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Hypothyroidism Attenuates SCH 23390-mediated Depression of Breathing and Decreases D1 Receptor Expression in Carotid Bodies, PVN and Striatum of Hamsters

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Abstract

Hypothyroidism can lead to depressed breathing. We determined if propylthiouracil (PTU)–induced hypothyroidism in hamsters (HH) altered dopamine D1 receptor expression, D1 receptor-modulated ventilation, and ventilatory chemoreflex activation by hypoxia or hypercapnia. Hypothyroidism was induced by administering 0.04% PTU in drinking water for three months. Ventilation was evaluated following saline or 0.25 mg/kg SCH 23390, a D1 receptor antagonist, while awake hamsters breathed normoxic (21% O₂ in N₂), hypoxic (10% O₂ in N₂) and hypercapnic (5% CO₂ in O₂) air. Relative to euthyroid hamsters (EH), HH exhibited decreased D1 receptor protein levels in carotid bodies, striatum, and hypothalamic paraventricular nucleus, but not in the nucleus tractus solitarius. Relative to EH, HH exhibited lower ventilation during exposure to normoxia, hypoxia, or hypercapnia, but comparable ventilatory responsiveness to chemoreflex activation. SCH 23390 decreased ventilation of EH hamsters exposed to normoxia, hypoxia, and hypercapnia. In HH SCH 23390 increased ventilation during baseline normoxia and did not affect ventilation during exposure to hypoxia and hypercapnia, resulting in reduced ventilatory responsiveness to chemoreflex activation by hypoxia and hypercapnia. Furthermore, in HH D1 receptor protein levels are decreased in several brain regions and within the carotid bodies. Moreover, D1 receptor-modulation of breathing at rest and during gas exposures were depressed in EH but not HH.

Keywords

hypothyroidism; carotid body PVN; NTS; hypoxia; hypercapnia; SCH 23390; propylthiouracil

1. Introduction

In the United States both overt and subclinical hypothyroidism are important endocrine disorders with a prevalence of 4.6% to 9.5% in adults (Canaris et al., 2000). Moreover, replacement therapy in hypothyroid patients is not always adequate to “normalize” function

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(Aoki et al., 2007; Liu et al., 2008). The depressive effect of hypothyroidism on control of breathing in conscious and sleeping individuals has been reported (Duranti R, 1993; Resta, 2004; Saarestranta and Polo, 2002). Moreover, severely hypothyroid patients may exhibit blunted ventilatory responses to hypoxia and hypercapnia and have a higher propensity to develop sleep apnea (Resta, 2004) that can be reversed by thyroxine replacement (Jha et al., 2006). There is evidence in rats and rabbits (Simsek et al., 2004) that hypothyroidism results in alterations in the function of the carotid body especially in response to hypoxia. For example, Olea and coworkers (Olea et al., 2007) made rats hypothyroid by administration of propylthiouracil (PTU) in their drinking water. Ventilation in response to hypoxia and hypercapnia was lower in the conscious hypothyroid rats than in controls. Moreover, when carotid bodies were studied *in vitro*, carotid sinus nerve activity and release of catecholamines in response to hypoxia, but not to hypercapnia, were lower in the PTU-treated rats. Thus, hypothyroidism may alter both central and carotid body modulation of ventilation.

There are several methods for inducing hypothyroidism in animal models including various genetic models that lack thyroid receptors or deiodenases, removing the thyroid glands, administration of anti-thyroid agents such PTU or methimazole, or diets low in iodine (Gong et al., 2010; Liu et al., 2003; Vaccari et al., 1990; Vallortigara et al., 2009). In some cases, combinations of treatments have been used. The advantage of using an agent such as PTU is that it can be administered in drinking water at various concentrations that may induce different levels of hypothyroidism (Goodman and Gilbert, 2007). Moreover, this is a method that has been used previously in our laboratory in hamsters (Varney and Schlenker, 2007).

A way that hypothyroidism may affect breathing is by altering the function of multiple neurotransmitter systems in the carotid body and within brain regions associated with control of breathing. For example, Schlenker and coworkers showed PTU-induced hypothyroidism in hamsters depressed the stimulatory effects of naloxone, a mu-opioid receptor antagonist, on ventilation (Schlenker, 1994). More recently, Varney and Schlenker reported that a specific serotonin 2A receptor antagonist (R)-(+)- α -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (MDL 100907) resulted in long-term depression of ventilation (a decrease in ventilation following intermittent hypoxia relative to baseline values) in euthyroid, but not in hypothyroid hamsters (Varney and Schlenker, 2007).

