Carbon monoxide is the heme oxygenase product with a pyretic action: evidence for a cGMP signaling pathway

ALEXANDRE A. STEINER AND LUIZ G. S. BRANCO
Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto and Departamento de Fisiologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14040-904 Ribeirão Preto, São Paulo, Brazil

Received 7 July 2000; accepted in final form 20 September 2000

Steiner, Alexandre A. and Luiz G. S. Branco. Carbon monoxide is the heme oxygenase product with a pyretic action: evidence for a cGMP signaling pathway. Am J Physiol Regulatory Integrative Comp Physiol 280: R448–R457, 2001.—We have recently reported that the central heme oxygenase (HO) pathway has an important role in the genesis of lipopolysaccharide fever. However, the HO product involved, i.e., biliverdine, free iron, or carbon monoxide (CO), has not yet been identified with certainty. Therefore, in the present study, we tested the thermoregulatory effects of all HO products. Body core temperature ($T_c$) and gross activity of awake, freely moving rats was measured by biotelemetry. Intracerebroventricular administration of heme-lysinate (152 nmol), which induces the HO pathway, evoked a marked increase in $T_c$, a response that was attenuated by intracerebroventricular pretreatment with the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), indicating that an HO product has a pyretic action in the central nervous system (CNS) of rats. Besides, heme-lysinate also increased gross activity, but no correlation was found between this effect and the increase in $T_c$. Moreover, intracerebroventricular biliverdine or iron salts at 152 nmol, a dose at which heme-lysinate was effective in increasing $T_c$, produced no significant change in $T_c$. Accordingly, intracerebroventricular treatment with the iron chelator deferoxamine elicited no change in basal $T_c$ and did not affect hemoglobin-induced pyresis. However, heme-induced pyresis was completely prevented by the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-d]pyrimidine (ODQ), but not by the enzyme heme oxygenase (HO) pathway, three members of which have been identified to date (HO-1, HO-2, and HO-3), with HO-1 and HO-2 being the most studied and best known (for review see Ref. 23). HO-2 is constitutively expressed throughout the body, including the central nervous system (CNS), whereas HO-1 is absent or expressed at low levels in tissues, but it can be overexpressed in response to a series of stimuli (10, 19, 20, 23, 30, 41), including lipopolysaccharide (LPS) (19, 20), which has been used frequently to induce fever in experimental animals (21).

Consequently, we hypothesized that CO could mediate and/or modulate LPS fever. In support, we recently reported that intracerebroventricular injection of the nonselective HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) attenuates intraperitoneal LPS fever in rats, suggesting that the central HO pathway has a role in fever generation (39). Additionally, we have observed (37, 39) that intracerebroventricular administration of heme-lysinate, which is known to induce the HO pathway (2, 28, 30, 35, 36, 41), elicits a rise ($\Delta T_c$) in body core temperature ($T_c$), which is reversed by pretreatment with ZnDPBG, indicating that an HO product has a pyretic effect in the CNS. To determine whether CO is the HO product involved, we then injected CO-saturated saline intracerebroventricularly and observed only a slight increase in $T_c$ ($\Delta T_c\sim 0.5^\circ C$ compared with the group that received heme-lysinate ($\Delta T_c\sim 1.5^\circ C$) (39). This less-pronounced effect of CO-saturated saline may have been due to 1) an insufficient amount of CO delivered to the brain, 2) a rapid diffusion of the gas out of the brain because this gas has a high affinity for hemoglobin, or 3) the fact that another HO product (biliverdine or iron) is involved. Taken together, these results imply that the central modulators (21), including nitric oxide (NO) (1, 13).

Recent evidence has accumulated that the gas carbon monoxide (CO) is an important signaling molecule, acting as a vasoactive substance and a neurotransmitter and/or neuromodulator (for a review see Refs. 9 and 17). A growing number of studies has indicated that CO, similarly to NO, acts mostly via activation of soluble guanylate cyclase (sGC), leading to a rise in cGMP levels (23, 26). Endogenous CO arises from the cleavage of the heme molecule yielding equimolar amounts of biliverdine, free iron, and CO, a process catalyzed by the enzyme heme oxygenase (HO) family, three members of which have been identified to date (HO-1, HO-2, and HO-3), with HO-1 and HO-2 being the most studied and best known (for review see Ref. 23). HO-2 is constitutively expressed throughout the body, including the central nervous system (CNS), whereas HO-1 is absent or expressed at low levels in tissues, but it can be overexpressed in response to a series of stimuli (10, 19, 20, 23, 30, 41), including lipopolysaccharide (LPS) (19, 20), which has been used frequently to induce fever in experimental animals (21).

