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Gaseous neurotransmitters and their role in anapyrexia

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1. ABSTRACT

Mammals keep their body temperature (Tb) relatively constant even under a wide range of ambient temperature variation. However, in some particular situations it may be beneficial to increase or to decrease Tb. For instance, under hypoxic conditions, a regulated drop in Tb (anapyrexia) takes place which has been reported to be crucial for survival in a number of different species. This review highlights major advances in the research about nitric oxide (NO) and carbon monoxide (CO- where data are relatively less abundant), before focusing on the role played by these gaseous neuromediators in thermoregulation, under the conditions of euthermia and anapyrexia. Available data are consistent with the notion that both NO and CO, acting on the CNS, participate in thermoregulation, with NO decreasing Tb and CO increasing it. However further studies are required before definitive conclusions can be made as to their physiological mechanisms of action.

2. INTRODUCTION

The efforts towards the identification and characterization of a potent vasodilating substance produced by endothelial cells in the 1980s ended up providing a new paradigm in research and human health and disease. These ground breaking studies revealed that an endothelial cell-derived relaxing factor (EDRF) was a soluble gaseous molecule (1) and not a peptide, protein, lipid mediator, or nucleic acid as one would normally expect. This fact gave origin to the concept that a gaseous compound may be a signalling molecule in biological systems. The impact of nitric oxide (NO) on biomedical research and applications to human diseases since its discovery has been astounding.

It is interesting to note that it was in 1968-1969, i.e., about 20 years before NO was identified as EDRF, when Tenhunen *et al.* (2, 3) reported that cells can produce another endogenous gaseous molecule by an endogenous

enzymatic reaction, i.e., the catalytic breakdown of heme by the microsomal heme oxygenase (HO) enzyme producing carbon monoxide (CO), as well as iron and bilirubin. This finding remained relatively quiescent, unnoticed by the scientific community for about 25 years. Possibly it was over looked because of the well known fact that CO administration in high amounts induces the formation of blood carboxyhemoglobin, causing tissue hypoxia and thus can be lethal.

Another interesting gaseous neurotransmitter is hydrogen sulphide (H_2S), a colourless irritant and asphyxiant gas with a noxious odour of rotten eggs. Exposure to high levels of H_2S can cause symptoms ranging from mild mucous membrane irritation to permanent neurological impairment and cardiopulmonary arrest (4). Recently, H_2S was found to be generated endogenously in mammalian tissues by two pyridoxal-5' phosphate-dependent enzymes, namely cystathionine-β-synthase and cystathionine-γ-lyase (5). The physiological role of H_2S has been addressed in the nervous, cardiovascular and gastrointestinal systems. Increasing evidence supports H_2S as a gaseous cellular messenger and it therefore joins the other gaseous mediators NO and CO. Additionally, cellular toxicity of H_2S may result from its capacity to inhibit cytochrome c oxidase, therefore reducing oxidative phosphorylation and leading to cellular hypoxia (6). Interestingly, Blackstone and cols reported that mice exposed to a low concentration of H_2S (80 p.p.m.) developed a suspended animation-like state with a sequential decrease in metabolic rate and body temperature (Tb) and recovered without adverse side-effects (7). Perhaps H_2S-induced hypothermia may become a powerful pharmacological tool to protect against severe trauma or disease.

In this review we will focus on the gases NO and CO, since over the years, evidence has been growing in support of the importance of them as neuromodulators involved in the regulation of Tb.

3. NITRIC OXIDE (NO)

It is currently known that endogenously formed NO arises from the catabolism of L-arginine, resulting in the formation of L-citrulline and NO, a reaction catalyzed by the enzyme NO synthase (NOS) (8). Active NOS enzymes are dimeric and their activity also requires other factors. Three major isoforms of nitric oxide synthase are known: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). nNOS and eNOS are constitutively expressed enzymes and their activity is Ca^{2+}-dependent. Expression of iNOS is induced in response to inflammatory stimuli (lipopolysaccharide (LPS), for instance); the enzyme is not regulated by Ca^{2+}, but has calmodulin (CaM) associated with it. After being synthesized, NO takes part in a wide number of physiological processes such as smooth muscle relaxation, blood pressure and volume regulation, platelet aggregation, immunomodulation, axon outgrowth and guidance, cellular growth, apoptosis, proliferation, differentiation, and neurotransmission. NO is produced on demand and is not stored as other messengers. However, NO complexes may exist as stored precursors to release NO (9).