Hypothyroidism also affects dopaminergic systems. For example, Crocker and Cameron (1988) reported that after 4 weeks of PTU-induced hypothyroidism, adult male rats exhibited similar striatal D1 receptor levels as euthyroid controls, but a greater increase in dopamine stimulated cyclic AMP levels. In contrast, induction of hypothyroidism by administration of methimazole in pregnant rats and then continuing administration of methimazole to the weanling offspring decreased both striatal D1 receptor protein levels and D1 stimulation of adenylate cyclase activity. Since induction of hypothyroidism prenatally and during critical periods of development can also have marked effects on brain morphology (Stoica et al., 2007), changes in D1 receptor levels may have been associated with this factor. Thus, both age and duration of hypothyroidism appear to affect D1 expression and function. How hypothyroidism influences D1-modulation of breathing is unknown.

In a previous study we assessed the role of D1 receptors on control of breathing in euthyroid hamsters using the brain permeant D1 receptor antagonist SCH 23390 (Bourne, 2001). SCH 23390, at a dose of 0.25 mg/kg, decreased ventilation in hamsters by decreasing frequency of breathing during exposure to room air, hypoxia, and hypercapnia (Schlenker, 2008). This

was the first study to evaluate the role of D1 receptors in control of breathing in a conscious animal.

In the present study, we evaluated the effects of PTU- induced hypothyroidism in adult hamsters on D1 receptor protein levels within the carotid bodies, paraventricular nucleus of the hypothalamus (PVN), striatum, and nucleus tractus solitarius (NTS) as well as D1 modulation of breathing during exposure of animals to normoxia, hypoxia, and hypercapnia. Although the NTS is well known as an integratory region associated with regulation of breathing (Mifflin, 1992; Nattie and Li, 2002), there is also evidence that the PVN and the striatum modulate ventilation (Evans et al., 1999; Reddy et al., 2005; Schlenker et al., 2001). We hypothesized that hypothyroidism would depress ventilation relative to euthyroid hamsters, that the ventilatory depression induced by blocking D1 receptors in normal hamsters would be blunted in hypothyroid hamsters, and that D1 receptors protein levels in carotid bodies, the PVN, striatum and NTS would be decreased by hypothyroidism. In addition we used immunohistochemistry to determine the location of D1 receptors in hamster carotid bodies.

2. Results

2.1 Body weights (BW), thyroxine levels, body temperatures, and metabolism

Body weights of the PTU-treated animals (134.3 ± 16.6 grams) were lower than in the euthyroid hamsters (146.8 ± 15.4 grams, $P < 0.05$). Thyroxine levels (in $\mu\text{g/dl}$) were lower in PTU compared to euthyroid hamsters (9.76 ± 2.12 versus 26.72 ± 5.17 , $P < 0.001$). Likewise body temperatures (in degrees Celsius) in the PTU-treated hamsters (34.2 ± 0.2) were significantly lower compared to the euthyroid animals (34.8 ± 0.2 ; $P < 0.02$). Moreover, SCH 23390 administration decreased body temperature significantly ($P < 0.05$) in the euthyroid hamsters to 33.5 ± 0.25 degrees Celsius, but had no effect on body temperature in the hypothyroid hamsters (33.8 ± 0.09).

Carbon dioxide production (in $\text{ml/min} \times 1000/\text{BW}$) was lower in the hypothyroid hamsters (13.2 ± 1.8) compared to euthyroid hamsters (21.7 ± 1.6 , $P = 0.004$). SCH 23390 decreased CO_2 production in the euthyroid hamsters to 12.7 ± 1.4 ($P = 0.02$), but had no effect on the hypothyroid hamsters (16.1 ± 0.83). No group or treatment effects were noted in oxygen consumption (data not presented).

2.2 D1 receptor protein levels in carotid bodies, NTS, striatum and PVN and immunohistochemistry of the carotid body

Both euthyroid and PTU-treated animals expressed D1 receptors in carotid bodies. In PTU-treated animals, carotid body D1 receptor protein levels were significantly lower than in euthyroid hamsters ($P < 0.0001$), but not different in the NTS (Fig.1). Moreover in the PVN and striatum D1 receptor protein levels were significantly decreased in the hypothyroid hamsters compared to levels in comparable regions in euthyroid animals ($P = 0.001$; Fig. 2). Only one band was noted for the antibodies used. Thus, relative to euthyroid state, hypothyroidism decreased expression of D1 receptor protein levels in the striatum, carotid bodies, and PVN, but not in the NTS. Moreover, D1 receptors appear to be located in the glomus cells of the carotid body as indicated by the tyrosine hydroxylase staining (Fig. 3) with expression of D1 protein being higher in carotid bodies from euthyroid relative hypothyroid hamsters, which corresponds to our Western blot findings.

In addition to using tyrosine hydroxylase as a marker for glomus cells, we also determined if hypothyroidism induced a difference in carotid body morphology and the relative to those of euthyroid hamsters. Cross sectional areas of carotid bodies (from four animals per group) were comparable in the two groups (means and (SD); euthyroid $= 8.92 \text{ mm}^2$ (1.37) and

hypothyroid = 8.41 mm² (0.20). In addition, the sum of the cross sectional area of glomus cells to the total carotid body cross sectional area was similar (Euthyroid = 0.290 (0.04) and hypothyroid = 0.288 (0.05) as was the number of glomus cells per area. Thus, the decrease in D1 expression noted by Western blot and immunohistochemistry analysis was not related to changes in the glomus cell levels or altered carotid body morphology.

