Hyperthermia stimulates nitric oxide formation: electron paramagnetic resonance detection of ·NO-heme in blood

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Hall, David M., Garry R. Buettner, Ronald D. Matthes, and Carl V. Gisolfi. Hyperthermia stimulates nitric oxide formation: electron paramagnetic resonance detection of ·NO-heme in blood. *J. Appl. Physiol.* 77(2): 548–553, 1994.—Previous experiments from our laboratory have demonstrated that severe hyperthermia results in a selective loss of splanchnic vasoconstriction. Using electron paramagnetic resonance spectroscopy to scan whole blood samples collected in vivo from the portal vein and femoral artery of conscious unrestrained rats, we observed an increase in the concentration of spectroscopy-detectable species in portal venous blood of all heat-stressed animals. These spectra consisted of at least three distinct species: one with a broad feature having an effective g factor for the unpaired electron (g) of 2.06 assigned to the copper-binding acute phase protein ceruloplasmin, and two with narrower features that evolved at core temperatures >39°C representing a semiquinone radical and ·NO-heme. This heat-induced signal displays the classic nitrogen triplet hyperfine structure (nitrogen hyperfine splitting constant = 17.5 gauss, centered at g = 2.012) that is consistent with a five-coordinate heme complex and is characteristic of an unpaired electron coupled to nitrogen in the ferrous ·NO-heme adduct $[(\alpha^{2+}NO)\beta^{3+}]_2$. The intensity of this signal increased approximately twofold as core temperature rose to >39°C, peaking 1 h post-heat exposure at greater than threefold basal concentration. This species was not seen in corresponding arterial blood samples. This is the first demonstration that whole body hyperthermia produces increased concentrations of radicals and metal binding proteins in the venous blood of the rat and suggests that severe hyperthermia stimulates an enhanced local release of ·NO within the splanchnic circulation.

hemoglobin; free radicals; rat

HEATSTROKE is a systemic disorder characterized by severe hypotension, delerium, and convulsions that can lead to tissue injury, organ dysfunction, coma, and death (30). The etiology of heatstroke is multifactoral and has been attributed to direct thermal injury to hypothalamic thermoregulatory control centers (20) and to circulatory failure (1). Proponents of both views agree that heatstroke results in shock and that the cardiovascular system plays a preeminent role in the process (12); however, the mechanism that initiates circulatory failure and results in the cascade of events leading to heatstroke is contentious. Evidence suggests that it may be due to myocardial failure (35) or peripheral vascular collapse (9).

The splanchnic circulation contains ~20% of the total blood volume and receives a similar portion of the cardiac output (26). The resistance to blood flow offered by the splanchnic vasculature represents a major portion of the total peripheral resistance, and this bed is intimately involved in determining normal systemic blood pressure. Although they did not measure splanchnic blood flow, Kielblock et al. (12) suggested that circulatory collapse with heatstroke can be attributed to splanchnic vasodilation. We confirmed this hypothesis (15) and demonstrated that the decline in splanchnic resistance in the hyperthermic rat is not the result of *1*) a decrease in sympathetic nerve activity, *2*) a reduction in circulating catecholamines, or *3*) a direct effect on the vascular contractile machinery (10). These data suggest that this dilation is mediated locally, possibly through the enhanced local release of or sensitivity to an endogenous vasodilator.

Nitric oxide (·NO) has been implicated in a number of diverse physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission, and immune regulation (22). The physiological actions of ·NO are consistent with those of endothelium-derived relaxing factor (24). Moreover, ·NO has been reported to modulate intestinal and microvascular permeability (17, 18) as well as participate in the pathophysiology of endotoxic and hemorrhagic shock (34). Because ·NO is a potent vasodilator and endotoxemia is proposed to play a role in the pathophysiology of heatstroke (3), we hypothesized that enhanced local release of ·NO would precede hyperthermia-induced dilation of the splanchnic bed. ·NO readily complexes with heme proteins, forming paramagnetic species (·NO-heme) that are observable at low temperatures with electron paramagnetic resonance (EPR) spectroscopy (32). Using this technology, the aim of this study was to investigate the mechanisms underlying the vascular dysfunction and decrease in thermal tolerance associated with heatstroke by addressing the following questions. *1*) Are there EPR-detectable changes in the concentration of ·NO-heme in the portal venous blood (PVB) of conscious unrestrained rats during heating? *2*) If there are changes in the concentration of ·NO-heme, at what colonic temperature (T_c) do they occur? *3*) Do any observable changes in ·NO-heme concentration represent purely local or systemic phenomenon?

