Interactions of Carbon Monoxide and Metabotropic Glutamate Receptor Groups in the Nucleus Tractus Solitarii of Rats

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ABSTRACT
Carbon monoxide has been shown to act as a neurotransmitter and neuronal messenger in the brain. Heme oxygenase catalyzes the conversion of heme to carbon monoxide and biliverdin. We have recently reported that carbon monoxide was involved in central cardiovascular regulation. Carbon monoxide modulated the baroreflex and may affect glutamatergic neurotransmission. In addition, metabotropic glutamate receptor agonists may be coupled to the activation of heme oxygenase in the nucleus tractus solitarii of rats. The present study was designed to investigate the possible interactions of carbon monoxide and metabotropic glutamate receptor groups in the nucleus tractus solitarii. Unilateral microinjection of several agonists for metabotropic glutamate receptor groups such as (R,S)-3,5-dihydroxyphenylglycine (DHPG) (group I) (0.03 nmol), 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC) (group II) (0.3 nmol), and L-(-)-2-amino-4-phosphonobutyric acid (L-AP4) (group III) (0.3 nmol) produced a significant decrease in blood pressure and heart rate. Among the metabotropic glutamate receptor agonists, prior administration of zinc protoporphyrin IX, an inhibitor of heme oxygenase activity, significantly attenuated the cardiovascular effects of APDC and L-AP4, and failed to attenuate the cardiovascular responses of DHPG. These results indicated interactions between carbon monoxide and group II and III metabotropic glutamate receptor groups in central cardiovascular regulation.

Carbon monoxide has been postulated to be a gaseous second neurotransmitter (Verma et al., 1993; Dawson and Snyder, 1994). In animals, the predominant source of carbon monoxide generation is from heme degradation. Heme oxygenase is the rate-limiting enzyme responsible for the catabolism of heme and subsequent production of carbon monoxide and biliverdin. Three isoforms of heme oxygenase have been identified. Heme oxygenase-1, induced by heme and numerous oxidative stressors, is enriched in spleen and liver. Heme oxygenase-2 is present abundantly in the brain and testis as a constitutive enzyme. Heme oxygenase-3 has been identified. Heme oxygenase-3 is present abundantly in the brain and testis as a constitutive enzyme. Heme oxygenase-3 has been identified. Heme oxygenase-3 has been identified. Heme oxygenase-3 has been identified.

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ABBREVIATIONS: ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol; DHPG, (R,S)-3,5-dihydroxyphenylglycine; APDC, 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate; L-AP4, L-(-)-2-amino-4-phosphonobutyric acid; mGlur, metabotropic glutamate receptor; ACPD, trans-(-)-1-amino-(1S,3R)-cyclopentanedicarboxylic acid; BP, blood pressure; HR, heart rate; MBP, mean blood pressure.
col (ZnDPBG) attenuated the depressor and bradycardic effects of hemin (Lin et al., 2003). It has been reported that systemic administration or direct microinjection into the nucleus tractus solitarii of ZnDPBG attenuates the baroreceptor reflex (Johnson et al., 1997; Lo et al., 2000). Taken together, these findings suggest that carbon monoxide within the nucleus tractus solitarii may play an important role in the regulation of cardiovascular function.

Most of the excitatory synapses in the central nervous system use glutamate as a neurotransmitter. Glutamate receptors have been reported to be present on baroreceptor afferent terminals in the nucleus tractus solitarii (Lewis et al., 1988), and transmitter-like release of glutamate occurs following electrical stimulation of the vagus nerve (Allchin et al., 1994) and baroreceptor activation after administration of phenylephrine (Lawrence and Jarrott, 1994). Glutamate activates ligand-gated cationic channels named N-methyl-d-aspartate, α-amino-3-hydroxy-5-methylisoxazole-4-propanate, as well as kainic receptors. However, glutamate also acts through a separate class of receptors, termed metabotropic glutamate receptors (mGluRs), that link to G-protein and intracellular calcium mobilization (Sugiyama et al., 1987; Furuya et al., 1989).