Consequently, we hypothesized that CO could mediate and/or modulate LPS fever. In support, we recently reported that intracerebroventricular injection of the nonselective HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) attenuates intraperitoneal LPS fever in rats, suggesting that the central HO pathway has a role in fever generation (39). Additionally, we have observed (37, 39) that intracerebroventricular administration of heme-lysinate, which is known to induce the HO pathway (2, 28, 30, 35, 36, 41), elicits a rise ($\Delta T_c$) in body core temperature ($T_c$), which is reversed by pretreatment with ZnDPBG, indicating that an HO product has a pyretic effect in the CNS. To determine whether CO is the HO product involved, we then injected CO-saturated saline intracerebroventricularly and observed only a slight increase in $T_c$ ($\Delta T_c\sim 0.5^\circ C$ compared with the group that received heme-lysinate ($\Delta T_c\sim 1.5^\circ C$) (39). This less-pronounced effect of CO-saturated saline may have been due to 1) an insufficient amount of CO delivered to the brain, 2) a rapid diffusion of the gas out of the brain because this gas has a high affinity for hemoglobin, or 3) the fact that another HO product (biliverdine or iron) is involved. Taken together, these results imply that the central

FEVER IS A MULTIMEDIATED process that involves the synthesis and release of endogenously formed mediators and is under the control of innumerable modulators and modifiers (21), including nitric oxide (NO) (1, 13).

Recent evidence has accumulated that the gas carbon monoxide (CO) is an important signaling molecule, acting as a vasoactive substance and a neurotransmitter and/or neuromodulator (for a review see Refs. 9 and 17). A growing number of studies has indicated that CO, similarly to NO, acts mostly via activation of soluble guanylate cyclase (sGC), leading to a rise in cGMP levels (23, 26). Endogenous CO arises from the cleavage of the heme molecule yielding equimolar amounts of biliverdine, free iron, and CO, a process catalyzed by the enzyme heme oxygenase (HO) family, three members of which have been identified to date (HO-1, HO-2, and HO-3), with HO-1 and HO-2 being the most studied and best known (for review see Ref. 23). HO-2 is constitutively expressed throughout the body, including the central nervous system (CNS), whereas HO-1 is absent or expressed at low levels in tissues, but it can be overexpressed in response to a series of stimuli (10, 19, 20, 23, 30, 41), including lipopolysaccharide (LPS) (19, 20), which has been used frequently to induce fever in experimental animals (21).

Consequently, we hypothesized that CO could mediate and/or modulate LPS fever. In support, we recently reported that intracerebroventricular injection of the nonselective HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) attenuates intraperitoneal LPS fever in rats, suggesting that the central HO pathway has a role in fever generation (39). Additionally, we have observed (37, 39) that intracerebroventricular administration of heme-lysinate, which is known to induce the HO pathway (2, 28, 30, 35, 36, 41), elicits a rise ($\Delta T_c$) in body core temperature ($T_c$), which is reversed by pretreatment with ZnDPBG, indicating that an HO product has a pyretic effect in the CNS. To determine whether CO is the HO product involved, we then injected CO-saturated saline intracerebroventricularly and observed only a slight increase in $T_c$ ($\Delta T_c\sim 0.5^\circ C$ compared with the group that received heme-lysinate ($\Delta T_c\sim 1.5^\circ C$) (39). This less-pronounced effect of CO-saturated saline may have been due to 1) an insufficient amount of CO delivered to the brain, 2) a rapid diffusion of the gas out of the brain because this gas has a high affinity for hemoglobin, or 3) the fact that another HO product (biliverdine or iron) is involved. Taken together, these results imply that the central

Address for reprint requests and other correspondence: L. G. S. Branco, Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto/USP, 14040–904 Ribeirão Preto, São Paulo, Brazil (E-mail: branco@forp.usp.br).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
In the endogenous CO and fever study, the HO pathway plays a significant role in fever and thermoregulation. This pathway has not yet been fully identified.

In fact, biliverdine and iron have been shown to have physiological actions. Iron can promote lipid peroxidation, modulate gene expression, and induce sGC in a direct manner. Biliverdine and its derivative bilirubin are antioxidative and as such may affect oxidative processes, possibly including those dependent on NO.

Therefore, the present study was designed to test the hypothesis that CO, rather than free iron or biliverdine, is a pyretic molecule in the CNS of rats. This was done by testing the thermoregulatory effects of all HO products. Moreover, to extend the knowledge about the participation of CO in the febrile response, the effect of intracerebroventricular ZnDPBG, an HO inhibitor, on intravenous LPS fever was verified because we had tested only intraperitoneal LPS previously, and several studies have already observed different results depending on the route of LPS administration.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on adult male Wistar rats weighing 250–300 g, housed at controlled temperature (26.0 ± 1.0°C), and exposed to a daily 12:12-h light-dark cycle with lights on at 6 AM. The animals were allowed free access to water and food. Experiments were performed between 8 AM and 4 PM.