METHODS

Procedures. Nine male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 320–350 g were anesthetized with pentobarbital sodium (50 mg/kg ip, Nembutal). A midline laparotectomy was performed, the portal vein was isolated, and a catheter (Silastic tubing over PE-10, Clay Adams, Parsippany, NJ) was filled with heparinized saline (100 U/ml) and placed in the portal vein through a tributary vessel. The distal end of the catheter was tunneled subcutaneously to the dorsal neck and was capped with a stainless steel stylette. Three of these rats were also fitted with a femoral artery catheter for collecting arterial blood samples. The midline incision was closed, and the animals were allowed to recover from surgery for 5 h. On recovery, a colonic thermocouple probe (36-gauge copper-constantan wire in PE-100 tubing) was inserted

6–7 cm beyond the anal sphincter to monitor T_c. Conscious unrestrained animals were heated in an environmental chamber (40°C dry bulb, 30% relative humidity) using a ramp protocol until T_c reached 41.5°C, and then the animals were removed from the heat and allowed to cool passively at an ambient temperature of 22–24°C. Blood samples were collected in sterile 1-ml Monoject syringes (5) at T_c values of 37, 39, 40, 41, and 41.5°C and at 1, 2, 3, and 24 h post-heat exposure. Aliquots (200 μl) were immediately delivered into quartz EPR tubes (3 mm ID) and were cooled to 77°K. Weight-matched sham-operated control rats (n = 3) were surgically prepared and instrumented as described above but did not undergo heating. PVB and femoral arterial blood samples were collected from these animals at time points corresponding to the T_c values of their heated counterparts.

EPR analysis. EPR spectra were recorded with an ESP 300 EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with an ER035M gaussmeter, ER4111VT variable temperature unit, and EIP-625A microwave frequency counter. Signal averaging (multiple scans of the same sample) was used to improve the signal-to-noise ratio. Sample volume and geometry were kept constant to allow for comparisons of radical concentration between samples. All data were collected at 100°K with spectra reported as the normalized average of 20 scans. EPR conditions were receiver gain 5.00×10^5, modulation frequency 100 kHz, modulation amplitude 4.0 gauss (G), microwave frequency 9.43 GHz, microwave power 10.0 mW, and scan rate 6.2 G/s.

RESULTS

Both arterial and venous blood samples collected before heat exposure ($T_c \approx 37$°C) displayed weak EPR signals for three distinct species having values of the g factor for the unpaired electron (g) of 2.06, 2.021, and 2.005 (Fig. 1, *trace a*). The broad feature at $g \approx 2.06$, suggestive of a transition metal, is typical of a g factor of the magnetic field perpendicular to the axis of symmetry for copper. To confirm the association of this feature with copper, we scanned PVB and femoral arterial blood samples collected from normothermic and hyperthermic rats in the 2,500- to 3,700-G range. Indeed, weak copper hyperfine splitting features were observed in the region for the g factor of the magnetic field parallel to the axis of symmetry. The concentration of this species increased in PVB during heating, as evidenced by a 20% increase in signal intensity at $T_c > 39$°C, then nearly doubled 1 h post-thermal stress (Fig. 1, *traces a–f*). Its concentration remained elevated in PVB up to 24 h post-heat exposure (Fig. 1, *trace h*) and was the only species that exhibited an enhanced concentration in arterial blood (Fig. 1, *trace i*). Centrifugation of PVB samples followed by EPR analysis of the plasma and cell fractions demonstrated that this copper signal is entirely localized to the plasma fraction (Fig. 2, *trace a*). An EPR signal nearly identical to this, identified as the copper-binding protein ceruloplasmin, has been observed in whole blood collected from patients undergoing intravenous nitroglycerin therapy (6); however, the g value was reported as 2.05. Note that, after separation of the plasma and cell fractions, the apparent g value is 2.05. For further comparison, we analyzed a sample of pure ceruloplasmin (Sigma Chemical, St. Louis, MO) (Fig. 2, *trace b*). These spectra are identical (within the noise), suggesting that the signal at g = 2.06 is attributable to a hyperthermia-induced increase

