Hypoxia transduction by carotid body chemoreceptors in mice lacking dopamine D2 receptors

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Hypoxia-induced dopamine (DA) release from carotid body (CB) glomus cells and activation of postsynaptic D2 receptors have been proposed to play an important role in the neurotransmission process between the glomus cells and afferent nerve endings. To better resolve the role of D2 receptors, we examined afferent nerve activity, catecholamine content and release, and ventilation of genetically engineered mice lacking D2 receptors (D2⁻/⁻ mice). Single-unit afferent nerve activities of D2⁻/⁻ mice in vitro were significantly reduced by 45% and 25% compared with wild-type (WT) mice during superfusion with saline equilibrated with mild hypoxia (Po2 ~50 Torr) or severe hypoxia (Po2 ~20 Torr), respectively. Catecholamine release in D2⁻/⁻ mice was enhanced by 125% in mild hypoxia and 75% in severe hypoxia compared with WT mice, and the rate of rise was increased in D2⁻/⁻ mice. We conclude that CB transduction of hypoxia is still present in D2⁻/⁻ mice, but the response magnitude is reduced. However, the ventilatory response to acute hypoxia is maintained, perhaps because of an enhanced processing of chemoreceptor input by brain stem respiratory nuclei.

hypoxia sensing; arterial chemoreceptors; chemoreception

Carotid body (CB) chemoreceptors are the primary sensing element of the cardiorespiratory system that transduce a decrease in arterial oxygen tension into increased spiking activity on the carotid sinus nerve. Increased spiking activity initiates a number of protective reflexes, including increased drive to breathe and arousal from sleep during acute hypoxia. Within the CB, afferent nerve fibers synapse with glomus cells (GCs), secretory cells that contain several neurotransmitters including catecholamines (CA), acetylcholine, adrenaline nucleotides, and neuromodulators. Biochemical and pharmacological experiments have suggested important regulatory roles for all of these purported transmitter substances.

The principal CA within the CB is dopamine (DA), which has been long proposed to play an important role in the neurotransmission between the GC and nerve terminals (26). DA is synthesized and stored in dense core vesicles of GCs (40, 42) and found in high concentrations in CBs of cat, rabbit, rat, and mouse (25, 48). Simultaneous measurement of nerve activity and DA secretion, with either radioactive labels or voltammetry, demonstrated proportional changes in the magnitude of DA secretion and afferent nerve activity during stimulation with hypoxia, hypercapnia, acidosis, and metabolic poisons (13, 18, 46, 47). In light of this similar time course, CA or chemicals coscored with CA may potentially be the principal factor(s) accounting for the increase in nerve activity, but this has not been definitively established. Under some experimental conditions, such as after loading with l-DOPA or after reserpine treatment, the magnitude of nerve activity is not directly proportional to the magnitude of DA secretion (Refs. 14, 15, 34; but see Ref. 26). This raises the question of the actual role of DA release by the GC as playing a major role in mediating the increase in nerve activity or acting as a secondary modulator of events leading to the increase in nerve activity.

