherwise indicated, a level of 5% was used to determine significance. Data are expressed as means $\pm$ S.E.M.

RESULTS

Injury model

The full-thickness thermal injury involving 20% of the total body surface area of the rats was non-lethal. One day after the injury and for the next 2–3 weeks, the damaged skin became dry and was not infected. The dry damaged skin detached 3 weeks after injury leaving an open wound. By 4 months after the injury, the wound had healed completely by wound contracture. No such changes were observed in the control rats.

Urinary nitrate excretion

Urinary nitrate excretion by injured rats given food and water *ad libitum* was dramatically reduced during the first 24 h, but during the next 2–3 weeks, it exceeded controls by 10-fold (Figure 1). Urinary nitrate excretion in injured rats that were starved but had access to water *ad libitum* decreased during the first 24 h, but increased to levels significantly higher than controls over the next 48–72 h (Figure 1, inset). Serum nitrate levels in injured rats exceeded control values by twofold at 24 h ($82.4 \pm 15.0 \mu\text{M}$ versus $43.2 \pm 1.1 \mu\text{M}$; $n = 6$; $P < 0.01$). During starvation, urinary nitrate excretion appears to peak by 48 h (Figure 1, inset) whereas in fed animals it appears to continue to rise for approx. 2 weeks (Figure 1). The difference between the two animal groups could be related to an impairment in arginine availability during prolonged starvation. These data suggest that thermal injury increases NO production *in vivo*.

It has been demonstrated that addition of NMA to the drinking water will inhibit the increased urinary nitrate excretion observed in mice injected with LPS [5]. The increased urinary nitrate excretion 11 days after thermal injury was inhibited by 50 mM NMA added to the drinking water (Figure 2), whereas ammonium acetate caused nitrate excretion to plateau. This latter effect may have resulted from prolonged fasting by these control animals because of the noxious smell and taste of ammonium acetate. This plateau response was similar to that seen in prolonged starvation (Figure 1, inset). These data suggest that thermal injury increases NO production by altering arginine metabolism *in vivo*.

There appeared to be no significant difference between the injured and control rats with respect to urinary creatinine excretion ($5.5 \pm 1.1 \text{ mg}/24 \text{ h}$ for fasting control rats compared with $2.0 \pm 2.3 \text{ mg}/24 \text{ h}$ for fasting injured rats and 1.1 ± 0.1 and $1.0 \pm 1.2 \text{ mg}/24 \text{ h}$ for the respective fed rats). Given that these

Figure 1 Urinary nitrate excretion by thermally injured fed and starved (inset) rats

Results are expressed as the difference in μmol of nitrate found in the urine collected from the injured rats after subtraction of the content in the urine of the controls for the same time point. The data are means $\pm$ S.E.M. ($n = 8$ –10 rats in each group). *Significantly different from the controls ($P < 0.01$).

Figure 2 Effect of NMA on urinary nitrate excretion in thermally injured fed rats

To measure the proportion of urinary nitrate derived from arginine metabolism, 50 mM NMA (●) was administered 10 days after injury to inhibit NO synthase [6]. Control thermally injured rats were given drinking water containing 50 mM ammonium acetate (○). The arrow indicates the time point at which NMA or ammonium acetate was first administered. Values for total urinary nitrate collected in 24 h are expressed as means $\pm$ S.E.M. ($n = 4$). *Significantly different from controls ($P < 0.01$).

Table 1 NO synthase activity in selected rat tissues after thermal injury

Results are means $\pm$ S.E.M. ($n = 4-6$). Control skin is normal skin, 1-day skin is virtually destroyed by the injury and 21-day skin is a healing wound composed of granulation tissue. * $P < 0.001$, † $P < 0.02$, ‡ $P < 0.003$, ** $P < 0.05$ compared with control.