2.3 Ventilation during exposure to normoxia

During the normoxic baseline condition (Fig. 4) ventilation was significantly lower in hypothyroid hamsters compared to euthyroid hamsters ($P < 0.05$) due to a decrease in both tidal volume ($P < 0.05$) and frequency of breathing ($P = 0.0001$). Whereas SCH 23390 increased ventilation in the hypothyroid animals ($P = 0.035$), SCH 23390 decreased minute ventilation in the euthyroid hamsters ($P = 0.035$). The decrease of ventilation in the SCH 23390-treated euthyroid hamsters was due to a decreased frequency of breathing ($P = 0.0005$). SCH 23390 had no effect on frequency of breathing in the hypothyroid hamsters. Tidal volume was not affected by SCH 23390 in the euthyroid hamsters, but it was increased in the hypothyroid animals ($P = 0.045$). Thus, in hypothyroid hamsters, long-term treatment with PTU decreased ventilation, and SCH 23390 had opposite effects on resting ventilation in the hypothyroid and euthyroid animals.

2.4 Ventilation during exposure to hypoxia

With saline treatment, during exposure of the hamsters to hypoxia, minute ventilation was decreased in hypothyroid compared to euthyroid hamsters (Fig. 5, $P = 0.03$) due to lower frequency of breathing and tidal volumes in the hypothyroid animals. However, ventilatory responsiveness to hypoxia (ventilation during hypoxia/ventilation during normoxia) was comparable between the two groups (2.2 ± 0.21 for the controls and 2.3 ± 0.24).

Ventilation was attenuated during hypoxia in SCH 23390-treated euthyroid, but not hypothyroid hamsters compared to respective saline controls ($P = 0.038$). In both groups SCH 23390 decreased frequency of breathing significantly (euthyroid $P = 0.0013$ and hypothyroid $P = 0.04$) in response to hypoxia. Interestingly, relative to saline, SCH 23390 administration decreased hypoxic ventilatory responsiveness only in the hypothyroid group (Fig. 5). Thus, saline-treated hypothyroid hamsters exhibited lower minute ventilation during hypoxia, and following SCH 23390 they exhibited lower hypoxic ventilatory responsiveness to SCH 23390 relative to euthyroid hamsters.

2.5 Ventilation during exposure to hypercapnia

During exposure to hypercapnia, minute ventilation of hypothyroid hamsters was lower, compared to euthyroid hamsters (Fig. 6). The hypothyroid hamsters exhibited lower tidal volumes compared to euthyroid animals ($P = 0.002$), but not breathing frequencies.

SCH 23390 administration decreased minute ventilation in the euthyroid hamsters during exposure to hypercapnia, ($P = 0.0125$), but had no effect on minute ventilation in the hypothyroid animals. Relative to saline treatment, both groups treated with SCH 23390 decreased frequency of breathing (euthyroid $P = 0.0004$, and hypothyroid $P = 0.01$). The decrease of frequency in the euthyroid hamsters was 46% and 19% in the hypothyroid animals. During exposure to hypercapnia, SCH 23390 had no effect on tidal volume in either group. Following saline treatment, hypercapnic ventilatory responsiveness (minute ventilation during hypercapnia/ventilation during air) was comparable between the groups (Figure 7). After administration of SCH 23390, hypercapnic ventilatory responsiveness decreased in both groups.

3. Discussion

The major findings of this study were that PTU-induced hypothyroidism in hamsters led to decreased body temperature, CO₂ production, and ventilation when animals were exposed to air, hypoxia, and hypercapnia. Moreover, relative to saline administration, SCH 23390 decreased CO₂ production and body temperature in the euthyroid hamsters, and ventilation during exposure to normoxia, hypoxia and hypercapnia. During normoxic air exposure, SCH 23390 actually increased minute ventilation in the hypothyroid animals. In hypothyroid hamsters following SCH 23390 treatment, hypoxic ventilatory responsiveness was decreased, whereas hypercapnic ventilatory responsiveness was decreased in both groups. The ventilatory results are summarized in Table 1. Another important finding of this study was that relative to euthyroid animals, D1 receptor protein levels were lower in the PVN, striatum, and carotid bodies of hypothyroid hamsters but not in the NTS.

In the PTU-treated hamsters the decreased D1 receptor protein levels paralleled the lower resting ventilation, as well as ventilation during exposure of animals to hypoxia, and hypercapnia. No such correlation existed with changes in chemoreflex responsiveness to hypoxia or hypercapnia in these animals. Interestingly, D1 receptor blockade decreased hypercapnic chemoreflexes in both normal and PTU groups to a similar degree, but decreased chemoreflexes to hypoxia only in the PTU group. Thus in euthyroid hamsters there was a close correlation between D1 receptor protein levels in the PVN, carotid bodies and striatum, but not NTS, and resting ventilation, no such correlation existed for chemoreflex responsiveness in the hypothyroid animals.