DA release within the CB may affect D1 and D2 receptors, located at both presynaptic and postsynaptic sites (23, 26). Denervation of nerve fibers results in a 60% decrease in spiroperidol and dopomeridone binding (11, 41), suggesting that 60% of the D2 binding sites were localized to the nerve fibers and 40% to the remaining CB cells. This was consistent with the observations in rat, rabbit, and cat that message for D2 receptors was present in both CB and petrosal ganglia, the site of the soma for the nerve fibers projecting to the CB (3, 4, 10, 19–22, 31). Although the presence of D2 receptors has been clearly established, the pharmacological role or importance has been more difficult to discern. Administration of exogenous DA to cat CB results in spiking inhibition at low doses and excitation at high doses (51). In contrast, DA administration to rabbit CB results only in excitation. Antagonism of D2 receptors with drugs such as haloperidol in vivo results in an increase in afferent nerve activity and an enhanced response to hypoxia (12, 39), suggesting an inhibitory role of DA in normal CB function. However, results have been inconsistent. In one study, administration of haloperidol to cat CB in vitro resulted in decreased spontaneous spiking activity (43). These disparate observations are difficult to synthesize into a coherent picture since multiple pathways are affected by the agonist or antagonist presentations. For instance, in addition to activation of D1 and D2 receptors in nerve endings and GC, exogenous DA in vivo affects vascular changes in the CB, resulting in altered oxygen gradients within the tissue. Haloperidol, in addition to blocking D2 receptors, also blocks calmodulin and BK-type Ca-activated K channels, both of which are present in CB cells (7).
The present experiments were designed to elucidate better the role of D2 receptors within the CB. This work takes advantage of genetically engineered mice that lack the D2 receptor (D2−/− mice). Work in other laboratories has demonstrated that these mice demonstrate an altered ventilatory response to hypoxia and an altered time course of ventilatory acclimatization to chronic hypoxia (32). Since measurements were limited to ventilatory responses of the intact animal, those investigators could not determine whether the alterations were due to vascular changes within the CB, changes in CB function per se, or central processing, which also utilizes dopaminergic mechanisms. Accordingly, we hypothesized that CB function of D2−/− mice would demonstrate an altered time course of nerve activity and secretory response to acute exposure to hypoxia. For this study we utilized a preparation that allowed single-axonal recording from mouse CB in vitro and electrochemical detection of CA secretion with carbon fiber voltammetry (47, 48).

MATERIALS AND METHODS

Animals. D2−/− and wild-type (WT; D2+/+) adult female mice (2–3 mo old) were used in the experiments detailed above. Both strains and their age-matched siblings were derived from breeding of D2+/− parents, as previously described (38). The genotype of each mouse was determined from DNA isolated from a tail clip, purified with phenol-chloroform, and analyzed by Southern blot analysis as previously described (27). Each animal was labeled with a unique telemetric marker along with its D2 genotype. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Valladolid. Body weights were 21.1 ± 0.4 g (n = 24) for WT mice and 24.5 ± 0.8 g (n = 20) for D2−/− mice. Because sex differences are established to alter CB CA turnover (44), experiments were only undertaken with female mice.

Tissue and organ harvest. Mice were deeply anesthetized with pentobarbital sodium (40 mg/kg ip), as evidenced by complete absence of a flexor withdrawal reflex. At this point, mice were decapitated. For nerve recordings, the chemoreceptor complex (carotid bifurcation-sinus nerve-petrosal ganglia) was dissected free and placed in cold, oxygenated Ringer saline solution, as previously described (16). In brief, the vagus nerve was isolated and followed to its junction with the nodose-petral ganglia complex. The glosso-pharyngeal nerve was cut central to the petrosal ganglia and reflected over the carotid bifurcation area removed en bloc. The complex was placed in dilute collagenase (type I, 0.8 mg/ml; Worthington Biochemical, Lakewood, NJ) and trypsin (0.2 mg/ml)-saline for 30 min with gentle agitation. Afterwards, the complex was transferred to a chamber containing ice-cold oxygenated (95% O2-5% CO2-balance N2) saline (in mM: 120 NaCl, 3 KCl, 2.1 CaCl2, 24 NaHCO3, 5 glucose) and cleaned of the surrounding muscle, vessels, and nerve fibers, leaving only the CB, sinus nerve, glossopharyngeal nerve, and petrosal ganglion. Measurement of DA content or measurement of release with amperometry did not require petrosal ganglion harvest, and for these experiments CBs were directly dissected free from the carotid bifurcation. Superior cervical ganglia were also isolated and dissected in cold solution.

Afferent unit recording protocols. For afferent unit recording, the chemoreceptor complex was transferred to a thermostabilized perfusion chamber (P-10, Warner Instruments, Hamden, CT) mounted on the stage of an inverted microscope. The chamber was perfused (2–3 ml/min) with saline, as described above, equilibrated with 21% O2-5% CO2-balance N2 at 37°C as control; both the saline reservoirs and perfusion lines were maintained at this temperature. Single-unit chemoreceptor recordings were obtained with small (30-μm tip diameter) glass pipettes advanced into the petrosal ganglia near the entry of the glossopharyngeal nerve fibers into the ganglion, where chemoreceptor activity is more abundant (16, 48). With application of small negative pressures to the pipette, a soma may be retracted into the pipette tip, allowing for extracellular recordings from the soma. The pipette voltage was amplified (NeuroLog, Digitimer, Welwyn Garden City, UK), filtered (1 kHz), digitized at 6 kHz (Axoscope, Axon Instruments, Molecular Devices, Wokingham, UK), and stored on a PC. Chamber oxygen tension was measured with a needle electrode (no. 760, Diamond Micro Sensors, Ann Arbor, MI) polarized to −0.8 V against an Ag/AgCl reference electrode placed in the chamber. The oxygen electrode current was also digitized in the same manner as above.