**Drugs**

Biliverdine and the HO inhibitor ZnDPBG were obtained from Porphyrin Products. Both biliverdine and ZnDPBG were dissolved in 50 mmol/l Na₂CO₃ and stored in the dark. LPS (from *Escherichia coli*, serotype 0111:B4), deferoxamine mesylate, ferric chloride (FeCl₃), and ferrous sulfate (FeSO₄) were purchased from Sigma Chemical (St. Louis, MO) and dissolved in pyrogen-free sterile saline. Heme-1-lysinate (38 mmol/l) was prepared as previously described. Heme-free preparations were used as amino acid (1-lysine) vehicle control solutions. The sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) was purchased from Trocris Cookson (St. Louis, MO) and dissolved in 1% DMSO in pyrogen-free sterile saline; 1% DMSO was used as vehicle control solution.

**Surgery**

Animals were anesthetized with 2,2,2-tribromoethanol (Aldrich) at the dose of 250 mg/kg ip and fixed in a stereotaxic frame. A stainless steel guide cannula (0.7 mm OD) was introduced into the right lateral cerebral ventricle (coordinates: A, −1.0 mm; L, −1.6 mm; D, 3.2–3.7 mm) (29). The displacement of the meniscus in a water manometer ensured correct positioning of the guide cannula in the lateral ventricle. The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion and infection. Immediately after, each animal was removed from the stereotaxic frame and a paramedian laparotomy was performed to insert a biotelemetry probe capsule (model ER-4000 temperature/activity; Mini-Mitter, Sunriver, OR) into the peritoneal cavity. The wound was then closed with skin sutures, and the implanted capsule allowed measurements of Tc and gross activity. The surgical procedure was performed over a period of 30–40 min. After surgery, animals were treated with 100,000 units of benzyl-penicillin and allowed to recover for 1 wk.

Animals designated to receive intravenous injections of LPS were submitted to a second surgery under anesthesia with 2,2,2-tribromoethanol, to implant a Sylastic catheter through the external jugular vein according to the technique described by Arms and Ojeda (4). After surgery, animals were treated with 100,000 units of benzyl-penicillin and allowed to recover for 4 days before experimentation. During this period the catheters were flushed daily with heparinized saline; on the day preceding the experiment heparinized saline was replaced with saline only.

**Tc and Gross Activity Measurements**

Tc was measured by biotelemetry. On the day before the experiment, fully conscious rats previously implanted with the telemetry probes were placed in the experimental cages. On the day of the experiment, Tc was measured by moving gently each cage on a Mini-Mitter receiver (model ER-4000, Mini-Mitter) each time Tc was to be measured, a procedure to which the animals had been trained during the 2 days preceding experimentation. Tc values were collected over a 5-min interval. The receiver was connected to a computer, in which the frequency emitted by the probe could be read. The frequency of each probe had a corresponding Tc value in a calibration table. The measurements were performed at 30-min intervals to minimize possible stress due to cage movement. Continuous monitoring of Tc was not possible because of technical difficulties, because at the time we had only one receiver used to collect data from six animals. Gross activity was determined simultaneously with Tc. Gross activity values were displayed directly in the computer without the need for previous calibration.

This methodology has been used in a previous study (37) and is validated by our observations of stable baseline Tc and gross activity values in animals that received no treatment or vehicle (see Fig. 2B). It could be argued that a 5-min interval is short for determination of gross activity. However, our results indicate that this period is long enough for discriminating increases in gross activity in our experimental protocols (see Fig. 3B).

**Experimental Protocols**

**Protocol 1**: determination of the effect of intracerebroventricular ZnDPBG on intravenous LPS-induced fever. Rats previously cannulated in the lateral cerebral ventricle were left undisturbed for at least 24 h before the experiment, after which initial Tc (Tcᵢ) was determined by three measurements made at 30-min intervals. Rats were then treated with an intravenous bolus injection of LPS (10 µg/kg). Two hours later, the animals were injected intracerebroventricularly with the HO inhibitor ZnDPBG (200 nmol in 4 µl) or the same volume of vehicle. Tc was measured at 30-min intervals for 5 h after intravenous administration of LPS.

**Protocol 2**: Determination of the central effect of the heme-lysinate preparation on Tc and gross activity. After Tcᵢ was determined, rats were treated intracerebroventricularly with
heme-lysinate (152 nmol in 4 μl) or the same volume of the t-lysine-vehicle mixture, and Tc, and gross activity were measured every 30 min for 5 h after the injections. This dose of heme-lysinate was chosen on the basis of previous studies (37, 39). A 10-μl Hamilton syringe and a dental injection needle (Missy, 200 μm OD) were used for all the intracerebroventricular injections. Injection was performed over a period of 1 min, and another minute was allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux.