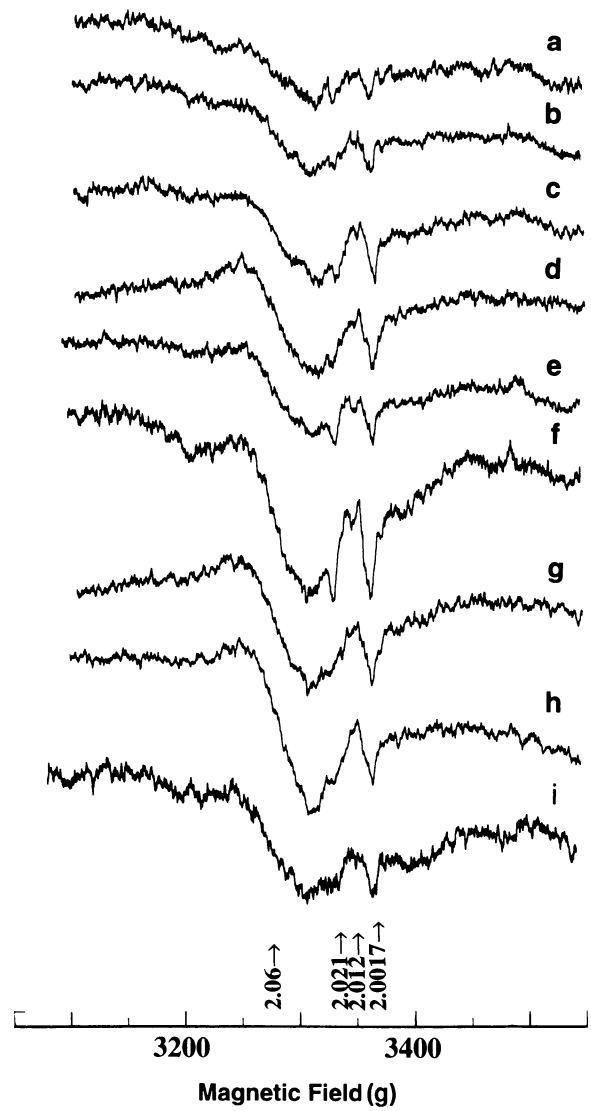

FIG. 1. Low-temperature (100°K) electron paramagnetic resonance (EPR) spectra recorded from portal venous and femoral arterial blood as function of core temperature. All spectra represent normalized sum of 20 scans. Rats were subjected to passive heating, and blood samples (200 μl) were drawn at colonic temperatures of ≈37, 39, 40, 41, and 41.5°C (*traces a–e*, respectively) or 1, 2, and 24 h post-heat exposure (*trace f–h*, respectively). *Trace i*: femoral arterial blood collected 1 h post-heat exposure. EPR conditions were receiver gain 5.00×10^5, modulation frequency 100 kHz, modulation amplitude 4.0 G, microwave frequency 9.43 GHz, microwave power 10.0 mW, and scan rate 6.2 G/s.

in the concentration of ceruloplasmin and that removal of the cell fraction eliminated the influence of other blood-borne radicals on the spectral conformation of this species.