	NO synthase activity (pmol of [14 C]citrulline/ 10 min per mg of protein)	
	Ca $^{2+}$ -dependent	Ca $^{2+}$ -independent
Brain		
Control	2608 $\pm$ 158	58 $\pm$ 31
1 Day	3509 $\pm$ 102*	77 $\pm$ 50
21 Day	3254 $\pm$ 68†	46 $\pm$ 15
Stomach		
Control	399 $\pm$ 13	86 $\pm$ 48
1 Day	563 $\pm$ 23*	115 $\pm$ 37
21 Day	755 $\pm$ 15*	69 $\pm$ 23
Caecum		
Control	455 $\pm$ 30	144 $\pm$ 7
1 Day	82 $\pm$ 17*	265 $\pm$ 12*
21 Day	616 $\pm$ 906	120 $\pm$ 36
Ileum		
Control	197 $\pm$ 13	112 $\pm$ 57
1 Day	345 $\pm$ 39†	82 $\pm$ 23
21 Day	265 $\pm$ 6†	53 $\pm$ 26
Liver		
Control	156 $\pm$ 81	336 $\pm$ 131
1 Day	514 $\pm$ 143**	508 $\pm$ 147
21 Day	595 $\pm$ 137†	139 $\pm$ 72
Kidney		
Control	106 $\pm$ 8	0
1 Day	63 $\pm$ 8†	11 $\pm$ 8*
21 Day	77 $\pm$ 5†	24 $\pm$ 7*
Spleen		
Control	20 $\pm$ 10	8 $\pm$ 4
1 Day	38 $\pm$ 5	10 $\pm$ 9
21 Day	17 $\pm$ 8	8 $\pm$ 2
Skin		
Control	294 $\pm$ 25	73 $\pm$ 70
1 Day	76 $\pm$ 43‡	31 $\pm$ 31
21 Day	19 $\pm$ 11*	462 $\pm$ 173**

values were within normal limits [6], the decreased urinary nitrate excretion during the initial 24 h possibly represents nitrate redistribution within an expanded extracellular space.

Tissue NO synthase activity

Direct measurements of both the Ca $^{2+}$ -dependent constitutive NO synthase and the Ca $^{2+}$ -independent inducible NO synthase were made in order to localize NO production 1 day and 3 weeks after injury (Table 1). Overall NO synthase activities were consistent with reported values [4].

A significant increase in Ca $^{2+}$ -dependent NO synthase was seen in the brain at both 1 day ($P < 0.001$) and 3 weeks ($P < 0.02$) after injury (Table 1). The Ca $^{2+}$ -independent activity was very low in the brain and probably reflects endothelial-cell enzyme [4].

Ca $^{2+}$ -dependent NO synthase activity was greatly increased in the stomach ($P < 0.001$) and ileum ($P < 0.002$) 1 day and 3 weeks after injury, whereas the activity in the caecum was decreased only on day 1 ($P < 0.001$) (Table 1). This pattern was

different from that seen after LPS treatment, in which Ca $^{2+}$ -dependent NO synthase activity increased significantly in both the ileum and caecum [4]. Production of this enzyme in the stomach and ileum may represent production by endothelial cells, intestinal epithelial cells and/or smooth-muscle cells. Local production of NO could be important in gut barrier function and/or intestinal motility. Ca $^{2+}$ -independent NO synthase activity in the stomach, caecum and ileum was not affected by injury, except a twofold increase 1 day after injury in the caecum (Table 1). It is entirely possible that the two enzyme activities are time dependent as previously demonstrated by LPS administration [4].

Detectable levels of Ca $^{2+}$ -dependent NO synthase activity were found in the liver even in control rats, and activity was increased after thermal injury at day 1 ($P < 0.05$) and 3 weeks ($P < 0.02$) (Table 1). Salter et al. [4], using [U- 14 C]arginine, could not detect any NO synthase activity in the liver; however, with the use of [guanidino- 14 C]arginine, Ca $^{2+}$ -dependent NO synthase activity was measurable. In contrast with the Ca $^{2+}$ -dependent enzyme, hepatic Ca $^{2+}$ -independent NO synthase activity was largely unaffected by injury and was actually decreased 3 weeks after injury. Other organs, including kidney (Table 1), spleen (Table 1), heart (results not shown) and lung (results not shown), exhibited low levels of this enzyme, and are not likely to be significant contributors to the NO production resulting from injury.

The Ca $^{2+}$ -dependent NO synthase in the injured skin demonstrated the greatest changes in activity, being dramatically reduced 1 day and 21 days after injury (Table 1). By contrast, the Ca $^{2+}$ -independent enzyme was greatly increased 21 days after injury (Table 1), and it is possible that the increases in urinary nitrate excretion seen *in vivo* after injury are related, in part, to this increased Ca $^{2+}$ -independent NO synthase activity in the wound area.