To date, eight different mGluRs have been identified, and they can be categorized into three groups based on their amino acid sequence identity. mGluRs are widely distributed in the central nervous system. A number of signal transduction pathways linked to mGluR activation have been identified (Chappak et al., 1990; Cartmell et al., 1992; Harvey and Collingridge, 1993). Nevertheless, the mechanisms underlying mGluR effects remain to be elucidated. All subgroups of mGluRs participate in cardiovascular and sympathetic regulations in the nucleus tractus solitarii of rats (Matsumura et al., 1999).

A recent study has provided evidence that a heme oxygenase product, such as carbon monoxide, may affect nucleus tractus solitarii glutamatergic neurotransmission and thus participate in cardiovascular control in conscious animals (Silva et al., 1999). Previously, we have also reported that carbon monoxide, involved in central cardiovascular regulation (Lo et al., 2000), interacted with adenosine (Lin et al., 2003), and mGluRs may couple to the activation of heme oxygenase in the nucleus tractus solitarii (Lo et al., 2002). In the present study, we examined further the role of mGluR groups in central cardiovascular regulation by carbon monoxide.

Materials and Methods

Materials. Experimental drugs such as urethane, L-glutamate, and heparin were obtained from Sigma-Aldrich (St. Louis, MO). Zinc protoporphyrin IX, (R,S)-3,5-dihydroxyphenylglycine (DHPG), 2R,4R-4-amino-2,4-dicarboxylate (APDC), and 3-aminophosphonobutyric acid (L-AP4) were obtained from Tocris Cookson Inc. (Bristol, UK). Zinc protoporphyrin IX was dissolved in 50 mM Na2CO3 (pH 8.8 to 9.4) immediately before use. All other drugs were dissolved in normal saline on the day of the experiment.

Animals. Male Sprague-Dawley rats (250–350 g) were obtained from the National Science Council Animal Facility and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan, ROC). The rats were kept in individual cages in a room in which lighting was controlled (12 h on/12 h off), and temperature was maintained at 23–24°C. The rats were given Purina Laboratory Chow and tap water ad libitum.

Experimental Procedures. All animal protocols have been approved by the Research Animal Facility Committee at Kaohsiung Veterans General Hospital. Humane treatment was administered at all times. Rats were anesthetized with urethane (1.0 g/kg i.p. and 300 mg/kg i.v. if necessary). During the whole study, the animals were fixed in the stereotaxic frame; the procedures were monitored carefully under surgical microscope. For nucleus tractus solitarii microinjection, the rats were placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA), with the head flexed downward at a 45° angle. The dorsal surface of the medulla was exposed by limited craniotomy, and the rats were rested for at least 1 h before experiment. Single-barrel glass cannulas were prepared (0.031-inch o.d., 0.006-inch i.d.; Richland Glass Co., Vineland, NJ) that had external tip diameters of 40 μm. The cannula was connected to a Hamilton microsyringe by polyvinyl tubing. The cannulas were filled with L-glutamate (0.154 nmol/60 nl, to functionally identify the nucleus tractus solitarii), DHPG (0.03 nmol/ml), APDC (0.3 nmol/ml), L-AP4 (0.3 nmol/ml), or different doses of zinc protoporphyrin IX. The cannula was lowered into the nucleus tractus solitarii with the anteroposterior coordinates, 0.0 mm; mediolateral, 0.5 mm; and vertical, 0.4 mm, with the obex as reference. The injection volume was monitored by estimating the stature of liquid in the single-barrel glass cannulas. The injection device was lowered into the nucleus tractus solitarii only at the time of injection. The preparation of animals for intranucleus tractus solitarii microinjection and the methods used in the localization of the nucleus tractus solitarii have been described previously (Tseng et al., 1996). In this study, each injection volume was restricted to 60 nl.