Like other free radicals, NO is highly reactive. *In vivo* concentrations of NO can range from low nanomolar to low micromolar. NO targets and cellular actions depend on its local concentrations and the availability of the target molecules. These include soluble guanylyl cyclase (sGC), the major NO receptor, which when activated by low nanomolar concentrations of NO, results in the elevation of intracellular cyclic GMP (cGMP) (10). This process is thought to be the major event triggered by low concentrations of NO. Other low NO concentration targets are transcription factors, cytochrome C oxidase, and catalase as well as thiol groups in various proteins which are nitrosated by NO on the cysteine residues (11). NOSs may also produce superoxide anion (O_2^{-}) or reactive nitrogen species other than NO (12). At higher concentrations, NO rapidly reacts with superoxide anion to form the very reactive peroxinitrite causing nitration of proteins involved in diverse cellular physiological processes (13, 14).

Therefore, the actions of NO can be classified into two different ways: cGMP-dependent and cGMP-independent. Here we review data providing solid evidence that when it comes to thermoregulation the effects of NO seem to be cGMP-dependent and involve the activation of sGC.

sGC is a heterodimer with alpha and beta subunits with a ferrous heme bound to histidine 105 of the beta subunit. In the absence of NO, sGC exhibits very low basal activity. Conformational changes following the binding of NO to heme result in marked activation of the enzyme. When NO binds to the ferrous iron, it changes the the enzyme, and activates the enzyme from 200- to 400-fold while decreasing the Michaelis Menton Constant (Km) for guanosine triphosphate as substrate (15). The resulting elevation in the intracellular cGMP concentrations triggers the activation of a number of signal transduction pathways that are responsible for regulating a number of physiological processes. cGMP can produce its effects by activating protein kinases that phosphorylate serine or threonine residues of a variety of proteins. Three isoforms of cGMP-dependent protein kinase (PKG) have been described, *i.e.*, 2 soluble isoforms and a particulated isoform. Protein phosphorylation can modify the structure and function of proteins or their enzymatic activity (*cf.*(16).

One may wonder how does an elevation of cellular cGMP levels resulting from the activation of sGC by NO regulate so many different physiological processes. One possible explanation may reside in the fact that there are a number of different cell types and their specific spatiotemporal regulation of sGC activity and the choice of signalling cascades regulated by NO-cGMP may also be involved. Thus, it sounds fairly suitable to discuss the regulation and localization of NOS activity. NOS regulation has been extensively studied (17). The expression of eNOS was first observed in endothelial cells

but later it was reported to be present in other cell types (18). All the three isoforms, nNOS, eNOS and iNOS, are expressed in other tissues such as the skeletal muscle (19) where NO may play a role by increasing contractile function (20) and altering glucose transport (21). In this context, NO arising from any NOS isoform in the skeletal muscle also may contribute to Tb regulation, once it is well established that shivering of skeletal muscle can be activated under cold conditions. Therefore, the hypothermic and antipyretic effects of nNOS inhibition by intraperitoneal injection of 7-nitroindazole (7-NI) observed in rats (22) might be, at least in part, due to decreased heat production from skeletal muscle.

There are examples for receptor stimulated activation of NOSs. Factors such as glutamate, histamine and acetylcholine have also been reported to stimulate the production of NO under a number of physiological conditions. Moreover, in endothelial cells, kinins can induce the synthesis of NO by stimulating B1 and B2 receptors (23) whereas steroid hormones such as estrogen can stimulate NO synthesis from eNOS by activating the G-protein $G_{\alpha}i$ (24). Estrogen as well as progesterone can also modulate NO effect on LPS-induced hypothermia in female mice (25). Ovariectomized inducible NO synthase knockout (KO) mice develop a more pronounced hypothermia after injection of a high dose of LPS than wild type ones, a response that is reverted after hormonal (estrogen and progesterone) replacement (25).

There is evidence that NOS activities may also be regulated through protein–protein interactions and lipid modifications. Such protein–protein interactions and posttranslational modifications are thought to allow NOS to act on specific intracellular compartments, permitting a spatial resolution of signals transmitted by NO to take place (26, 27). As recently reported in CNS neurons nNOS is recruited to the site of the NMDA receptor activation through its protein–protein interactions (28), and NMDA receptors are known to be involved in thermoregulation (29).

A recent study reported that localized activation of sGC can also be achieved by means of protein–protein interactions. The interaction of the synaptic protein PSD-95 with sGC is dependent on the recruitment of sGC to the synaptic membranes, where nNOS is located (30). At the synaptic membrane proteins form a signalling complex with the cytoplasmic domain of the NMDA receptor. Although the recruitment of sGC to the site of NO synthesis by PSD-95 is well accepted, the contribution of such a phenomenon to the overall activation of sGC and elevation in cellular cGMP levels observed after NMDA receptor activation is not completely understood (*cf.* (31).