In another set of experiments, Tci was measured and animals were injected with ZnDPBG (200 nmol in 4 μl) or its vehicle (Na2CO3, 50 mM). Thirty minutes later, heme-lysinate (152 nmol in 4 μl) was injected into the lateral ventricle of both groups. Another group of animals received only intracerebroventricular ZnDPBG or its vehicle. Tc, and gross activity were measured every 30 min for a period of 5 h after the last injection.

Protocol 3: determination of the central effect of biliverdine on Tc. After Tci was determined, animals received an intracerebroventricular injection of biliverdine (152 nmol in 4 μl) or its vehicle (Na2CO3, 50 mM) and Tc was measured every 30 min for a period of 5 h. This dose of biliverdine was chosen because heme-lysinate at the same dose has been shown to increase Tc by ~1.5°C.

Protocol 4: determination of the central effects of free iron on Tc. The Tci of animals prepared as described was measured, and the rats were injected intracerebroventricularly with the iron chelator deferoxamine (250 μg in 2 μl) or its vehicle (pyrogen-free sterile saline); Tc was determined as in protocol 1. The dose of deferoxamine used in the present study was about 1,000-fold higher than the minimal effective dose habitually used (5) to guarantee that all free iron in the brain was chelated.

To determine the role of free iron in the rise in Tc induced by intracerebroventricular heme, another group of animals was pretreated with deferoxamine (250 μg in 2 μl) or its vehicle 30 min before intracerebroventricular administration of heme-lysinate (152 nmol in 4 μl). Tc was determined as in protocol 1. Ferrous and ferric salts were also administered intracerebroventricularly to confirm a possible thermoregulatory effect of iron. Accordingly, rats were treated intracerebroventricularly with ferric chloride (FeCl3, 152 nmol in 4 μl) or ferrous sulfate (FeSO4, 152 nmol in 4 μl), and Tc was measured for 5 h. This dose was chosen because heme-lysinate at the same dose has been shown to increase Tc by ~1.5°C.

Control animals received intracerebroventricular injections of sodium salts of the anions used, i.e., NaCl (saline) and Na2SO4.

Protocol 5: determination of the effect of the sGC inhibitor ODQ on the intracerebroventricular heme-induced rise in Tc. After Tci was measured rats were injected intracerebroventricularly with ODQ (1 μg in 4 μl), a sGC inhibitor (11, 27) or its vehicle (4 μl of 1% DMSO in saline). The dose of ODQ was chosen on the basis of previous studies (11, 15, 46) and because when preliminary doses were tested the dose of 1 μg intracerebroventricularly produced the most consistent and repeatable results. Tc was measured for 5 h after the injections.

Another group of animals also received intracerebroventricular ODQ (1 μg in 4 μl) or its vehicle (4 μl of 1% DMSO in saline), but now heme-lysinate (152 nmol in 4 μl) was administered intracerebroventricularly 30 min later. Tc was measured for 5 h.

**RESULTS**

In all experimental protocols, Tc ranged from 36.5 to 37.6°C during the control period, and no difference in Tci values was observed among the different groups; Tci values are shown in the figures. During the experiments, room temperature was 26.2 ± 0.7°C.

**Protocol 1: Effect of Intracerebroventricular ZnDPBG on Intravenous LPS-Induced Fever**

Intravenous injection of pyrogen-free sterile saline caused no change in Tc, whereas LPS (10 μg/kg) evoked a fever that started 1.5 h after injection. Intracerebroventricular administration of ZnDPBG (200 nmol) 2 h after intravenous LPS significantly attenuated the febrile rise in Tc compared with the group that received the vehicle of ZnDPBG. These data are plotted in Fig. 1.

---

Fig. 1. Effects of intracerebroventricular (icv)-injected zinc deuteroporphyrin 2,4-bis glycol [ZnDPBG, 200 nmol, a heme oxygenase (HO) inhibitor] or its vehicle (Na2CO3, 50 mM) on the body core temperature (Tc) of conscious rats previously injected intravenously (iv) with saline or lipopolysaccharide (LPS, 10 μg/kg). Data are expressed as changes from Tci, relative to their initial levels (Tci; see text for details). Arrows, times of injections. Values are means ± SE; number of animals in parentheses. Intravenous LPS caused a significant (P < 0.05) increase in Tc, but saline did not affect Tc. Intravenous LPS-induced fever was attenuated (P < 0.05) by intracerebroventricular ZnDPBG, whereas Na2CO3 did not affect the response.
Protocol 2: Effect of Intracerebroventricular Heme-Lysinate on $T_c$ and Gross Activity