3.1 Effects of hypothyroidism on metabolism and ventilation

The results of this study indicate that PTU treatment of hamsters for three months induces hypothyroidism as manifested by decreases in T4 levels, body temperature, CO₂ production, body weight, baseline normoxic ventilation, and decreased ventilation during exposure to hypoxia and tidal volume during hypercapnia relative to euthyroid hamsters. The decreased ventilation may be result of lower body temperature and lower CO₂ production. In previous studies we noted decreased CO₂ production and lower oxygen consumption in hypothyroid relative to euthyroid hamsters (Schlenker et al., 1994).

In the present study when we evaluated hypoxic and hypercapnic responsiveness, which were comparable in hypothyroid to those observed in euthyroid hamsters. This contrasts with finding in hypothyroid hamsters in a previous study from our laboratory (Schlenker, 1994) where ventilatory responsiveness to hypoxia and hypercapnia were lower in hypothyroid compared to euthyroid hamsters. Interestingly, those hamsters were obtained from another vender (Sasco, Omaha), while hamsters in the present study were obtained from Harlan.

Some, but not all of our results are similar to those reported by Simsek and coworkers (Simsek et al., 2004) in hypothyroid rabbits. One group (known as the responders) exhibited the blunted hypoxic and hypercapnic ventilatory responses due to both decreased peripheral and central chemoreception capabilities. Interestingly another subgroup of rabbits did not show these blunted responses following induction of hypothyroidism by methimazole administration for 10 weeks. The major difference between the two groups was the level of thyroid stimulating hormone elevation which the authors speculated may have affected thyroid regulating hormones in brain regions associated with control of breathing such as the retrotrapezoid nucleus. A similar finding of subsets of hypothyroid patients was reported by Duranti and coworkers (Duranti R, 1993). One group was clearly hypothyroid, but had normal ventilatory responses to hypercapnia and normal respiratory muscle function. In that study thyroxine replacement therapy improved ventilatory drive, improved respiratory

control in some patients, but did not normalize respiratory muscle strength in hypothyroid patients who had low maximal inspiratory pressures at baseline. Thus, hypothyroidism may affect respiratory muscle function and chemoreceptor regions associated with respiratory drive, but results are not consistent in all hypothyroid subjects possibly due to the extent and duration of the hypothyroidism. Likewise in two studies in hypothyroid hamsters (the present study) and a past study (Schlenker, 1994) we observed disparate results in chemoresponsiveness to hypoxia and to hypercapnia.

3.2 Effects of hypothyroidism on D1 receptor protein levels

This is the first report to demonstrate that hypothyroidism decreased D1 receptor protein levels in the carotid body, stratum, and the PVN. In hamster carotid bodies, D1 receptors are located in glomus cells as indicated by tyrosine hydroxylase staining in adjacent sections. Hypothyroidism did not affect TH expression in the carotid body as indicated by our quantification of glomus cell TH levels relative to total carotid body cross sectional area. Moreover, in an unpublished study of carotid bodies in hypothyroid and euthyroid hamsters, using Western blot (Schlenker and Sykora, unpublished observations) levels of tyrosine hydroxylase relative to actin levels were comparable between the two groups ($n=8$ per group, values for hypothyroid hamsters were 2.23 ± 0.08 and euthyroid hamsters 2.28 ± 0.11 , $P=0.77$). The fact that carotid body morphology, but function may not be normal was also noted in a study by Kline and coworkers (Kline et al., 2002) in mice partially defective in hypoxia-inducible factor 1 α (HIF). They also found similar carotid body morphology, but lower isolated carotid body function in response to hypoxia in the HIF heterozygotes relative to wild type controls.

In the present study we also did not observe differences in D1 receptor expression in the NTS. Since this region is involved in integration of respiratory inputs from areas such the carotid body and affects control of breathing, these results may appear surprising. However, in a recent study (Schlenker, 2010) we also did not see differences D1 receptor protein levels in the medial, lateral or commissural areas of the NTS in dystrophic hamsters that exhibits a form of euthyroid sick syndrome hypothyroidism. However, chronic administration of an angiotensin AT1 receptor blocker increased levels of D1 in the commissural and medial regions of the NTS and also increased ventilation in dystrophic hamsters. Thus, D1 receptor proteins in the NTS do not appear to be affected by hypothyroidism in hamsters.