The features at g = 2.021 and 2.005 have more narrow line widths typical of free radicals. The deflection at g = 2.005 (peak-to-peak EPR spectral line width ≈ 10 G) is characteristic of a semiquinone free radical (33). The intensity of these signals increased in PVB as T_c rose, and new features became apparent at g = 2.012 and 2.0017 (Fig. 1, *traces a–f*). The concentration of this new heat-induced species increased dramatically at $T_c > 39$°C, as evidenced by a twofold rise in spectral intensity of the features at g = 2.021, 2.012, and 2.0017 (Fig. 1, *traces a–e*). The increase in concentration is even more dra-

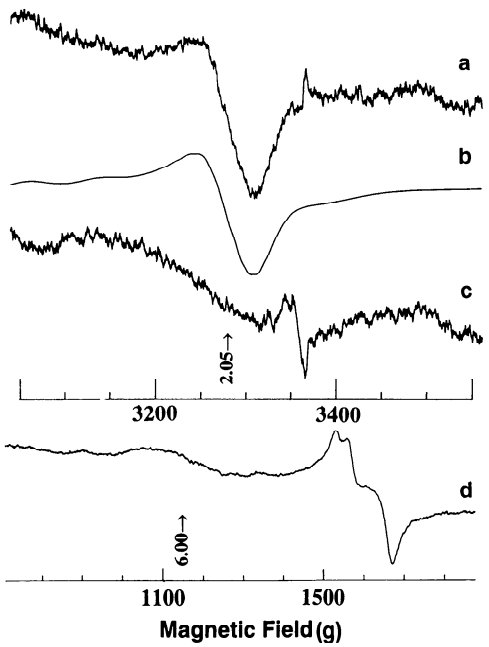

FIG. 2. Low-temperature (100°K) EPR spectra recorded from whole venous blood. *Trace a*: 1 h post-heat exposure, plasma fraction. *Trace b*: pure ceruloplasmin. *Trace c*: 1 h post-heat exposure, cell fraction. *Trace d*: 1 h post-heat exposure, scan centered at 1,400 G. EPR conditions were same as those in Fig. 1.

matic 1 h post-heat exposure (Fig. 1, *trace f*) as evidenced by a threefold rise in the intensity of these three features. The concentration of this species remained elevated for 2 h post-thermal stress and did not diminish until 3 h post-thermal stress. Aside from small differences in the noise level, spectra collected at $T_c > 39°C$ were essentially the same in all nine animals. EPR analysis of PVB plasma and cell fractions showed that this heat-induced species is completely localized to the cell fraction (Fig. 2, *trace c*). If this new species is an ·NO-heme complex, then three nitrogen hyperfine splitting features, representative of an unpaired electron localized around a nitrogen atom, should be present at g = 2.022, 2.012, and 2.002. This would be equivalent to a nitrogen triplet with a hyperfine splitting constant of 17.5 G. Indeed, the spectral location and apparent triplet structure of this heat-induced species are consistent with ·NO-heme; however, its interpretation is complicated because the lines at g = 2.012 and 2.0017 overlap those from the semiquinone signal. Further interpretation would require a method of decreasing the concentration of semiquinone radicals in our samples.