DISCUSSION

NO, which is a noxious highly reactive free-radical gas, is a major messenger molecule which regulates immune function and blood vessel dilation, serves as a neurotransmitter in the brain and peripheral nervous system, and is involved in wound healing [7-10]. As it spontaneously oxidizes to its inactive stable end products within 6-10 s, its sphere of influence is limited to autocrine and paracrine effects in NO synthase-containing tissues. This phenomenon explains why mammals excrete more nitrate than they ingest particularly when inflammation is present [11]. We hypothesized that thermal injury might alter NO production *in vivo*, and have measured the end product of NO production, i.e. nitrate, in the urine as well as NO synthase activity in various tissues.

Five other studies have demonstrated increased urinary excretion of nitrate after various treatments. Wagner et al. [11] demonstrated that the injection of rats with LPS increased urinary nitrate excretion, which persisted for 3-4 days. Stuehr and Marletta [12] reported that mice injected with LPS or *Mycobacterium bovis* excreted more nitrate in the urine for 10-15 days; they also demonstrated that macrophages were the primary immune cell responsible for this increase *in vivo*. Granger et al. [5] demonstrated that *M. bovis* infection increased urinary nitrate excretion in mice for 20 days. The inclusion of NMA, a specific inhibitor of NO synthase, in the drinking water blocked the increased urinary nitrate excretion thereby demonstrating that all the nitrate was coming from arginine. Smith et al. [13] later showed that the infliction of a circular full-thickness dermal wound, 3.8 cm 2 in area, resulted in an 88% increase in urinary

nitrate excretion in rats which persisted for 27–32 days. Finally, Becker et al. [1] reported that, after a 30% total body surface area burn in 300 g Lewis rats, there was a significant increase in urinary nitrate excretion which persisted for the 8 days of the experiment.

The wound itself could be an important long-term contributor of NO. Wound healing proceeds in an orderly fashion [14]: the trauma occurs; this is followed by platelet aggregation and then clotting. During the first day, neutrophils and lymphocytes infiltrate the wound. These are followed by macrophages, and then finally by fibroblasts, smooth-muscle cells and epithelial cells [14]. In control rats, the wound is normal skin. After injury, Ca^{2+} -dependent NO synthase activity had decreased on day 1 ($P < 0.003$) and 3 weeks later ($P < 0.001$). The injured skin was essentially dead, and the low NO synthase activity observed probably comes from any remaining endothelial cells and platelets. At 3 weeks, the granulating wound is primarily endothelial cells, inflammatory cells and fibroblasts. The endothelial cells are probably major contributors of Ca^{2+} -independent NO synthase activity in the healing wound [7]. Although not directly measured in this study, it is likely that the major contributor to the increased NO after thermal injury is the activated circulating macrophage.

The present study shows that thermal injury dramatically changes urinary nitrate excretion by altering NO production. The pattern of activity of NO synthase is different from that reported after LPS administration to rats [4]. LPS induces very substantial production of Ca^{2+} -independent NO synthase in the spleen, lung and liver; however, this response was not seen after burn injury. LPS had no effect on Ca^{2+} -dependent NO synthase in the brain, but thermal injury appeared to induce this enzyme activity. This induction is probably related to endothelial and/or neuronal NO synthase activity, although this was not measured directly. It is also possible that the differences seen may be temporal ones, in that the current study involved changes that persisted for 12 weeks whereas, after LPS administration, the maximum effects appear to occur within 6 h.

Cytokines are considered to be intimately involved in the modulation of expression of Ca^{2+} -dependent and -independent NO synthase and they have been shown to be present within tissues after burn injuries. We have measured the expression of interleukin-1 (IL-1) in thermally injured rats; this response was very different from that produced by LPS administration [15].

Thermal injury induced marked increases in IL-1 α in the liver and lung within 30 min with maximal levels reached at 2.5 h. Levels in the spleen, ileum and thymus were not detectable. In contrast, endotoxin challenge resulted in dramatic elevation of IL-1 α levels in liver, lung, spleen, ileum, thymus and plasma, whereas the skin maintained its usual large amounts [15,16].

In thermal injury, NO probably plays a major role in non-specific immune defence and as a vasodilator and potentiator of oedema. These clinical features are dramatically changed by thermal injury in a prolonged and sustained fashion. It is possible that a better understanding of NO production in patients with burn injury may lead to more effective treatment.

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