After a single-unit chemoreceptor recording was obtained, as evidenced by an irregular spontaneous discharge pattern and unity of action potential height and confirmed by an increase in spontaneous activity following switching of the perfusate to saline equilibrated with 0% O2-5% CO2, data acquisition was begun (48). Recordings were obtained over 60 s before stimulus presentation during superfusion with saline equilibrated with 20% O2-5% CO2-balance N2. Stimuli presented were switching the perfusate to saline equilibrated with 5% O2-5% CO2-balance N2 or with saline equilibrated with 0% O2-5% CO2-balance N2. In general, the hypoxia perfusate remained on for 4 min, although in some instances the stimulus was terminated earlier because of a loss of spontaneous activity.

Occurrence times for single-unit activities were detected off-line with a program designed to detect transition events (pCLAMP6, Axon Instruments, Molecular Devices). The program produced a file of times and event amplitudes that was used to compute event frequencies, as well as to confirm that the events were produced by a single unit, based on uniformity of action potential amplitudes. Unit spiking rates were quantified for (1) baseline spiking activity, as the mean spiking rate averaged over 1 min during superfusion with saline equilibrated with 20% O2-5% CO2-balance N2, (2) peak spiking rate, averaged over 3 s during superfusion with saline equilibrated with 5% O2 or 0% O2-5% CO2-balance N2, and (3) rate of spike increase, as the rate of increase between the start of increased spiking activity during hypoxia perfusion and the peak of activity during hypoxia perfusion.

Measurement of catecholamine content. After dissection, CBs were homogenized in 200 μl of cold 0.1 M perchloric acid containing 0.01% EDTA with glass-to-glass homogenizers. Samples were centrifuged (10,000 g, 10 min), and 20 μl of supernatant was injected in an HPLC-ED system composed of a Milton Roy CM 400 pump (Riveria Beach, FL), a C18 column (Waters, Madrid, Spain), a U6k injector (Waters), and a LC-4A electrochemical detector equipped with an LC-17 cell that contains a glassy carbon electrode (Bioanalytical System, West Lafayette, IN) at a holding potential of +0.65 V. The mobile phase was composed of (in mM) 10 NaH2PO4, 0.6 sodium octansulfonate, and 0.1 EDTA, with 16% MET-OH, pH 3.2 (adjusted with concentrated phosphoric acid). The signal coming out of the detector was fed to an analog-to-digital converter controlled by PeakSimple Chromatography System software (Buck Scientific, East Norwalk, CT). Identification and quantification were done against analytical standards. Superior cervical ganglia were homogenized in 200 μl of cold 0.1 M perchloric acid containing 0.01% EDTA with glass-to-glass homogenizers and processed according to a similar protocol.

Measurements of catecholamine secretion. After dissection, CBs were exposed to a diluted enzymatic solution [Tyrode-bicarbonate equilibrated with 95% O2-5% CO2 containing 0.1% collagenase (type I, Worthington Biochemical)] for 30 min at 35°C to facilitate penetration of the carbon fiber electrode into the tissue. CBs were transferred to the perfusion system mounted on the stage of an inverted microscope, as described above. The CA electrode was a 5-μm carbon fiber (proCFE, Dagan Instruments, Minneapolis, MN) polarized with a patch amplifier (EPC-7, List, Darmstadt, Germany) to +200 mV against an Ag/AgCl reference electrode. This potential is slightly above the oxidation potential for DA as determined in a previous study (47).
Free tissue CA was estimated based on the electrode current and calibrated against a known concentration (2 μM) of DA applied to the superfusate, as previously described (47).