A search in the chemical literature revealed that the EPR spectral line shape of ·NO-heme complexes is pH dependent. At alkaline pH, ·NO binding to α- and β-chains is equivalent. At acidic pH, this is not the case (21, 25). Lowering the pH of PVB samples should change the tertiary and quaternary structure of heme proteins such that, at resonance, the ·NO-heme triplet centered at g = 2.012 is enhanced (21, 32, 25). Moreover, acidification should decrease the concentration of semiquinone radicals in solution by enhancing their rate of dismutation and subsequent decay (2). Therefore, we titrated PVB collected from hyperthermic animals with acetic acid to a final concentration of 90 mM, decreasing the pH from 7.4 to 4.6. Figure 3, *trace a*, represents PVB collected 1 h post-heat exposure. Decreasing the pH of this sample produced a signal, centered at g = 2.012, with a distinct three-line multiplet structure and a hyperfine splitting constant of 17.56 G (Fig. 3, *trace b*). These data are equivalent to the g values predicted for ·NO-heme and are consistent with EPR spectra representative of a five-coordinate ·NO-heme complex derived from ·NO-hemoglobin (·NO-Hb) (13, 14, 16, 21, 32, 34). For further comparison, we produced ·NO-heme in vitro by first purging vacutainer test tubes containing whole blood with argon gas and then very briefly exposing the contents to ·NO. The EPR spectrum from this sample (Fig. 3, *trace c*) displays the classic anisotropic spectrum of five-coordinate ·NO-Hb with the nitrosylated ferrous α-subunits $[(\alpha^{2+}NO)\beta^{3+}]_2$ (14, 16, 21, 25). Acidification enhanced the high-field nitrogen triplet feature, producing a stronger five-coordinate ·NO-heme signal (Fig. 3, *trace d*). Comparing the high-field triplet in *trace b* with the high-field

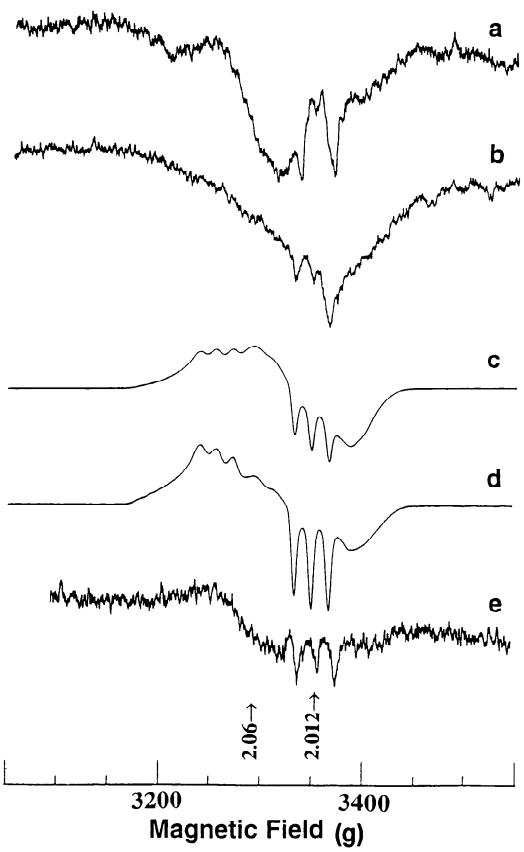

FIG. 3. Low-temperature (100°K) EPR spectra recorded from whole venous blood. *Trace a*: portal venous blood from heat-stressed rat collected 1 h post-heat exposure. This spectrum consists of ≥3 species. Broad feature at g = 2.06 is consistent with g feature for copper. Region around 2.012 contains 2 species, ·NO-heme with nitrogen hyperfine splitting constant = 17.56 G and semiquinone radical at g = 2.005 that overlaps high-field line of ·NO-heme. *Trace b*: sample from *trace a* treated with acetic acid to final concentration of 90 mM. *Trace c*: standard ·NO-heme generated by exposing whole blood to ·NO. *Trace d*: sample from *trace c* treated with acetic acid to final concentration of 90 mM. *Trace e*: portal venous blood from rat with core temperature >42.5°C. EPR conditions were same as those in Fig. 1.

triplets in *traces c* and *d* shows that all three exhibit three-line multiplet structures, centered at $g = 2.012$, with hyperfine splitting constants of 17.5 G. These g values and hyperfine splitting constants are identical (within the noise). The identification of this spectrum as ·NO-Hb is further corroborated by our observation of a signal at $g = 6$ (Fig. 2, *trace d*), which is typical for the high-spin Fe(III)heme in methemoglobin (14). When ·NO is bound to heme(II), exposure to oxygen results in the formation of Fe(III)heme (16); therefore, when ·NO complexes with heme in red blood cells under normoxic conditions, both ·NO-Hb and Fe(III)heme are formed (6). These data demonstrate the presence of enhanced concentrations of ·NO complexed with heme in the PVB of hyperthermic rats and that its concentration markedly increases at $T_c > 39°C$. Furthermore, this signal can be interpreted as a five-coordinate ·NO-heme complex that is most likely derived from ·NO-Hb.