Expression of sGC may also be regulated posttranscriptionaly. At least in smooth muscle cells, a cyclic AMP (cAMP)-dependency (32) has been reported. There is evidence to support the notion that the regulation of mRNA for sGC α and β subunits is dependent on both cAMP and cGMP (33). Consistent with the putative role of cAMP and cGMP, the degree of phosphorylation, dephosphorylation and the protein–protein interactions also have an effect on the activity of sGC (34). It is important to mention that, once again, the possible physiological implication of such processes remains unknown, including regarding thermoregulation.

In summary, the signal diversity and specificity observed for NO synthesis and regulation of sGC activity includes multiple factors, which provides room for a scenario where a high degree of spatial and temporal resolution exists. Such signals downstream of cGMP can be transmitted by downstream effectors. Cyclic GMP regulates these processes by means of three direct effectors: 1) PKG, 2) cyclic nucleotide phosphodiesterases (PDEs) and 3) cyclic nucleotide gated ion channels. In turn, each of these effectors can transmit their signals to a number of intracellular signaling molecules (regulating neurotransmission, for instance). It is important to mention that all of these effectors of cGMP have been found to be expressed in brain (as well as other tissues) and their role in regulating nervous system functions is in general relatively well studied. Unfortunately, once again, the knowledge of their physiological role (including thermophysiology) remains poorly understood. PKG was one of the first proteins to be identified as a target of cGMP (35). It is very well established that activation of PKG by cGMP is a major mechanism by which NO relaxes smooth muscle tissue. Latter, following the discovery of the activation of sGC by the NO donor sodium nitropruside, it has been established that cGMP synthesized is activated downstream to neurotransmitter action (36). PKG has been reported to be widely expressed in many parts of the brain. To date, two isoforms of PKG have been reported, PKGI (PKG-Iα and PKG-Iβ) and PKG-II (37, 38). Both PKG-I and PKG-II contains catalytic and regulatory domains. Among these, PKG-I is primarily cytosolic, where as PKG-II is generally found in membrane associated form. PKG-I has recently been reported to play a role in inflammation in mice (39).

Cytosolic cGMP can change neuronal excitability by activating cyclic nucleotide gated ion (CNG) channels and thus play a crucial role in the signal transduction pathway involved in the modulation of various functions by NO (40). Activation of these channels causes cell depolarization and excitation of neurons. Moreover, there are reports suggesting that they may permit a significant Ca^{2+} influx that may influence synaptic function (41). Although the expression of CNG channels have also been reported in several parts of the brain including hippocampus the exact mechanisms of their regulation or their physiological function is not known.

Cyclic nucleotide signalling is modulated not only by cAMP and cGMP, but also by the rate of cyclic nucleotide degradation via phosphodiesterases (PDEs) (42). The PDE superfamily includes a number of subfamilies (about 11) with around more than 50 enzyme species. Many PDEs have been reported to be expressed in the CNS (43) including not only by several cGMP-specific PDEs, but also by dual-substrate PDEs that hydrolyse both cAMP and cGMP. The scenario looks far from simple, as cGMP may also inhibit or activate specific PDE subtypes by

binding to their regulatory domains. Thus, the nucleotide may actually affect its own intracellular concentration. Finally, an unique characteristic has been reported: there seem to be a cross-talk between the Ca^{2+} and cyclic nucleotide signalling pathways once they can be activated by the binding of Ca^{2+}/calmodulin (44).

As mentioned above, localization of NO production within the CNS, similarly to its regulation, may have important implications. The tissue distribution of NOS may mediate multiple effects. For instance, NOS inhibition in the anteroventral preoptic region (AVPO) of the rat brain results in an increased febrile response indicating an antipyretic role of the NO-cGMP pathway in the AVPO (45), whereas the NOS pathway in *organum vasculosum laminae terminalis* has been reported to play a pyrogenic role in rabbits (46). Indeed, Feleder *et al* have recently suggested a mechanism for the antipyretic activity of NO in the AVPO since they demonstrated an inhibitory modulation by NO on LPS-induced norepinephrine release in the preoptic region of guinea pigs (47). As to the systemic effect of NO, Kozak and Kozak assessed the different roles of NOS isoforms in fever using NOS gene-deficient mice (48) and they found that NO was indeed a regulator of fever, but its action would differ depending on the pyrogen used and the NOS isoform. More recently, we used iNOS-KO mice to study the role of NO in the tolerance to LPS, and we found that NO arising from the iNOS isoform modulates LPS tolerance in mice (49). Recently, mice lacking all the three NOSs have been generated (50). Data regarding Tb regulation using this animal are eagerly awaited with interest.