**Whole body plethysmography.** Mice were located in individual cylindrical chambers (chamber volume ~1 liter) that were connected to a multichannel pump (air supply unit oxylet 4 LE400-4; Letica Scientific Instruments, Barcelona, Spain). A fraction of the outflow for each chamber was directed to a flow head (MLT 10L; ADInstruments, Castle Hill, Australia). In each experiment the flow delivered to the spirometer was adjusted to obtain an adequate signal (oscillations related to the respiratory movements) in the recording computer. PO2 of the chamber was recorded by an oximeter (Oxydig; Dräger Safety, Luebeck, Germany), and under the present conditions (gas flow 1 l/min) it took ~3 min to reach the delivered O2 percentage. Before the hypoxic test, the animals were in the chambers for 30 min to allow acclimation and to acquire basal ventilation during the last 5 min.

During the hypoxic test, the animals were in the chambers for 30 min to allow acclimation and to acquire basal ventilation during the last 5 min. During the hypoxic test, an atmosphere of 10% O2 was bled into the superfusate, as previously described (47).

**RESULTS**

**Chemoreceptor afferent activity.** Single-unit recordings were obtained from 15 axons of 8 WT and 13 axons of 7 D2−/− chemoreceptor complex preparations. Figure 1 represents the recorded nerve activity of chemoreceptor units from WT and D2−/− mice during superfusion with normoxic saline (solution equilibrated with 20% O2-5% CO2-balance N2; PO2 in the chamber ~150 Torr) and during superfusion with moderate hypoxia (5% O2-5% CO2-balance N2; PO2 in the chamber ~50 Torr; Fig. 1A) or severe hypoxia (0% O2-5% CO2-balance N2; PO2 in the chamber ~20 Torr; Fig. 1B) for 4 min. Baseline chemoreceptor nerve activity was not different between WT and D2−/− strains [WT 0.17 ± 0.04 Hz (n = 26), D2−/− 0.15 ± 0.03 Hz (n = 23)] (Fig. 2). However, peak discharge activity was significantly lower in D2−/− mice during stimulation with moderate hypoxia [WT 19.2 ± 2.2 Hz (n = 11), D2−/− 10.0 ± 2.6 Hz (n = 10); P < 0.02] (Fig. 2). The rate of rise of chemoreceptor activity was not different between the groups [WT 0.21 ± 0.03 Hz/s (n = 11), D2−/− 0.21 ± 0.09 Hz/s (n = 10)]. Peak discharge activity during stimulation with severe hypoxia was significantly larger in WT mice compared with D2−/− mice [WT 24.7 ± 2.2 Hz (n = 15), D2−/− 17.8 ± 1.5 Hz (n = 13); P < 0.02] (Fig. 2). Again, the rate of rise in chemoreceptor activity was not different between groups [WT 0.63 ± 0.10 Hz/s (n = 15), D2−/− 0.48 ± 0.09 Hz/s (n = 13)].

**Catecholamine secretion in response to hypoxia.** After finding that the chemoreceptor nerve activity in response to moderate and intense hypoxia in mice lacking D2 receptors is lower than in WT mice, we investigated whether the lower response in hypoxia (5% O2-5% CO2-balance N2; PO2 in the chamber ~50 Torr) or severe hypoxia (0% O2-5% CO2-balance N2; PO2 in the chamber ~20 Torr) compared with D2−/− mice (26).
could be the result of the lower secretory activity in response to hypoxia. In this context we used the secretion of CA from the in vitro-superfused CB as an index of the secretory response. Data from this group of experiments were obtained from 18 CBs harvested from 12 WT mice and 13 CBs harvested from 8 D2−/− mice. In some CB preparations the same hypoxic test was undertaken twice, and although the second trial produced smaller responses, differences were not significant between first and second responses (P > 0.05); hence the data from the first and second responses were pooled.