Given the similarity between our spectra and those observed during endotoxic (13, 34) and hemorrhagic shock (34) in the rat, we attempted to induce cardiovascular shock by heating rats ($n = 2$) to $T_c > 42.5°C$. At this T_c, animals were unresponsive to external stimuli and died <40 min after sample collection. EPR spectra collected from these blood samples (Fig. 3, *trace e*) are notably similar to the ·NO-Hb EPR spectrum reported by Westenberger et al. (34) in venous blood collected from rats during hemorrhagic shock. Note that the triplet centered at $g = 2.012$ now dominates these spectra, which suggests that the concentration of semiquinone radicals is decreased or that the concentration of ·NO-heme is proportionally large enough that its spectra overshadows other features. Also, the g values and hyperfine splitting constants of the triplet centered at $g = 2.012$ are identical (within the noise) to those of ·NO-heme produced in vitro (Fig. 3, *trace c*) and those of acidified PVB collected 1 h post-heat exposure (Fig. 3, *trace b*). These data are consistent with the premise that severe heat stress results in shock and suggest that ·NO released from the splanchnic vasculature plays a role in this process.

DISCUSSION

Our observation of increased concentrations of EPR-detectable complexes in the portal circulation of hyperthermic rats provides exciting new information regarding the pathophysiology of heatstroke and opens a new avenue of research into the mechanisms and consequences of intravascular radical generation during whole body hyperthermia. For the first time, we have demonstrated that severe heat stress results in increased concentrations of metal-binding proteins and semiquinone radicals in the splanchnic circulation of the rat. Their source remains obscure, but cellular electron transport chains have been established as a major site of semiquinone radical production during periods of enhanced metabolism and/or oxidative stress (33). The idea that hyperthermia produces cellular oxidative stress is novel and warrants further investigation. Even more exciting is the observation that high elevations of T_c produced a markedly enhanced concentration of ·NO complexed with heme in the PVB of all nine of the hyperthermic animals we studied. Moreover, this ·NO-heme signal demonstrated a remarkable uniformity among all heat-stressed animals, consistently increasing at $T_c > 39°C$ with sustained elevations up to 2 h post-heat exposure. This change in ·NO-heme concentration is localized to the splanchnic circulation, as corresponding arterial blood samples did not prominently display this signal (Fig. 1, *trace i*). Thus, these results promote the hypothesis that enhanced release of ·NO within the splanchnic circulation precedes splanchnic vasodilation and suggest that this phenomenon may contribute to local vascular dysfunction in the heat-stressed rat.

The enhanced ·NO-heme signal that we observed in hyperthermic animals is attributed primarily to increased "production" of ·NO; however, it is possible that a portion of this signal could be attributed to increased scavenging of ·NO by deoxyhemoglobin. Both Fe(II)- and Fe(III)-Hb exhibit a high affinity for ·NO (31). The ratio of the rates of ·NO uptake and release for Fe(II)-Hb is five to six orders of magnitude greater than that of oxygen (31). The association rate constants with deoxyhemoglobin are comparable ($\sim 5 \times 10^7\ M^{-1} \cdot s^{-1}$), but the dissociation rate constants are substantially different ($\sim 10^{-5}\ s^{-1}$ for ·NO and $\sim 20\ s^{-1}$ for O_2; Ref. 31). Therefore, an increase in the concentration of deoxyhemoglobin would produce an increase in ·NO-heme concentration in PVB. However, the strongest ·NO-heme signal that we observed occurred 1 h post-thermal stress and persisted at a level greater than twofold that observed under normothermic conditions for another 1 h. At this time, T_c had declined to pre-heat stress levels. Preliminary results from our laboratory indicate that arterial oxygen saturation is also normal 1 h post-heat exposure, arguing against enhanced scavenging of ·NO by deoxyhemoglobin as an important mechanism in our system. A logical progression of experiments designed to extend these observations would be to study changes in blood gas concentration across the splanchnic bed during hyperthermia and to correlate these data with indexes of cellular hypoxia. This project is currently in progress.