Besides the CNS, the NOS enzymes are widely distributed throughout the body (51). Specifically regarding thermoregulation, we and others have provided evidence that NO plays differential thermoregulatory effects by acting on the periphery and on the CNS. This notion is based on the opposite results obtained by injecting pharmacological modifiers of the NO pathway systemically or intracerebroventricularly (icv) (52, 53).

3.1. NO in the periphery

Studies on rats exposed to ambient temperatures of 25 ± 2ºC have shown that the systemic inhibition of NO synthesis using L-arginine analogues at doses ranging from 10 to 40 mg/kg decreases Tb (10, 22, 51-53), despite the fact that NOS inhibitors should decrease cutaneous heat loss because it causes vasoconstriction of both large and small vessels, including the superficial vascular beds. Thus, it has been suggested that NO synthesis inhibition is likely to reduce Tb by causing a failure of thermogenic mechanisms. Actually, inhibition of NO synthesis has been shown to impair brown adipose tissue thermogenesis (54). It is important to mention that Steiner *et al* (55) demonstrated that intravenous infusion of L-NAME decreases Tb of rats exposed to ambient temperature of 24^{o}C but has no effect on rats at 31^{o}C. Therefore, the hypothermic effect of this NOS inhibitor in the periphery may be the result of the impairment of the increased thermogenesis under subthermoneutral condition. It seems that nNOS is at least one NOS isoform involved in thermogenesis since intraperitoneal administration of the nNOS inhibitor 7-NI at the dose of 30 mg/kg evokes a drop in Tb of rats similar to that obtained with nonselective NOS inhibitors (22, 54).

On the other hand, rabbits at 24ºC exhibit a rise in Tb when treated systemically with L-NAME, while a drop in Tb is observed after intravenous infusion of the NO donors SIN-1 and SNAP. In this case, the NO-induced changes in the Tb are mainly mediated by changes in respiratory heat dissipation instead of cutaneous heat loss and metabolic heat production (56). Taken as a whole, those results would lead one to conclude that the thermoregulatory effect produced by systemic inhibition of the NO pathway depends on the prominent thermoregulatory effector mechanism in the tested species. However, the authors of the latest study (56) suggested that the effect of the intravenous infusion of the NO donors on respiratory rate of rabbits may be centrally mediated. This notion might be supported by the facts that icv application of small doses of SIN-1 (57) presents the same effect as intravenous infusion of this NO donor (56). Moreover, the intravenous administration of L-NAME might also have a central effect since at least for anesthetised dogs, cats and pigs, intravenous injected L-NAME is capable of inhibiting brain NOS activity (58). If this hypothesis is corrected, the Mathai and colleagues' results (56) agree with the hypothermic effect of NO acting on the brain (see item 2.2 NO in the CNS). More studies are certainly necessary to make this issue clearer.

Recently, the physiological roles of constitutively expressed NOS isoforms in humans, *in vivo*, have been assessed. 7-NI attenuates cutaneous vascular conductance increases in response to whole-body heat stress, but not during local skin warming. These opposite effects of 7-NI on two NO-dependent processes may suggest that the nNOS isoform affects NO increases and hence vasodilatation during centrally mediated, reflex responses to whole-body heat stress, but not during locally mediated, axon reflex responses to local skin warming (59).

The involvement of peripheral NO in febrigenic signaling to the brain has been proposed because peripherally administered NOS inhibitors attenuate LPS-induced fever (53). However, this hypothesis finds no support in the literature and it is suggested that, in this case, NOS inhibition in the periphery attenuates fever by suppressing thermogenesis in brown fat of animals that have been tested in a subneutral ambient temperature (55).

3.2. NO in the CNS

NO acting on the brain has important thermoregulatory effects. A number of studies have observed that icv injection of about 250 µg/animal of L-NAME causes a slight increase in the Tb of rats, indicating that central NO plays a tonic role by reducing Tb (53, 60), although opposite results have been observed in birds (61). At least in mammals, the hypothermic role of NO in the central sites is likely to be mediated by activation of sGC and consequent rise in the intracellular levels of cGMP since icv administration of the sGC inhibitor ODQ (1 µg)

elevates Tb similarly to NOS inhibitors. One could argue that ODQ could also affect Tb by inhibiting CO-dependent sGC activity. However, this is unlikely since we observed that central inhibition of the CO pathway causes no change in Tb (45).