The level of free CA in the tissue, as measured with carbon fiber voltammetry, increased in response to hypoxia (Fig. 3). The magnitude of increase was significantly greater in D2−/− compared with WT mice (Fig. 4A). In response to moderate hypoxia applied over 3 min, free tissue CA increased to 0.9 ± 0.3 μM (n = 10) in WT mice compared with 2.1 ± 0.4 μM (n = 10) in D2−/− mice (P < 0.05). In response to severe hypoxia over 3 min, free tissue CA increased to 4.8 ± 0.6 μM (n = 35) in WT mice compared with 8.3 ± 1.0 μM (n = 24) in D2−/− mice (P < 0.01). The increase in the evoked CA secretion in D2−/− mice was lower in response to severe hypoxia (76%) than to moderate hypoxia (116%), which could reflect a lower modulation exerted by D2 receptors during severe hypoxia. The time to peak of the hypoxia-evoked secretory response was significantly shorter in D2−/− mice (Fig. 4B). In the 7 of 10 preparations that responded to moderate hypoxia, peak CA levels were reached at 178.6 ± 17.8 s in the evoked responses of WT mice (n = 7) compared with 129.1 ± 9.8 s (n = 10) in D2−/− mice (P < 0.02).

Similarly, in response to severe hypoxia, CA in the tissue reached a peak at 113.9 ± 2.0 s (n = 24) in D2−/− mice compared with 131.4 ± 6.3 s (n = 35) in WT mice (P < 0.05). In contrast to the response to hypoxia, CA in the tissue reached a peak at 170.3 ± 13.5 s (n = 35) in WT mice (P < 0.05) or depolarizing (1.5 min with solution containing 35 mM KCl) stimuli. Comparisons of the peak responses (A) and of the time to peak after the start of the stimuli (B) are shown. Data represent means ± SE for n indicated in parentheses. *P < 0.05, **P < 0.02.

Catecholamine content. After finding an increase in hypoxia-induced CA secretion in D2−/− mice, we investigated CA content in the respective mice. DA and norepinephrine content were not different between WT and D2−/− CBs (Fig. 5). As previously reported, DA is the primary CA in the mouse CB (48). Nonchemoreceptor structures were also not different. Norepinephrine content of the superior cervical ganglia was 44.0 ± 5.6 pmol (n = 8) for WT and 39.1 ± 2.6 pmol (n = 8) for D2−/− mice. DA content of the superior cervical ganglia was 3.7 ± 0.3 pmol (n = 8) for WT and 3.5 ± 0.3 pmol (n = 8) for D2−/− mice.

Ventilatory response to hypoxia. A final group of experiments was done to investigate whether the observed changes in chemoreceptor activity in response to hypoxia are translated to ventilatory reflexes. For this purpose 15 mice of both strains (WT and D2−/−) were individually placed in a whole body plethysmograph, in which they were unrestrained and free to move about the chamber. After acclimatization to the chamber for 30 min, acute hypoxia was induced by flushing the chamber for 12 min with a hypoxic (10% O2) atmosphere. WT and D2−/− mice did not differ in basal respiratory frequency or in increase in respiratory frequency in response to acute hypoxia (Fig. 6).
DISCUSSION

In this work we examined the role of D2 receptors in CB secretory and afferent nerve responses to hypoxia as well as the ventilatory response to acute hypoxia. Peak nerve activities during hypoxia were decreased in D2−/− mice compared with WT mice, and the rate of hypoxia-stimulated CA release was enhanced. However, the ventilatory response to acute hypoxia was not significantly changed in D2−/− mice. These observations lead to the general conclusion that D2 receptors contribute but are not essential to CB function or hypoxia transduction and play a role in modulating or shaping the secretory response to hypoxia.

In the past we had postulated (26) important roles of DA, and D2 receptors in particular, in initiating and modulating the organ response to hypoxia. This was largely based on the unequivocal demonstration of DA synthesis within the organ and its release in direct proportion to afferent nerve activity. While D2 receptors are often associated with inhibitory processes, some excitatory effects have been ascribed to D2 receptors in the central nervous system (30), and exogenous application of DA is sometimes excitatory to afferent nerve activity (35). However, the present demonstration that nerve activity is well maintained in the absence of D2 receptors requires some modification of this postulate. Several other neurotransmitters, including substance P, acetylcholine, ATP, and serotonin, have recently been shown to be important in mediating nerve excitation and transmission between the GC and nerve ending (35, 36, 52). Elimination of postsynaptic D2 receptors in the knockout mouse may have eliminated only one of the excitatory drives, perhaps accounting for the observed decrease in peak afferent nerve activity during stimulation with moderate or severe hypoxia. Alternatively, DA, perhaps acting through D2 receptors, has been shown to modulate the release or effect of other excitatory transmitters. For instance, in one study, application of exogenous DA did not modify the rate of basal discharges in the carotid nerve but did modify the subsequent excitatory responses to exogenous acetylcholine (1).