These experiments are necessarily observational in nature; consequently, the mechanism(s) of production and site of ·NO release remain obscure. However, evidence is accumulating that during severe heat stress, as blood flow is shunted away from the splanchnic region to facilitate heat transfer, direct thermal injury or ischemia-reperfusion-induced damage to the intestinal mucosa allows bacterial endotoxins to escape the intestinal lumen and enter the portal circulation where they stimulate a cascade of cardiovascular events similar to those observed during septic shock (3). In support of this model, previous investigations have documented endotoxemia and elevated plasma levels of tumor necrosis factor-α and interleukin-1β in fatal (7, 11) and nonfatal (3) cases of classic heatstroke. Ultramarathon runners, candidates for exertional heatstroke, have also been found to be endotoxemic (4). Putative ·NO-Hb EPR signals with g values, hyperfine structures, and splitting constants nearly identical to those of the signal we observed have been reported in rat models of endotoxin

shock (34) and after tumor necrosis factor and interleukin-1 challenge (13). These cytokines play a prominent role in expanding the acute immune response after antigen challenge, and both induce the calcium-independent isoform of nitric oxide synthase (NOS) in target cells. Inducible NOS produces ·NO continuously, leading to prolonged smooth muscle relaxation, reduced responsiveness to vasoconstrictors, and possibly tissue damage (22). This induction occurs in both endothelial and smooth muscle cells as well as in activated leukocytes. We have shown that there is dramatic mucosal damage to the small intestine of the rat after heat stress (28), and we have speculated that the cardiovascular derangements characteristic of prolonged heat exposure are in part due to splanchnic endotoxemia. The results of this investigation support this postulate and suggest that the increased ·NO production seen after heat stress could be mediated through an inducible isoform of NOS.

Although we have speculated that hyperthermia leads to splanchnic endotoxemia, the mechanism by which gut permeability is enhanced during heat stress remains undefined. However, increasing evidence supports a role for endogenous ·NO production in the control of both intestinal and microvascular permeability. Kubes (17) demonstrated that infusion of the ·NO synthesis inhibitor nitro-L-arginine methyl ester into the mesenteric artery of cats significantly increased ischemia-reperfusion-induced intestinal permeability. In a subsequent study, Kubes et al. (23) reported that exogenous sources of ·NO attenuated reperfusion-induced mucosal barrier dysfunction independent of alterations in intestinal blood flow. These data suggest that enhanced production of ·NO, through a constitutive NOS, acts to protect the gastric mucosa during periods of ischemic challenge. Possible mechanisms by which ·NO is protective may involve several avenues of cytotoxic superoxide anion scavenging (27) and inhibitory effects on inflammatory cells (8, 18, 19, 29). In our experiments, the observed ·NO-heme signal could therefore be a marker of increased ·NO release within the splanchnic region as part of a mechanism designed to protect activated vascular cells from reactive oxygen species damage, modulate leukocyte diapedesis, or preserve the integrity of the gastric mucosa.

In conclusion, we have shown that prolonged heat exposure stimulates the formation of EPR-detectable species within the splanchnic circulation, including an ·NO-heme complex that is consistent with a five-coordinate valency hybrid ·NO-Hb. The concentration of this species quantitatively increases in PVB at $T_c > 39°C$ and remains elevated for >2 h post-heat exposure. Although these experiments do not allow for specific conclusions concerning the mechanism(s) of formation or the site of release for the ·NO-heme we observed, our data are consistent with the tenet that splanchnic endotoxemia plays a role in the pathophysiology of heatstroke and suggest that enhanced ·NO release may be protective after heat stress.

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