Intracerebroventricular administration of the nonselective HO inhibitor ZnDPBG (200 nmol) caused no significant change in $T_c$ or gross activity (Fig. 2). On the other hand, intracerebroventricular heme-lysinate (152 nmol) produced a highly significant increase in $T_c$, which was already elevated 30 min after injection and continued to rise until 2 h, after which a plateau phase was reached. No change in $T_c$ was observed after intracerebroventricular injection of heme-free lysinate control solution (Fig. 3A). Moreover, we also observed that intracerebroventricular heme-lysinate produced a rapid elevation in gross activity, which returned completely to baseline values 3 h after injection; lysinate (vehicle) preparations did not alter gross activity (Fig. 3B). The areas under the $T_c$ (thermal index) and gross activity (gross activity index) curves were calculated for each animal to compare the magnitude of the increases in $T_c$ and in gross activity evoked by heme-lysinate, from which a linear correlation was obtained. As shown in Fig. 3C, there was no correlation between the rise in $T_c$ and gross activity induced by intracerebroventricular injection of heme-lysinate ($r^2 = 0.08588$).

Pretreatment with ZnDPBG significantly attenuated the elevation in $T_c$ (Fig. 3A) and completely abolished the increase in gross activity (Fig. 3B) induced by intracerebroventricular heme-lysinate, whereas pretreatment with the ZnDPBG vehicle did not affect the responses.

Protocol 3: Effect of Intracerebroventricular Biliverdine on $T_c$

Neither intracerebroventricular administration of biliverdine (152 nmol) nor its vehicle altered $T_c$ of rats. These data are depicted in Fig. 4.

Protocol 4: Effects of Brain Iron on $T_c$

Intracerebroventricular injection of the iron chelator deferoxamine (250 μg) caused no change in $T_c$ (Fig. 5A) and did not affect the increase in $T_c$ produced by intracerebroventricular heme-lysinate (Fig. 5B).

Moreover, we observed that saline or ferric chloride (152 nmol) administered intracerebroventricularly caused no change in $T_c$ of rats (Fig. 6A). As to the ferrous ion, Fig. 6B shows that the control injection of sodium sulfate per se produced a significant long-lasting elevation in $T_c$, an effect that did not differ from that of ferrous sulfate (152 nmol). In short, the presence of ferrous iron did not produce any thermoregulatory effect.

Protocol 5: Effect of sGC Inhibitor ODQ on Intracerebroventricular Heme-Induced Rise in $T_c$

As shown in Fig. 7A, the vehicle of ODQ (1% DMSO) injected intracerebroventricularly did not affect the $T_c$ of rats, whereas intracerebroventricular ODQ (1 μg) elicited a slight rise in it, which continued until the end of the experiment.

Moreover, we observed that 1% DMSO did not alter the intracerebroventricular heme-induced rise in $T_c$, but intracerebroventricular pretreatment with ODQ completely prevented the thermoregulatory response to heme-lysinate. These results are plotted in Fig. 7B.

DISCUSSION

The major finding of the present study is that CO is the HO product with a pyretic action in the CNS of rats, acting through a cGMP-dependent pathway. In support, we observed that intracerebroventricular injections of the HO products biliverdine or iron salts had no thermoregulatory effects and that the iron chelator agent deferoxamine did not affect either basal $T_c$ or the increase in $T_c$ evoked by activation of the HO pathway using heme-lysinate, suggesting that biliverdine and iron did not affect thermoregulation by acting in the
Moreover, it was demonstrated that intracerebroventricular administration of the sGC inhibitor ODQ completely abolished the rise in \( T_c \) evoked by heme-lysinate, indicating that an HO product has a pyretic role in the CNS acting through a cGMP-dependent pathway. Because CO has been shown to produce most of its actions via activation of sGC (23, 26), these data strongly imply that CO is the HO product with a pyretic action in the CNS.

Since the beginning of the 1990's, a growing body of evidence has given support to the physiological actions of the gaseous compound CO, which has been shown to be a vasoactive substance and to act as a neurotransmitter/neuromodulator (for review, see Refs. 9 and 17). HO is the enzyme responsible for CO biosynthesis and has been found to be expressed in the CNS of several species, including rats (10, 19, 20) and humans (41). Accordingly, evidence has accumulated that the HO/CO pathway has a substantial role in the control of blood pressure (for review, see Ref. 17) and of the neuroendocrine function (24), but until recently no report existed about the participation of the HO-CO pathway in thermoregulation. However, we have recently demonstrated (39) that intracerebroventricular injection of the nonselective HO inhibitor ZnDPBG causes no change in basal \( T_c \) (a fact confirmed in the