It is interesting to note that central NO has been shown to play a role in reducing sympathetic tonus by acting on several brain sites, including an important autonomic nucleus, the paraventricular nucleus, and posterior hypothalamus and the nucleus tractus solitarius (62). Since sympathetic fibers play a key role in both increasing nonshivering thermogenesis and evoking vasoconstriction of the superficial vascular beds, responses which lead to an increase in Tb, it is suitable to propose that a reduction in the sympathetic outflow by centrally acting NO may be responsible for the hypothermic action of NO in the CNS.

The cellular role of NO in regulating thermoregulatory pathways, even after small, circumscribed injections may be quite complex. For example in addition to the post synaptic effects on neurons described above, activation of the NO pathway is associated with the release of almost every amino acid transmitter (63). In particular, an elegant study by Bains and Ferguson (64) have revealed that NMDA receptors activation in type I neurons of the paraventricular nucleus of the hypothalamus (65, 66), causes not only depolarization of some cells, but also induces inhibitory postsynaptic potentials (IPSPs), dependent on GABA, in 40 % of those cells. This increase in GABAergic signaling is mediated by NO, a fact that lead the authors to suggest a role for NO as an intermediary in the control of neuronal excitability (64). Given the suggested inhibition of thermogenesis by the preoptic GABAergic neurons via dorsomedial hypothalamus or *raphe pallidus* (67), one might predict that NO would cause hypothermia by acting on those preoptic cells. Moreover, one could speculate that NO may influence other GABAergic transmission throughout the medial hypothalamus (68), and the important projections from the paraventricular nucleus to brainstem and spinal cord autonomic areas (69, 70) as these neuron are thought to inhibit downstream thermoregulatory pathways.

4. HEME-OXYGENASE (HO)

Heme-oxygenase catabolizes heme into CO, biliverdin (which is rapidly converted to bilirubin), and free iron (which leads to the induction of ferritin, an iron-sequestering protein). The amount of data reported for the HO pathway is not as extensive as for NO pathway. This section discusses briefly some biochemical characteristics of the HO pathway as well as highlights the few existing data about HO as an interesting enzyme involved in thermoregulatory pathways.

HO was first purified from rat liver (71). It is now clear that two isoenzymes of HO exist i.e., the original enzyme was designated HO-1 and the second isoenzyme was designated HO-2. These isoenzymes are the products of two distinct genes, but share approximately 40% amino acid sequence homology (72). HO-1 is the product of only one transcript, but HO-2 is encoded by two transcripts from one gene (72). HO-2 is constitutively expressed throughout the body, including in the CNS, but its role in cells is not well understood. HO-2 may play a role in epidermal cells, germ cell development, and signal transduction in neural tissues (73).

In contrast, HO-1 is a 32-kDa protein sparsely found in other tissues (73), but may be overexpressed in response to a series of stimuli including heme but also nonheme stimuli such as heavy metals, hormones, LPS, cytokines (at least interleukin 10), oxidants (hydrogen peroxide) (74), and hypoxia (75). This diversity of HO-1 inducers has provided further support for the speculation that HO-1, besides its role in heme degradation, may play a vital function in maintaining cellular homeostasis (76). HO-1 activity can be increased in whole animal tissues by treating the animals with its natural substrate heme, as well as other stimuli such as cytokines and LPS. Moreover, data obtained from experiments using deficient HO-1 (*hmox-1* $^{-/-}$) mice suggest that HO-1 may be a key molecule in the host's defense, once the *hmox-1* $^{-/-}$ mice seems to exhibit increased susceptibility to inflammation and ischemic injury (77, 78).

CO is continuously synthesized endogenously by HO-1 and HO-2. Endogenous CO produced by heme catabolism has clear physiological roles in eukaryotic cells. Both endogenous and exogenous CO seems to play an important role as an inflammatory (79) and anti-hyperalgesic (80) agent by relatively undetermined physiological mechanisms. CO arising from HO action in the CNS may play an important role in fever generation (81).

In mammals, the other product of HO activity biliverdin is then converted to bilirubin by the cytosolic enzyme biliverdin reductase and bilirubin is then conjugated by UDP-glucuronyl transferase before being excreted into the bile. Most of the bilirubin formed *in vivo* is derived from hemoglobin released from aging or damaged erythrocytes (82). In culture, a number of cell types (hepatic, renal, testicular, brain, etc) catalyze heme degradation to biliverdin (73). Our results indicate that not only billiverdin, but also, free iron, seem to play no role in Tb maintenance, fever or LPS-tolerance (83, 84).