Few studies have investigated the effects of hypothyroidism on D1 receptors in the PVN or carotid bodies, however several studies have examined the effects of hypothyroidism on D1 receptors expression and function in the striatum, a brain region also involved in regulation of breathing (Evans et al., 1999). Cameron and Cocker noted that relative to euthyroid rats animals made hypothyroid by administration of 200mg/kg PTU for 4 weeks showed no difference in the number, binding sites or affinities of striatal D1 receptors. In homogenates of D1 stimulation-induced striatal tissue cAMP levels were lower in hypothyroid relative to euthyroid rats (Cameron and Crocker, 1987). In another study using the same model to induce hypothyroidism described in their previous paper, Cocker and Cameron noted that the behavioral responses (assessed at the time needed to move from a center grid in an open field apparatus) of hypothyroid hamsters to SCH 23390 were lower than that of euthyroid controls (Crocker and Cameron, 1988). Vaccari and coworkers reported that prenatal and neonatal hypothyroidism in rats, decreased striatal D1 receptors levels and second messenger activity (Vaccari et al., 1990). The length of hypothyroidism may also affect expression of dopamine receptors in the striatum. For example in rats, if hypothyroidism was maintained for 10 weeks instead of 4 weeks, D2 receptors in the striatum were increased (Crocker, et al., 1986). Interestingly, preliminary studies from our laboratory also noted that following 12 weeks of PTU treatment striatal D2 receptors were also increased in

the striatum and the carotid body, but not in the NTS (unpublished data). Thus, the length of time animals remain hypothyroid, the dose of the agent used to induce hypothyroidism, and the time during development when hypothyroidism was induced, and the region of the brain examined may all affect expression and function of dopamine receptors.

3.3 Dopamine D1 receptor modulations of metabolism and breathing: potential mechanisms

In this study in the air-breathing euthyroid hamsters the decrease of ventilation following SCH 23390 administration may have been related to the decreased CO₂ production and decreased body temperature. The latter finding was also noted in a previous study (Schlenker, 2008). However, the relative increase in ventilation in the hypothyroid hamsters appears not to be metabolically driven, since SCH 23390 had no effect on O₂ consumption or CO₂ production. Interestingly in a previous study in hamsters (Schlenker, 2008), a significant effect on body temperature, but not on CO₂ production was noted, but frequency of breathing (but not ventilation) was also lower following SCH 23390 treatment. Whether the difference could have been due to a circannual effect (Summer: this study, Winter: previous study), is not clear.

We had hypothesized that both D1 receptor protein levels and chemoreflex ventilatory responses to D1 receptor antagonist would be decreased in hypothyroid animals. Acute SCH 23390 treatment reduced hypoxic chemoreflex responsiveness only in hypothyroid animals. However, hypercapnic chemoreflex responsiveness to D1 receptor blockade did not differ between euthyroid and hypothyroid groups. These results suggest that the contribution of D1 receptors blockade by SCH 23390 to hypoxic chemoreflex responsiveness is impaired in hypothyroid hamsters, but their contribution to hypercapnic chemoreflex is not affected. One factor that may affect the responsiveness of the receptors is the release of dopamine. Preliminary results from our laboratory (Schlenker and Forster) indicate that during acute exposure to hypoxia dopamine turnover is less in hypothyroid in various brain regions including the striatum and parabrachial nuclei. Peterson and coworkers also found decreased dopamine release in the striatum of hypothyroid rats (Peterson et al., 2006). To determine dopamine release during exposure to hypoxia, microdialysis or a more rapid voltammetric method to determine dopamine release (Greco et al., 2006).

To investigate whether having lower levels of dopamine may be important in the responsiveness to hypoxia and hypercapnia, in a separate study we gave euthyroid and hypothyroid hamsters a D1 receptor agonist, SKF 38393. There was a dose dependent increase in ventilation and increase in the hypothyroid hamsters that was not seen in the euthyroid hamsters (unpublished observations, Schlenker). In that study although hypercapnic ventilatory responsiveness was increased in response to SKF 38393 in hypothyroid hamsters, no increase in hypoxic ventilatory responsiveness was noted. Taken together with the present study, these data suggest that D1 receptors in hypothyroid hamsters in some regions (possibly hypercapnic sensors) may actually be more sensitive to dopamine, but that the amount of dopamine released during hypoxia may be reduced relative to euthyroid animals. This combined with the reduction in D1 receptors would result in less of a stimulation of lower amount of D1 receptors.

Few studies have investigated the role of D1 receptors on the ventilatory response to hypercapnia. Lalley (Lalley, 2004) examined the role of D1 receptors on ventilation in anesthetized and unanesthetized decerebrate cats and noted that D1 receptor agonists given intravenously stimulated frequency of breathing of cats in air and during exposure to CO₂. These effects were blocked by SCH 23390. In the present study while D1 receptors contributed to the hypercapnic reflex in euthyroid hamsters, the impaired expression of D1 receptors in hypothyroidism did not influence ventilatory responsiveness to CO₂.

Paradoxically, in hypothyroid hamsters treated with SCH 23390 during normoxia, tidal volume and minute ventilation increased but these parameters decreased in SCH 23390-treated euthyroid animals. Srinivasan and coworkers suggested that one way that SCH 23390 might enhance normoxic ventilation (Srinivasan et al., 1991). They noted that acute intravenous administration of SCH 23390 into rabbits resulted in enhanced phrenic nerve activity and increased release of substance P from the NTS. Whether hypothyroid hamsters release more substance P in the NTS needs to be evaluated.