Fig. 3. A: effects of intracerebroventricularly injected heme-lysinate (152 nmol) or its vehicle (L-lysine preparation) on the \( T_c \) of conscious rats. The effect of intracerebroventricularly injected ZnDPBG (200 nmol, an HO inhibitor) or its vehicle (Na$_2$CO$_3$, 50 mM) on \( T_c \) of conscious rats injected intracerebroventricularly with heme-lysinate (152 nmol) is also shown. Data are expressed as changes from \( T_c \) relative to their \( T_{ci} \) (see text for details). B: effects of the same treatments as in A on gross activity of conscious rats. Data are expressed as absolute values. C: correlation between the rise in \( T_c \) and in gross activity evoked by intracerebroventricular heme-lysinate. Arrows, times of injections. Values are means ± SE; number of animals in parentheses. Intracerebroventricular injection of heme-lysinate elicited a significant \( (P < 0.05) \) increase in \( T_c \) and gross activity, whereas no change was observed after intracerebroventricular L-lysine. Pretreatment with ZnDPBG attenuated \( (P < 0.05) \) the elevation in \( T_c \) and prevented \( (P < 0.05) \) the rise in gross activity evoked by heme-lysinate. Regression analysis showed that there is no correlation between the increases in \( T_c \) and gross activity (\( r^2 = 0.08588 \)).
present study, Fig. 2A), but significantly attenuates the fever evoked by intraperitoneal LPS, showing that the central HO-CO pathway has no tonic role in the maintenance of basal $T_c$, but has an important role in the genesis of intraperitoneal LPS fever in rats. We now extend this effect to a fever evoked by intravenous LPS (Fig. 1). In support of this finding, it has been reported that HO-1 is overexpressed in response to LPS (19, 20) or to the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor-α (32), all of which have been shown to be involved in fever (21). Several mechanisms through which peripherally administered LPS signals the brain to produce fever have been proposed (for review, see Ref. 6), and different mechanisms have been found depending on the route of pyrogen administration (14, 22). Therefore, based on the fact that ZnDPBG attenuates the fever produced by either intraperitoneal or intravenous LPS, it is tempting to speculate that the HO/CO pathway is likely to be acting in the CNS in a common final pathway for both routes of LPS administration.

Acute overload of heme preparations such as heme-lysinate have been used by us (37, 39) and others (18) to induce the HO pathway in vivo, which is certainly an important tool to investigate the physiological actions of this pathway. In agreement with our previous obser-

**Fig. 5.** A: effects of intracerebroventricularly injected deferoxamine (250 μg, an iron chelator) or its vehicle (saline) on the $T_c$ of conscious rats. B: effects of intracerebroventricularly injected deferoxamine (250 μg) or its vehicle (saline) on $T_c$ of conscious rats injected intracerebroventricularly with heme-lysinate (lys; 152 nmol). Data are expressed as changes from $T_c$ relative to $T_{ci}$ (see text for details). Arrows, time of injections. Values are means ± SE; number of animals in parentheses. Intracerebroventricular deferoxamine caused no significant change in $T_c$ nor did it affect the rise in $T_c$ evoked by heme-lysinate.

**Fig. 6.** A: effects of intracerebroventricularly injected FeCl$_3$ (152 nmol) or its vehicle (saline) on the $T_c$ of conscious rats. B: effects of intracerebroventricularly injected FeSO$_4$ (152 nmol) or its control solution (Na$_2$SO$_4$, 152 nmol) on $T_c$ of conscious rats. Data are expressed as changes from $T_c$ relative to $T_{ci}$ (see text for details). Arrows, times of injection. Values are means ± SE; number of animals in parentheses. Neither FeCl$_3$ nor its vehicle caused any change in $T_c$, whereas both FeSO$_4$ and its control solution elicited a significant increase in $T_c$ ($P < 0.05$). There was no difference between the thermoregulatory response to FeSO$_4$ and its control solution.
heme overload in the brain produced a rapid rise in $T_c$, a response that was attenuated by pretreatment with the nonselective HO inhibitor ZnD-PBG (Fig. 3A), indicating that an HO product has a pyretic action in the CNS of rats. It is important to point out that $T_c$ was already elevated 30 min after intracerebroventricular heme-lysinate. Because most evidence supports that heme overload activates the HO pathway by inducing the transcription of HO-1 (2, 28, 30, 35, 36, 41) this result would imply that HO-1 is induced rapidly. In fact, there are data showing that HO-1 overexpression reaches maximum values within 1 h after the application of the stressing stimuli (10, 23). However, we believe that an increase in the activity of HO-2 in response to an excess of substrate, according to Michaelis-Menten kinetics, may also possibly explain the rapid elevation in $T_c$ after intracerebroventricular heme-lysinate. In agreement with our findings, a recent study (12) has observed that intracerebroventricular injection of hemoglobin increases $T_c$ of rabbits, but in that study the authors did not investigate whether this effect was related to the HO pathway.