To investigate the effect of preadministration of the heme oxygenase inhibitor zinc protoporphyrin IX on cardiovascular responses to DHPG in the nucleus tractus solitarii, animals were first injected with DHPG (0.03 nmol) into the unilateral nucleus tractus solitarii. The rats were then allowed to rest for at least 30 min until the BP and HR had returned to basal levels. The changes in BP and HR were then monitored by microinjection of the same doses of DHPG 10 min after intranucleus tractus solitarii administration with different doses (0.1, 0.33, and 1 nmol) of zinc protoporphyrin IX or vehicle (50 mM Na2CO3). Similar experimental procedures were used to study the effects of pretreatment with the zinc protoporphyrin IX (0.1, 0.33, or 1 nmol) on APDC (0.3 nmol) or L-AP4 (0.3 nmol) into the nucleus tractus solitarii in different groups of animals.

Statistics. A paired t test (before and after pretreatments), unpaired t test (for control and study group comparisons), or repeated-measures analysis of variance followed by Dunnnett’s test for significant differences was applied to compare group differences. Differences with a P value of less than 0.05 were considered significant. All data were expressed as means ± S.E.M.

Results

Previously, we have shown that intranucleus tractus solitarii microinjection of trans-(a)-1-amino-(1S,3R)-cyclopentanedicarboxylic acid (ACPD), an agonist for mGluRs, caused

| TABLE 1 | Effect of inhibition of carbon monoxide generation with intranucleus tractus solitarii administration of zinc protoporphyrin IX (ZnPPIX) on the cardiovascular effects of the metabotropic glutamate receptor agonists |
|-----------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------|
|                | Control | ZnPPIX (1 nmol) |                |                   |                   |                   |
|                | ΔMBP     | ΔHR               | ΔMBP | ΔHR               |                   |                   |
| mm Hg         | beats/min | mm Hg   | beats/min | mm Hg | beats/min | mm Hg | beats/min |
| DHPG       | –33 ± 2  | –49 ± 7          | –29 ± 3 | –39 ± 7          |                   |                   |
| APDC        | –43 ± 3  | –71 ± 5          | –6 ± 2* | –8 ± 4*          |                   |                   |
| L-AP4       | –41 ± 4  | –78 ± 10         | –8 ± 2* | –28 ± 9*         |                   |                   |

* Significantly different from corresponding control response (n = 10), P < 0.05.
hypotension and bradycardia (Lo et al., 2002). However, the cardiovascular effects of ACPD were significantly attenuated by pretreatment with ZnDPBG (Lo et al., 2002). Microinjection of three mGluR group agonists (DHPG, APDC, and L-AP4) produced depressor and bradycardic responses (Table 1; Figs. 1 and 2). To test which groups of mGluRs linked to the cardiovascular effects of carbon monoxide in the nucleus tractus solitarii of rats, we used the heme oxygenase inhibitor zinc protoporphyrin IX on the cardiovascular responses of group I, II, and III mGluR agonists.

Prior administration of zinc protoporphyrin IX (1 nmol) or vehicle did not modify the depressor and bradycardic effects of the group I mGluR agonist DHPG (Table 1) (from $-33 \pm 2$ mm Hg and $-49 \pm 7$ beats/min to $-29 \pm 3$ mm Hg and $-39 \pm 7$ beats/min, respectively). Figure 1A showed significant attenuation of the group II mGluR agonist APDC-induced cardiovascular effects by previous intranucleus tractus solitarii administration of zinc protoporphyrin IX (0.33 nmol). Pretreatment with different doses of zinc protoporphyrin IX (0.1, 0.33, or 1 nmol) attenuated the depressor and bradycardic responses to APDC dose dependently, as shown in Fig. 1B ($P < 0.05$ compared with control value).