3.4 Future studies

A challenge in determining where SCH 23390 is acting in the present study is that it was given systemically. Thus, it may have acted on several sites including the carotid body and central regions associated with control of breathing. To investigate where and how modulation of breathing may occur future studies will inject D1 receptor agonists and antagonists microinjected into specific brain regions such as the striatum and PVN in conscious hypothyroid and euthyroid animals (Schlenker et al., 2001) in euthyroid and hypothyroid hamsters. These approaches would be valuable in dissecting central versus peripheral aspects of the D1 mediated signaling pathways in hypothyroidism.

Additional studies of isolated carotid bodies from euthyroid and hypothyroid hamsters using calcium signaling to indicate activation could be used to determine relative effects of D1 receptor activation during exposure to hypoxia and hypercapnia in these animals. These would be important to dissect central versus peripheral effects of D1 modulation of breathing in hypothyroid animals.

In summary, in hamsters, hypothyroidism induced by PTU administration decreased ventilation and decreased D1 receptor expression in the carotid body, PVN and striatum (but not NTS), but did not alter chemoreflex responsiveness to hypoxia or hypercapnia. SCH 23390 decreased body temperature, CO₂ production and ventilation in euthyroid hamsters during exposure to air, and decreased ventilatory responsiveness to hypercapnia. In the hypothyroid hamsters, SCH 23390 treatment increased minute ventilation during air exposure, whereas both hypoxic and hypercapnic responsiveness was decreased. Thus, while hypothyroidism in hamsters decreased D1 receptor protein levels in carotid bodies, PVN and striatum, the contribution of D1 receptor function to the control of breathing in air, during hypoxia and hypercapnia in hypothyroidism is complex and cannot be easily explained by impaired expression of the receptor alone.

4. Experimental Procedure

4.1 Animals

Seven to eight week old male golden Syrian hamsters were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The hamsters were divided into two groups. Sixteen hamsters received tap water to drink and constituted the euthyroid group. The other sixteen animals received 0.04% PTU in tap water to induce hypothyroidism for three months. Animals were housed four per cage, exposed to 14 hours of light and 10 hours of dark, and received rodent pellets (8604, Harlan Sprague Dawley, Inc.). Both food and fluids (water or PTU) were available *ad libitum*. Three days prior to the experiments, hamsters were acclimated to the barometric chamber for 30 minutes each day. The University of South Dakota Animal Care and Use Committee approved all procedures used in this study.

4.2 Barometric Chamber and respiratory and metabolic measurements

Respiratory measurements were made in a 20.2 × 7.9 cm Plexiglas cylindrical chamber using the barometric method (Schlenker, 1984). Ports allowed air or test gases to enter and

exit the chamber. Fractional contents of oxygen and CO₂ entering and exiting the chamber were measured with Vacu-Med Model 17515A gas analyzers. Barometric pressure was measured using a W.M. Welch (Chicago, IL) mercury barometer. Airflow rate through the chamber was measured using a Gilmont rotameter. Chamber temperature was determined using a Fisher Scientific digital thermometer. Relative humidity within the chamber averaged 50%. Inspiratory and expiratory times were measured from 10 breaths and were averaged for each portion of the study and used to calculate frequency of breathing by adding inspiratory and expiratory times and dividing the sum into 60 (sec/min). The minute ventilation was determined by multiplying frequency by tidal volume. Both tidal volume and minute ventilation were normalized by body weight (variable*1000/BW). Oxygen consumption and CO₂ production were determined by the flow through method (Schlenker, 1984) and corrected by Standard temperature pressure dry (STPD) and body weight.

4.3 Protocol and T4 measurements

On the day of an experiment the hamster was weighed and then injected subcutaneously with approximately 0.2 ml of the saline or a 0.25 mg/kg dose of SCH 23390 (SCH 23390, Tocris Bioscience) in saline. This dose of SCH 23390 was used in a previous study (Schlenker, 2008). A lower dose was shown not to affect ventilation in the hamster. Then the hamster was placed into the chamber for 30 minutes during room air exposure and during the last minute metabolic rates, frequency, tidal volume, and minute ventilation were determined. This initial time was selected from a study by Kilts and colleagues (Kilts et al., 1985) who demonstrated that 0.3 mg/kg SCH 23390 reached high levels in the rat brain by 15 minutes and these levels were maintained at this level for over one hour.

The hamster was then exposed to hypoxia (10% oxygen in nitrogen) for 5 minutes. During the last minute of exposure to hypoxia, tidal volume, frequency and minute ventilation were determined. Following hypoxia, the chamber was washed out with room air, ventilation determined and the hamster was exposed to 5 minutes of 5% CO₂ in oxygen. During the last minute of exposure to hypercapnia, tidal volume, frequency and minute ventilation were determined. Afterwards the hamster was removed from the chamber and body temperature was measured using a Sontek BAT-12 thermometer and a Physiotemp thermocouple. Each animal received two treatments: saline and SCH 23390 at approximately the same time of day. The time between the treatments for each animal was less than seven days.