4.1. Carbon Monoxide (CO)

Two major sources of CO in biological systems have been reported, one is HO-dependent, and the other is HO-independent, i.e., due to the photo-oxidation and the auto-oxidation of organic molecules, phenols, and flavenoids and the peroxidation of lipids as a result of severe stress, which may not be achieved under physiological conditions (85). However, the fast increase of CO that take place *in vivo* is only due to the induction of HO (either HO-1 or HO-2) (86). Because the major source of endogenous produced CO is the degradation of heme by HO, it is now clear that CO may work as an important cellular signal molecule. Evidence exists that CO, similarly

Table 1. Effect of agents that activate and inhibit the NO and CO pathways in the central nervous system on the reduction of body temperature (Tb) during hypoxia and other stressful stimuli

Mediator	Tested species	Pharmacological agent(s) and local of injection	Effect on hypoxic drop of Tb	Effect on drop of Tb induced by other stimuli*	References
NO	rat	L-NAME (icv)	Inhibit		(121)
	rat	L-NMMA (intra-PO)	Inhibit		(109)
	rat	L-NAME (icv)		Inhibit (insulin)	(119)
	rat	L-NAME and 7-NI (icv)		Inhibit (2-DG)	(118)
	toad	L-NMMA (icv)	inhibit		(122)
CO	rat	ZNDPBG (icv)	Intensify		(88)
	rat	ZNDPBG (icv)		Intensify (insulin)	(90)
	rat	CO-saturated saline (icv)		Inhibit (insulin)	(90)
	rat	ZNDPBG (icv)		Intensify (2-DG)	(89)
	rat	ZNDPBG (intra-LC)		Inhibit (restraint stress)	(93)
	rat	heme-lysinate (intra-LC)		Intensify (restraint stress)	(93)

It can be noted that studies approaching the action of NO and CO on specific regions of the brain as well as the neural circuits involving these gaseous neurotransmitters during anapyrexia are still scarce. Icv= intracerebroventricular injection; intra-PO= injection in the preoptic region; intra-LC= injection in the *locus coeruleus*; 2-DG= 2-deoxy-dglucose, * The specific stimulus is identified between parentheses.

to NO, activates sGC leading to a rise in cGMP levels, which may account for a number of its physiological effects (71). It is interesting to note that NOS is a heme containing enzyme. It has been proposed that some NO effects can be duplicated by CO, including action of certain neurotransmitters could be regulated by both molecules (87). Such interaction between NO and CO may be responsible for thermoregulatory mechanisms which seems to be a fairly ripe research area for scientists interested in thermoregulation.

We have provided evidence for the thermoregulatory role of CO in the CNS. Experiments using icv injection of agents that activate or inhibit the HO-CO pathway indicate that CO in the CNS increases Tb and acts as a pyrogenic molecule (51, 81, 84, 88-90). It has been shown that the nonselective HO inhibitor, ZNDPBG, inhibits fever induced by endotoxin (81) while the activation of the HO/CO pathway by heme lisinate abolishes the tolerance to LPS (84). This pyrogenic effect of CO has been confirmed by others (91). In fact, CO seems to exert a key function to prevent excessive decreases in Tb during stressful situations such as the reduction of glucose availability (89, 90). However, it is interesting to note that the effect of CO on Tb depends on the brain site of action. Ravanelli *et al.* (92) reported that microinjections in an important noradrenergic nucleus in the brain stem, the *locus coeruleus*, of ZNDPBG increases and of heme lisinate decreases LPS-induced fever in rats, indicating an antipyretic rather than a pyretic effect of HO-CO pathway specifically acting on this site. This effect is suggested to be dependent on cGMP since ODQ counteract the antipyretic effect of heme lisinate (92). Corroborating these data, the same authors used similar pharmacological tools to demonstrate the participation of the HO-CO-cGMP pathway in the *locus coeruleus* in the induction of hypothermic response to restraint stress (93); Table 1).

5. ANAPYREXIA: REGULATED HYPOTHERMIA

Anapyrexia has been defined in the Glossary of Terms for Thermal Physiology (94), (Gk. ana—reverse, pyretos—fever) as is a pathological condition in which there is a regulated decrease in Tb, distinct from hypothermia in that thermoregulatory responses indicate a defence of the lower level of Tb. Although a recent review (95) suggested that this term is not suitable because this response seems to be incompatible with a single set-point model of Tb control and has a strong dependence on ambient temperature, it is clear that if many animal species exposed to a variety of hostile stimuli (hypoxia, hypercapnia, dehydration, starvation and hypoglycemia), have the chance to decrease their Tb, they will, and the outcome of this is to improve their survival (51, 52). Further experiments are therefore urgently needed to understand the mechanisms involved in this phenomenon, such as the determination of the threshold Tbs for autonomic thermoeffectors activation in all of these different situations.