Similarly, prior treatment with zinc protoporphyrin IX also significantly attenuated the cardiovascular effects of group III mGluR agonist L-AP4 (Fig. 2A). In addition, pretreatment with different doses of zinc protoporphyrin IX (0.1, 0.33, or 1

![Fig. 1. The cardiovascular effects of microinjection of APDC into the nucleus tractus solitarii before and after zinc protoporphyrin IX. A, tracings show cardiovascular effects of microinjection of APDC (0.3 nmol) into the nucleus tractus solitarii before and after zinc protoporphyrin IX (1 nmol) in anesthetized rats. APDC and zinc protoporphyrin IX were injected at the indicated time points. Blood pressure BP, mean blood pressure (MBP), and HR recordings were made at a paper speed of 3 mm/min. Horizontal bar represents time period of 5 min. B, comparative MBP and HR effects of APDC (0.3 nmol) by the heme oxygenase inhibitor zinc protoporphyrin IX on intranuclear tractus solitarii administration of the substances. APDC was injected in the absence (control) or presence of vehicle or zinc protoporphyrin IX. Each point represents the average of eight rats. *, $P < 0.05$, significantly different from corresponding control.](image-url)
nmol) also attenuated the depressor and bradycardic responses to L-AP4 (P < 0.05 compared with control value, Fig. 2B). The attenuated cardiovascular effects of zinc protoporphyrin IX on APDC or L-AP4 reached a maximum effect in 10 min and lasted for at least 60 min (data not shown). However, prior administration of vehicle did not modify the cardiovascular effects of APDC or L-AP4 (Figs. 1B and 2B).

**Discussion**

Previously, we have shown that microinjection of hemin (Lin et al., 2003) or hematin (Lo et al., 2000), a substrate for carbon monoxide production, into the nucleus tractus solitarii induced depressor and bradycardic effects. These effects of hemin require heme oxygenase because prior administration of the heme oxygenase inhibitor, zinc protoporphyrin IX or ZnDPBG significantly suppressed the cardiovascular effects of hemin (Lin et al., 2003). Microinjection of the mGluR agonist, ACPD, into the nucleus tractus solitarii produced depressor and bradycardic effects (Lo et al., 2002). These results were similar to previously published results which suggested that the excitatory amino acid, L-glutamate, and carbon monoxide may be potential neurotransmitters of baroreceptor information in the rat nucleus tractus solitarii (Talman et al., 1980; Lo et al., 2000). During the experiment, we noticed that microinjection of zinc protoporphyrin IX into the nucleus tractus solitarii produced decrease in arterial pressure and heart rate, which was opposite to the findings in conscious rats as described previously (Silva et al., 1999). Such observations also were found in our previous reports (Lo et al., 2000, 2002; Lin et al., 2003). The decreasing responses are nonspecific effects because, apparently, they are also observed with the solvent. The phenomenon is not known and is not necessarily inhibition of a putative tonic effect of carbon monoxide.
Prior administration of zinc protoporphyrin IX significantly attenuated the cardiovascular effects of intranucleus tractus solitarii microinjection of ACPD. Such findings are consistent with the possibility that the function of heme oxygenase may be linked to mGluR neurotransmission and its effects on blood pressure (Lo et al., 2002). However, the mGluR groups of mGluRs in central cardiovascular regulation remained to be determined. In the present study, we investigated the cardiovascular effects of carbon monoxide and groups of mGluRs in central cardiovascular regulation. Consistent with this idea, microinjection of three mGluR agonists (DHPG, APDC, and L-AP4) produced depressor and bradycardic responses (Table 1; Figs. 1 and 2). The results suggested that each receptor group has a role in central cardiovascular regulation.