At the end of the study, hamsters were deeply anesthetized. The brain and carotid bodies was quickly removed, frozen on dry ice, and stored at -80 degrees Celsius. Trunk blood was collected for measurement of thyroxine (T₄) levels. The blood was spun down, the serum extracted, and frozen at -20 degrees Celsius for thyroxine determination. Thyroxine levels were measured in duplicate using a solid-phase competitive ELISA kits according to the manufacture's protocol (T₄ kit Diagnostic Systems Laboratories, Inc. Webster, TX).

4.4 Western blot analysis for D₁ dopamine receptor protein levels in carotid bodies and brain regions

The protein in carotid bodies was extracted with the lysing buffer (10 mM Tris, 1 mM EDTA, 1 % SDS) plus protease inhibitor cocktail (100 ml/ml). Following a centrifugation at 12000 g for 20 min at 4 °C, the protein concentration in the supernatant was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). The protein sample was mixed with loading buffer containing β-mercaptoethanol and heated at 100°C for 5 min. Equal amount of protein was loaded. Protein was fractionated using in a 10% polyacrylamide gel along with molecular weight standards and transferred to PVDF membrane. For carotid bodies, the membrane was probed with mouse anti D₁ dopamine receptor antibody raised against the last 123 C-terminal amino acids of D1DR of rat origin

(Santa Cruz, CA, USA, 1:500) and a peroxidaseconjugated goat anti-mouse IgG (Pierce Chemical, Rockford, IL, USA). The signal was detected using enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL, USA) and the bands were analyzed using UVP BioImaging Systems. Protein loading was evaluated by probing all Western blots with mouse anti- β -tubulin antibody (Santa Cruz, CA, USA, 1:1,000) and normalizing D₁ dopamine receptor protein intensities to that of β -tubulin. There was no difference in the level of expression of β -tubulin between normal and PTU-treated groups.

To determine D₁ receptor protein levels in the three brain regions, brains were sliced at 300 μ m and punches made using a 20-gauge stainless steel needle according to the hamster brain stereotaxic atlas (Morin, 2001) in the striatum, PVN, and NTS. Brain punches were frozen at -80 degrees Celsius until they were analyzed using the Western blot methods described above except that the D₁ receptor antibody used was a rabbit polyclonal antibody from Abcam (ab40653, 1:4,000) made against a sequence corresponding to amino acids 9–21 of Human Dopamine Receptor D₁. Protein levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH 1:2,000).

4.5 Immunohistochemistry of the carotid body for tyrosine hydroxylase (TH) and D₁ receptor protein

In a separate study carotid bodies from PTU-treated and euthyroid hamsters were placed in 4% paraformaldehyde and then embedded in paraffin. Seven to 10 μ m thin slices were cut and mounted on slides. Prior to conducting studies for immunohistochemistry for tyrosine hydroxylase of D₁, sections were deparaffined, and placed in a sodium citrate solution that was heated in a vegetable steamer for antigen retrieval. For immunohistochemistry for TH and D₁, slices were incubated in H₂O₂ in 0.1 M phosphate buffered saline (PBS), blocked using goat serum for 1 hour (for D₁ antibody only), and then incubated in primary antibodies (monoclonal anti D₁ receptor antibody produced in rat D2944, Sigma-Aldrich, 1:500 and TH monoclonal anti-TH antibody produced in mouse, T2928, Sigma-Aldrich, 1:7500). After incubation overnight for TH or 2 days for D₁, tissues were processed using appropriate secondary antibodies (Vector), placed in Avidin **Biotin Complex** and then developed using **3,3'-diaminobenzidine** and H₂O₂. Control slices received no primary antibody.

To quantitate the size of carotid bodies and glomus cell clusters, NIH ImageJ software (rsb.info.nih.gov/ij/) was used. The largest cross sections of carotid bodies were determined from each hamster and the cross sectional areas of all glomus cell clusters were measured in that carotid body. Then the ratio of the sum of glomus cells to carotid body cross sectional area was calculated. In addition, the number of glomus cell clusters per cross sectional area were evaluated.

4.6 Statistical Analysis

Statistical analysis of the ventilatory and metabolic rate data consisted of two-way analysis of variance (ANOVA) to test the effects of treatment (PTU versus tap water) and drug administration (saline or SCH 23390). If the result was significant ($P < 0.05$), post hoc Holm-Sidak tests for paired and unpaired comparisons were conducted. Body weight, body temperature, thyroxine levels, carotid body quantification data, and Western blot data were analyzed using unpaired Student's *t* tests. If the data were skewed, we used nonparametric tests such as the Mann-Whitney test (for unpaired comparisons) or the Wilcoxon sign rank test (for paired comparisons). Data were analyzed using Statmost programs (Dataxiom Software Inc. Los Angeles). All data are presented as means and standard error of the mean unless specified.