In 1943, Fay reported a beneficial effect of hypothermia in septic patients (96). Now a days, hypothermia is still thought to be beneficial in certain clinical settings such as acute brain injury (97). Considering that anapyrexia is a regulated response, the mechanisms underling this phenomenon may give insights to improve therapeutic hypothermia that usually requires pharmacologic intervention to blunt thermoregulatory defences, such as intense vasoconstriction and vigorous shivering. These responses are likely to be injurious to patients since they may be accompanied by hypertension, tachycardia and activation of sympathetic nervous system (52, 98).

Hypoxia comprises the anapyretic stimulus most studied and reviewed (51, 99, 100). Environmental conditions may impose a reduction in oxygen availability for living organisms. Examples include exposure to a hypoxic environment (reduced O_2 partial pressureresulting from high altitude, burrows and oxygen-deprived water habitats (52, 101). Pathologies such as, obstructive sleep apnea and chronic obstructive pulmonary disease are examples of conditions in which patients suffer from hypoxia (52, 102).

It is well established that hypoxia-induced anapyrexia occurs in fish, amphibians, reptiles, mammals, birds and even in a unicellular organism, the *Paramecium*

(52, 99). Among mammals, hypoxic-anapyrexia has been extensively studied in laboratory rats, mice, hamsters and guinea pigs (cf.(52). The data obtained are consistent with the notion that hypoxia-induced anapyrexia is a beneficial response due to a decreased metabolic rate, an improved oxygen extraction in the lungs, attenuated energetic costly responses like hyperventilation and increased cardiac output, inhibited thermogenesis, increased heat loss and survival rates, preserved brain ATP levels, and shifted thermoneutral zone to lower temperatures (51, 52, 99). Barros *et al* (103) compared the thermoneutral zone during normoxia and hypoxia in the Canadian golden-mantled ground squirrels and emphasized that the Tb drop induced by hypoxia represents a regulated phenomenom.

Both *in vitro* and *in vivo* toxicity of many environmental chemicals and drugs like heavy metals, methylmercury, pesticides and ethanol is directly proportional to temperature (98). Moreover, there is evidence that the drop in Tb induced by intoxication is beneficial to survival since the lethality of most toxic agents increases with rising temperature (104). It seems not to be known if toxic agents-induced hypothermia is mediated by gaseous neurotransmitters.

6. MECHANISMS (MEDIATORS, NUCLEI AND PATHWAYS)

The mechanisms of anapyrexia are of intense interest to physiologists. Despite this fact, hypoxic anapyrexia remains a phenomenon poorly understood if we consider the whole thermoregulatory system (sensors, afferent and efferent pathways, CNS integration). There is evidence that the drop in metabolic rate in response to hypoxia, unlike the ventilatory response to hypoxia, does not depend on the activation of peripheral chemoreceptors (105).

Several substances, among them NO and CO, have been suggested as putative mediators of anapyrexia, and they appear to act in the CNS to drive adequate thermoeffectors. Even in toads, NO has been demonstrated to be a mediator of hypoxic anapirexia (106) Table 1). Other substances, such as dopamine, serotonin, adenosine, vasopressin, lactate, ethanol, have been tested for their involvement in the development of hypoxia-induced anapyrexia in mammals. This issue is the focus of previous reviews (51, 52, 99, 107).

The effect of hypoxia on Tb seems to be mediated, at least in part, by the activation of the serotonin- and dopamine-cAMP and NO-cGMP pathways in the AVPO (cf. (107). The increased intracellular levels of these two second messengers might cause an elevation in the thermal sensitivity of preoptic warm-sensitive neurons (108) leading to inhibition of thermogenesis and activation of heat loss, and finally resulting in Tb reduction. The first studies (10) suggesting a participation of cyclic nucleotides in the regulation of Tb were reported in the 1970s. More specifically, these studies suggested that administration of cAMP analogs that mimic cAMP-like effects into the preoptic region (PO) which is the presumed brain Tb controlling site, increased Tb. However, these observations started to be contested in 1984, when it was reported that intra-PO administration of cAMP and cGMP analogs to rabbits produces a rapid decrease in Tb followed by a feverlike response (*cf*.(10). Interestingly, the fever, but not the decrease in Tb, was abolished by treatment with paracetamol, indicating that cAMP and cGMP reduce Tb by acting on the PO and that the pyretic effect of intra-PO cAMP observed in previous studies is likely to result from a local inflammatory response produced by the injection procedure (*cf.* (10).