The mGluRs are a family of receptors that are coupled to various effector systems via GTP-binding proteins. Eight subtypes of mGluR have been identified so far (Knopfel et al., 1995; Pin and Duvoisin, 1995), and they can be categorized into three groups based on their amino acid sequence homology, pharmacology, and signal transduction pathways. Heme oxygenase inhibitor has been reported to block the effects of mGluR activation, and carbon monoxide is thought to be primarily responsible for mediating glutamate action at metabotropic receptors (Glaum and Miller, 1993b). In the present study, we found that the cardiovascular effects of APDC, a group II mGluR agonist, were blocked by zinc protoporphyrin IX in a dose-dependent manner (Table 1; Fig. 1). Similarly, pretreatment with zinc protoporphyrin IX attenuated the depressor and bradycardic effects to intranucleus tractus solitarii administration of L-AP4, a group III mGluR agonist (Table 1; Fig. 2). Nevertheless, prior administration of the same dose of zinc protoporphyrin IX did not affect the depressor and bradycardic responses of group I mGluR agonist DHPG (Table 1). Such observations suggest that group II and III mGluRs in some way works through the heme oxygenase/carbon monoxide pathway.

Group I receptors are generally located at postsynaptic sites, whereas group II and III receptors can occur at both presynaptic and postsynaptic sites (Baude et al., 1993). Presynaptic mGluRs regulating the release of excitatory amino acids have been demonstrated in in vitro release studies in various brain slice and synaptosomal preparations (Herrero et al., 1994; Lombardi et al., 1994, 1996; East et al., 1995). The presynaptic mGluRs are likely to be localized on glutamatergic vagoafferent terminals in the nucleus tractus solitarii (Lawrence and Jarrott, 1994), whereas postsynaptic mGluRs could be associated with GABAergic interneurons (Glaum and Miller, 1993a; Beart et al., 1994) or aspartate-utilizing neurons/terminals (Beart et al., 1994) in the nucleus tractus solitarii. Group I mGluRs are localized in the peripheral plates of postsynaptic densities (Baude et al., 1993) and contribute to the regulation of synaptic plasticity.

Group I mGluR is coupled to phosphatidylinositol hydrolysis/Ca²⁺ signal transduction, whereas group II and group III mGluRs are coupled to the inhibition of cAMP synthesis (Nakanishi, 1992). Therefore, the fact that heme oxygenase inhibitor significantly attenuated the cardiovascular effects of group II and III mGluRs in the nucleus tractus solitarii of this study suggests that the relationship between group II, III, and the heme oxygenase/carbon monoxide system may be caused by the locations of these receptors and the inhibition of adenylyl cyclase. On the contrary, the fact that heme oxygenase inhibitor failed to attenuate group I mGluR cardiovascular effects may suggest that group I mGluR activation does not interact directly with the heme oxygenase/carbon monoxide system. The data presented herein may represent evidence for physiologically relevant interactions between mGluR groups and carbon monoxide within the nucleus tractus solitarii.

In our studies, L-glutamate and the agonists of mGluR (Lo et al., 2002) or mGluR groups (Table 1; Figs. 1 and 2) produced depressor and bradycardic responses, which was different from the findings in conscious rats (Silva et al., 1999). We assumed that the anesthetics we used during the study might have produced the difference. Silva et al. (1999) showed that carbon monoxide may exert effects on cardiovascular functions by interacting with mechanisms underlying L-glutamate neurotransmission. Based on that finding, our previous study clarified the relationship between carbon monoxide and mGluRs (Lo et al., 2002). To inquire further into the underlying neurotransmission, we investigated the interactions of carbon monoxide and mGluR groups in this study. We provide evidence that heme oxygenase might be activated by stimulation of group II and III mGluRs to produce carbon monoxide to participate in central cardiovascular regulation of rats.

In conclusion, the present study showed that the heme oxygenase inhibitor zinc protoporphyrin IX-attenuated cardiovascular effects of APDC and L-AP4 are relatively specific and provides evidence that heme oxygenase might be activated by stimulation of group II and III mGluRs to produce carbon monoxide to participate in central cardiovascular regulation of rats.

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