An alternative pathway by which D2 receptor activation may enhance the responsiveness to other excitatory transmitters lies in the voltage dependence of Na channels present in chemoreceptor nerve terminals. The steady-state inactivation characteristics of petrosal chemoreceptor neurons place more than half the Na channels in the inactive state at a resting potential of −70 mV (9). Chemoreceptor nerve terminals are reported to be even more depolarized (28). Thus under resting conditions most Na channels in chemoreceptor nerve terminals would be inactivated and unable to support action potential generation. Therefore “inhibitory” transmitters, such as DA acting through D2 receptors, may potentially hyperpolarize nerve terminals, placing more Na channels in the closed state compared with the inactivated state, thus lowering the voltage threshold for action potential generation.

The best-established role of the D2 receptor lies in feedback modulation of GC secretion, and this likely accounts for the enhanced CA secretion observed in D2−/− mice. D2 message is present in GC (3, 19). Blockade of D2 receptors enhances the hypoxia-induced release of CA in mature rabbit CB (6). This is likely modulated through Ca channels since exogenous DA reduces GC Ca currents (8) and reduces the increase in intracellular Ca caused by hypoxia (37). A similar feedback inhibition has been reported in other tissues (49). Thus the present observation, demonstrating enhanced CA release in D2−/− mice, is consistent with the role of presynaptic D2 receptors acting as elements mediating feedback inhibition.

In addition to D2 receptors, some actions of DA may be affected by D1 or D3 receptors, but the presence and role of D1 receptors is not well resolved. In one study the message for D1 receptors was present in both CB and petrosal ganglia (5), but others failed to detect the message with in situ hybridization (10) or conventional RT-PCR (23). The original positive result may have been due to the presence of the message in nerve fibers, as suggested by Gauda (23) or to its presence in nonchemoreceptor tissue such as vascular tissue (2). Almaraz et al. (2) demonstrated that D1 receptor activation changed tissue cAMP levels but was without effect on GC function. D3 receptors, which are part of the D2 family, have been recently detected in goat CB (50). However, the potential role of these receptors is not currently known.

Our observation of normal ventilatory response to acute hypoxia in D2−/− mice is consistent with that previously reported by Huey and Powell (31). At the moderate PO2 levels used in their study and the present study, the ventilatory response was normal in D2−/− mice. At more severe hypoxic levels that were not tested in the present study, D2−/− mice showed an enhanced ventilatory response to hypoxia (31). D2−/− mice also demonstrated an altered ability to acclimatize to continuous hypoxia (33). These changes cannot be attributed solely to the CB since hypoxia-induced release of DA is present in the brain stem regions important for respiratory rhythm generation and modulation (24). Acute blockade of central D2 receptors depresses the ventilatory response to acute hypoxia (29). This suggests that chronic ablation of D2 receptors, as is present in our D2−/− mice, results in several compensatory changes. For instance, acute blockade of D2 receptors results in enhanced peripheral chemoreceptor activity, while chronic blockade (i.e., knockout) results in lower chemoreceptor activity. Similarly, acute blockade of central D2 receptors results in a reduced ventilatory response to acute hypoxia, while chronic blockade results in normal or enhanced response to acute hypoxia. Thus compensatory mechanisms that may come into play in the knockout model will need to be identified before the physiological response can be properly understood in this model.

In conclusion, our results demonstrate an excitatory role, albeit small, of D2 receptors in mouse CB, although it is
unresolved whether this is a direct effect or D₂ modulation of other neurotransmitters. In addition, these receptors modulate the magnitude of hypoxia-induced CA release, likely through presynaptic receptors on DA-synthesizing cells. These results differ somewhat from those observed during acute blockade of D₂ receptors in vivo, suggesting that vascular effects or compensatory changes have a significant role in the different models.

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