More recently studies using small volume microinjections have confirmed that intra-PO administration of cAMP and cGMP analogs that activate protein kinases A and G, respectively, produces a decrease in the Tb of rats (109, 110). Consistent with this notion, cGMP increases the thermosensitivity of warm-sensitive preoptic neurons, an effect that seems to be associated with increased heat loss mechanisms and a decrease in Tb (108). Moreover, the use of small volume microinjections also permitted the identification of the AVPO as the preoptic site most sensitive to the thermoregulatory effects of cyclic nucleotides (45, 110). Actually, we have shown that the activation of cAMP- and cGMP-dependent pathways in the AVPO mediates hypoxia-induced anapyrexia. Inasmuch as the rise in cAMP during anoxia seems to be under the control of the monoaminergic system (111), whereas rises in cGMP may be driven by NO. It is interesting to note that recently Wright *et al.* (108) showed, using immunohistochemistry, that rostral hypothalamic neurons contain cGMP, guanylate cyclase, and CNG A2 (an important cyclic nucleotide-gated channel). They also measured extracellular electrophysiological activity from different types of neurons in rat hypothalamic tissue slices in response to 8-bromo-cGMP (a membrane-permeable cGMP analog). The cGMP analog decreases the spontaneous firing rate in 45% of temperature-sensitive and -insensitive neurons, an effect that is likely due to cGMP-enhanced hyperpolarizing K(+) currents (108). The authors suggested that a decreased PO activity induced by cGMP may attenuate thermoregulatory responses leading to hypothermia in a cold or neutral ambient. Moreover, 8-bromo-cGMP increases warm sensitivity of non-PO thermosensitive neurons (dorsal, lateral and posterior to the PO), which might contribute to the cGMP effect on Tb (108).

Evidence indicates that, in addition to their role in nociceptive and stress responses, endogenous opioids are involved in thermoregulation during hypoxia (51, 112, 113), as well as during euthermia (113). We recently demonstrated that the kappa opioid receptors in the PO are involved in the induction of anapirexia (114)**.** Perhaps kappa receptors could decrease Tb during hypoxia by a mechanism dependent on NO/cGMP, since it is known that these receptors activate the NO pathway in the CNS to cause hypothermia in rats (115).

Besides the effect of the above mentioned agents which decrease Tb during hypoxia, it has been suggested that there are agents that counteract this effect, possibly

preventing excessive decreases in Tb (88). Indeed, the endogenously produced gas CO acting on the CNS has been reported to play a counter-regulatory effect during hypoxia-induced anapyrexia since icv injection of ZNDPBG intensify this response (88) Table 1). However, this result needs to be interpreted with caution because there is no data about the role of CO acting on specific sites in the CNS during hypoxia. In fact, different effects may be observed depending on the brain region considered. For instance, while icv injection of agents that inhibit or activate the HO-CO-cGMP pathway indicates a pyretic role of CO in the CNS in the LPS-induced fever (a response that is considered to be the opposite of anapyrexia), the contrary result, i.e., an antipyretic action, is demonstrated specifically in the LC [see the item "Carbon monoxide (CO)"] and no effect is observed in the AVPO (116).

As cited above, some stressful situations such as the drop in glucose availability decreases Tb, which constitutes a beneficial response since energy is saved because of the reduction in metabolism (117). In this case, a similar scenario concerning the participation of NO and CO in the CNS in both hypoxia and low glucose-induced Tb drop is observed (Table 1). We demonstrated that NO in the CNS is essential for hypothermic responses observed after 2-deoxy-dglucose injection (2-DG; a non-metabolizable glucose analogue) as well as after insulin infusion (118, 119). Because 2-DG impedes intracellular glucose utilization, the resultant reduction in Tb, which might be an anapyretic response (*cf.* (52), is a result of the intracellular rather than extracellular glucose deficiency. This stimulus seems to activate nNOS since icv injection of 7-NI inhibits 2-DG-induced Tb decrease (118)**.** The opposite effects were reported for CO, i.e., this gas in the CNS counteracts the hypothermic response to 2-DG injection (89) and to insulin infusion (90)**.**

7. CONCLUSIONS

The data indicate overwhelming evidence that NO and most likely CO play important roles in neuronal function. At the synaptic and cellular level, they have well described actions. However, in the context of neuronal circuits and neuronal control of autonomic function evidence is still fragmentary, which is even more incomplete when it comes to H_2S. In particular, the existing data about the neurochemistry responsible for Tb regulation are far from enough to provide a clear scenario. More experimental data remains urgently needed. Recent reports have added important details about the afferent pathways to the brain signalling during fever (120). The tools (from biochemistry, pharmacology and genetics, mainly regarding NO) are available for testing their effect on thermoregulation and future research is eagerly awaited on this topic.

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