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Research Highlights

- > Hypothyroid hamsters exposed to air, hypoxia, and hypercapnia decreased ventilation.
- > Blocking D1 receptors only decreased breathing in euthyroid hamsters.
- > Hypothyroidism decreased D1 receptor protein in striatum, PVN and carotid bodies.
- > Functional D1 receptor modulation of breathing is altered in hypothyroidism.

Figure 1.

Representative Western blots and analysis of D1 protein levels in the nucleus tractus solitarius (A, NTS n=3 per group) and carotid bodies (B, n=8 per group). Lanes 1–3 are from euthyroid hamsters and 4–6 from hypothyroid hamsters. The graphs below summarize the results of data from NTS (C) and carotid bodies (D) of euthyroid (black bars) and hypothyroid (gray bars) hamsters. Values are means $\pm$ SEM. The asterisk denotes significant differences in D1 receptor protein levels between the two groups.

Figure 2. Representative Western blots and analysis of D1 protein levels in the paraventricular nucleus of the hypothalamus (A, PVN, n=3 per group) and striatum (B, n=3 per group)). Lanes 1–3 are from euthyroid hamsters and 4–6 from hypothyroid hamsters. The graphs below summarize the data from the PVN (C) and striatum (D) of euthyroid (black bars) and hypothyroid (gray bars) hamsters. Values are means $\pm$ SEM. The asterisk denotes significant differences in D1 receptor protein levels between the two groups.

Figure 3. Photomicrographs of tyrosine hydroxylase (TH) expression and D1 receptor expression (D1 receptor IHC) in euthyroid (A and C) and hypothyroid (B and D) hamster carotid bodies. The brown staining indicates the location and relative intensity of staining for TH or D1 receptor antigen. The scale bar = 100µm.

Figure 4.

Effects of saline and 0.25 mg/kg SCH 23390 (SCH) on minute ventilation (A), tidal volume (B) and frequency of breathing (C) in control, euthyroid (black bars) and PTU-treated hypothyroid (gray bars) hamsters exposed to air. Values are means $\pm$ SEM of 8 hamsters per group. Asterisks denote significant differences due to hypothyroidism compared to euthyroidism. The pound signs denote significant effects of SCH relative to saline in each group.

Figure 5.

Effects of saline and 0.25 mg/kg SCH 23390 (SCH) on minute ventilation (A), tidal volume (B) and frequency of breathing (C) in control, euthyroid (black bars) and PTU-treated hypothyroid (gray bars) hamsters exposed to hypoxia. Values are means $\pm$ SEM of 8 hamsters per group. Asterisks denote significant differences due to hypothyroidism compared to euthyroidism. The pound signs denote significant effects of SCH relative to saline in each group.

Figure 6. Effects of saline and 0.25 mg/kg SCH 23390 (SCH) on minute ventilation (A), tidal volume (B) and frequency of breathing (C) in control, euthyroid (black bars) and PTU-treated hypothyroid (gray bars) hamsters exposed to hypercapnia. Values are means $\pm$ SEM of 8 hamsters per group. Asterisks denote significant differences due to hypothyroidism compared to euthyroidism. The pound signs denote significant effects of SCH relative to saline in each group.

Figure 7. Effects of saline and 0.25 mg/kg SCH 23390 (SCH) on Hypoxic (A) and Hypercapnic (B) responsiveness in control, euthyroid (black bars) and PTU-treated hypothyroid (gray bars) hamsters. The pound signs denote significant effects of SCH relative to saline in each group.

Table 1

Summary of relative effects of PTU and SCH23390 on Frequency (F), Tidal volume (VT), and Minute Ventilation (VE) in Hypothyroid and Euthyroid Hamsters during exposure to Air, Hypoxia, and Hypercapnia

Air	Hypothyroid vs. Euthyroid	SCH23390 vs. Saline
F	↓	Euthyroid ↓; hypothyroid ↔
VT	↓	Euthyroid ↔; hypothyroid ↑
VE	↓	Euthyroid ↓ hypothyroid ↑
<u>Hypoxia</u>		
F	↓	Euthyroid ↓↓; hypothyroid ↓
VT	↓	Euthyroid and hypothyroid ↔
VE	↓	Euthyroid ↓; hypothyroid ↔
Responsiveness (ΔVE)	↔	Euthyroid↔; hypothyroid↓
<u>Hypercapnia</u>		
F	↔	Euthyroid↓↓; hypothyroid ↓
VT	↓	Euthyroid and hypothyroid ↔
VE	↓	Euthyroid ↓; hypothyroid ↔
Responsiveness (ΔVE)	↔	Euthyroid↓; hypothyroid ↓

Effects of PTU (Hypothyroid vs. Euthyroid) or SCH23390 vs. Saline on ventilatory parameters in hamsters. Responsiveness (ΔVE) is defined as the ventilation during exposure to hypoxia or hypercapnia divided by the preceding ventilation when the animal was breathing air. The symbols are as follows: ↓ decreased a parameter, ↑ increased a parameter, and ↔ had no effect on the parameter.