It is important to emphasize that the effective dose of heme-lysinate and ZnDPBG, when administered systemically, is 45 $\mu$mol/kg (18), indicates that the thermoregulatory effects observed after intracerebroventricular injection of the ~80-fold smaller amount (152 and 200 nmol, respectively) are centrally mediated and not due to a systemic action of the drugs. Interestingly, we have recently observed that intraperitoneal administration of ZnDPBG at the dose of 45 $\mu$mol/kg per se decreases $T_c$ of euthermic rats (unpublished observations). Because intracerebroventricular injection of ZnDPBG causes no change in $T_c$, it seems that the HO-CO pathway may also have a thermoregulatory role in peripheral tissues independently of central control. Currently, a more careful and detailed study on this subject is being performed in our laboratory.

Besides increasing $T_c$, heme overload in the brain also led to an elevation in gross activity, a response that also seems to be mediated by an HO product because it was reversed by ZnDPBG (Fig. 3B). ZnD-PBG per se did not affect gross activity (Fig. 2B). This effect is likely to be associated with the activation of the HO pathway in brain regions other than those involved in thermoregulation. In fact, HO has been demonstrated to be expressed in the brain cortex (10, 41), where it could affect motor control. One could therefore speculate that the rise in $T_c$ produced by heme-lysinate is associated with the elevated gross activity. Nevertheless, this is not the case because we found no correlation between the increase in $T_c$ and gross activity after intracerebroventricular injection of heme-lysinate (Fig. 3C), suggesting that these effects are independent and may result from the action of an HO product in different brain regions. Anyway, because the understanding of the mechanisms underlying gross activity control was not the aim of the present study, this parameter was not considered further in the subsequent experimental designs.

At this point, it is clear that an HO product has a pyretic role in the CNS of rats, but the HO product involved still remains uncertain. All the products of HO, biliverdine, free iron, and CO, have been considered to have physiological actions, which will be discussed.

Biliverdine and its derivative bilirubin are well characterized by their antioxidant properties (40) and thus may interfere with oxidative processes to affect $T_c$. Accordingly, Riedel and Maulik (31) have proposed that the alteration of the redox state of the brain by free radicals produced after LPS administration could have a major role in fever. Moreover, biliverdine could possibly also increase the half-life of the free radical NO, which has been shown to have thermoregulatory actions in the CNS (1, 13, 38). However, we observed that intracerebroventricular administration of biliver-
dine, at the same dose at which heme overload produced a marked increase in $T_c$, caused no alteration in basal $T_c$ (Fig. 4). Therefore, biliverdine is unlikely to be the HO product with a pyretic action in the CNS.

In regard to free iron, it is known that the degradation of heme produces iron, but whether this iron is released in the ferrous (20) or in the ferric state (18, 33) remains controversial. The ferrous form of free iron may catalyze the formation of free radicals (43), which in turn could affect $T_c$ (31). Moreover, iron can also activate sGC in a direct manner (34) or even stimulate the overexpression of HO-1 (2, 8). Indeed, iron has been shown to be a modulator of gene expression (44). Despite this evidence, we observed that iron overload with both the ferric and ferrous forms did not affect $T_c$ compared with the group that received the vehicles (Fig. 6). One could therefore argue how can iron overload not affect $T_c$ if it may induce HO-1? The answer to this question may reside in the fact that this effect has been observed mostly in vitro (8) and the only study in which this effect was assessed in vivo demonstrated that HO-1 protein is increased by iron overload in the lungs but not in the heart or liver (2); according to the present results, it is likely that this effect is also not present in the brain. To further investigate the thermoregulatory role of free iron, we also used the iron chelator deferoxamine (5, 18). Figure 5 shows that intracerebroventricular treatment with deferoxamine did not affect either basal $T_c$ or the heme-induced rise in $T_c$, emphasizing that free iron is not the HO product with a pyretic action. With these results, it becomes also interesting to note that HO induction after heme overload has been reported to afford protection against oxidative damage and may thus counteract the effects of the free iron produced (30).

It is important to emphasize that sodium sulfate (the control salt for the intracerebroventricular injected ferrous sulfate) per se elicited an increase in $T_c$, an effect not altered by ferrous sulfate (Fig. 6B). The reason for this hyperthermic effect of the anion sulfate is unknown, but because sulfate is primarily an intracellular anion an increase in extracellular levels of this anion may affect the excitability and transport mechanisms across the CNS cell membranes. Accordingly, a sulfate-bicarbonate exchange in liver plasma membrane vesicles (25) and an electrogenic cotransport of sodium and sulfate in oocytes (7) already have been found. Whether these transport systems also are present in the brain still remains to be assessed.

Finally, the fact that neither biliverdine nor free iron has any thermoregulatory role in the CNS of rats leads to the rationale that CO is likely to be the HO product with a pyretic action. Because the majority of CO actions have been shown to be mediated by activation of sGC, we tested the effect of the sGC inhibitor ODQ (11, 27) on the rise in $T_c$ evoked by heme overload. Intracerebroventricular administration of ODQ only led to a slight increase in $T_c$ (Fig. 7). Assuming that all sGC activity in the CNS comes from the action of the gaseous compounds NO and CO and that inhibition of the central NO pathway causes a slight elevation in $T_c$ (1, 38), whereas inhibition of the CO pathway did not affect $T_c$ (39, Fig. 1), this result is not surprising. Moreover, we observed that intracerebroventricular pretreatment with ODQ completely blocked the elevation in $T_c$ evoked by intracerebroventricular heme, showing that this effect is mediated through a cGMP-dependent pathway. This result confirms that CO is the HO product with thermoregulatory effects because 1) CO exerts most of its actions through a cGMP-dependent pathway (23, 26) and 2) biliverdine, which is an antioxidative (40) and could activate sGC by increasing the half-life of NO, and iron, which could activate sGC in a direct manner (34), have no thermoregulatory role in the CNS of rats (Figs. 4–6). Therefore, CO is the only HO product to activate sGC and consequently to evoke an elevation in $T_c$.

In summary, the present data support the pyretic role of HO-derived CO in the CNS of rats by showing that the other HO products, biliverdine and free iron, have no thermoregulatory role. Moreover, this study also demonstrates that the pyretic effect of CO is dependent on activation of sGC, with consequent production of cGMP, and extends the participation of the central CO pathway mediating a fever evoked by LPS administered intravenously.

**Perspectives**

It is now almost 10 years since the first studies were published, suggesting that the gaseous compound CO could have a physiological action, acting similarly to NO (for review see Refs. 9, 17, and 23). Nowadays, CO is recognized as an important modulator of several physiological systems, among them the cardiovascular and endocrine. Immunohistochemical data had accumulated that HO-2 is widely expressed in the brain thermoregulatory areas, such as the hypothalamus (10, 41), and that HO-1 is overexpressed in the brain after LPS administration (19, 20), although probably not in the hypothalamus (16). However, only a year ago we first reported that the central HO/CO pathway has a role in fever genesis (39), an action that we now show to be dependent on CO with subsequent activation of sGC. Certainly, an immunohistochemical study searching for cGMP staining in distinct thermoregulatory brain areas after intracerebroventricular administration of heme-lysinate as well as LPS would be useful in determining the specific site where CO plays its pyretic role. Pharmacological manipulation of the HO/CO pathway in specific nuclei of the CNS by using microinjection techniques would also contribute to the knowledge related to the role of CO in fever. Furthermore, evidence about the HO isoform involved in thermoregulation would be important. Yet, it is tempting to speculate that even though CO appears to be the only pyretic product of HO, a modulatory or synergistic effect of the other HO products on the pyretic action of CO may not be excluded.

The mechanism by which CO participates in fever generation as well as the position of CO in fever cascade also remains poorly understood and explored. In
this context, we have recently observed that CO mediates fever through a pathway independent of cyclooxygenase (37), which is considered the proximal mediator of fever in the CNS (6, 21). Certainly, information is still needed to definitely establish CO and also NO in the context of the mechanisms involved in the febrile response, possibly providing the basis for development of new and useful strategies for pharmacological modulation of fever.

We thank Mauro F. C. Silva for technical assistance.

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and PRONEX. A. A. Steiner was the recipient of a FAPESP graduate scholarship.

REFERENCES

3. Aronson AL, Chretien ML, McLaughlin BE, Vreman HJ, Anning PB, Chen Y, Lamb NJ, Mumby S, Quinlan GJ, Arms PG and Ojeda SR. The iron chelator desferrioxamine (desferal) retards 6-hydroxy-

Methylene blue inhibits stimulatory effect of sodium nitroprusside but not of 3-morpholinol

13. Meier PJ, Valantinas J, Hugentobler G, and Rahm I. Bi-
carbonated sulfate exchange in canalicul ar rat liver plasma mem-
treatment: protective role of heme oxygenase-1 induction. Bio-
17. Pagnini G and Watson C. The Rat Brain in Stereotaxic Coor-
21. Schmidt HHHW. NO, CO and HO: endogenous soluble guany-
23. Shibahara S. Heme oxygenase - regulation of and physiological implication in heme catabolism. In: Regulation of Heme Protein


