Targeted deletion of the neutral endopeptidase gene alters ventilatory responses to acute hypoxia in mice

H. Grasemann, B. Lu, A. Jiao, J. Boudreau, N. P. Gerard, and G. T. De Sanctis. Targeted deletion of the neutral endopeptidase gene alters ventilatory responses to acute hypoxia in mice. J. Appl. Physiol. 87(4): 1266–1271, 1999.—Neutral endopeptidase (NEP) is one of the major endopeptidases responsible for the inactivation of substance P in the carotid body, a neurotransmitter shown to be important in the transduction of hypoxic stimuli. Ventilatory responses to acute hypoxia were measured by indirect plethysmography in unanesthetized, unrestrained wild-type mice and in mice in which the NEP gene was deleted (NEP -/-). Ventilation was measured while the animals breathed room air: 12% O2 in N2 and 8% O2 in N2. Deletion of the NEP gene caused marked alterations in both the magnitude and composition of the hypoxic ventilatory response to both 8% O2 in N2 and 12% O2 in N2, compared with the wild-type mice (C57BL/6J) on the same genetic background as the NEP -/- mice. Treatment of C57BL/6J mice with thiorphan, a NEP inhibitor, resulted in a greater ventilatory response to 8% O2 because of a significantly greater shortening of expiratory time. The results of these studies demonstrate that NEP plays an important role in modifying the expression of the ventilatory response to acute hypoxia.

SP, a tachykinin, has evoked considerable attention with regard to its role in chemoreception (2, 4–7, 21, 22, 24). SP-like immunoreactivity has been demonstrated in the carotid body (13, 22), and intra-arterial administration of SP causes a dose-dependent increase in carotid sinus nerve discharge in cats (18). Additionally, administration of SP antagonists markedly attenuates the hypoxia- but not CO2-induced excitation of the carotid body (23). Furthermore, administration of SP into the ventrolateral medulla oblongata has an excitatory effect on tidal volume (VT) and minute ventilation (VE) (3).

Whereas numerous studies have investigated the role of neuropeptides in chemoreception, the metabolic fate of these neuropeptides has only recently been investigated in the carotid body. An extensive investigation of the biochemical, immunologic, and hydrolytic functions of peptidases has recently been published by Kumar (11). In this study, it was demonstrated that neutral endopeptidase (NEP) is the major peptidase in the chemoreceptor tissue of the carotid body and is believed to represent the major endopeptidase responsible for the degradation of SP (11). The importance of NEP in setting the sensitivity of the carotid body was demonstrated in experiments in which dose carotid body administration of the NEP inhibitor phosphoramidon was found to significantly potentiate the carotid body response to hypoxia (12). As these observations suggest that NEP may play an important role in setting the level of sensitivity of the carotid body to hypoxia, we investigated acute hypoxic ventilatory responses in mice with a targeted deletion of the NEP gene (16). We hypothesized that the targeted deletion of the NEP gene would result in a diminished degradation of SP in the peripheral and/or central chemoreceptors and an augmented ventilatory response to acute hypoxia. Furthermore, we have also evaluated the effects of thiorphan, a NEP inhibitor, on in vivo ventilatory responses to acute hypoxia.

METHODS

Animals. Male C57BL/6J wild-type (WT) mice, obtained from Jackson Laboratories, that were 8–12 wk of age were compared with sex- and age-matched NEP gene knockout mice (NEP -/-) (16) bred onto the same genetic background (C57BL/6J). All NEP -/- mice were bred and raised in a barrier facility. Sentinel animals housed in the same facility were periodically screened for infections by routine serology. A separate cohort of sex- and age-matched C57BL/6J mice was used in a second series of experiments investigating the...
### Table 1. Respiratory parameters in wild-type and NEP -/- mice measured during normoxia and hypoxia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type mice (21% O₂)</th>
<th>12% O₂</th>
<th>8% O₂</th>
<th>NEP -/- mice (8% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, breaths/min</td>
<td>188 ± 13</td>
<td>245 ± 14</td>
<td>224 ± 12</td>
<td>210 ± 6</td>
</tr>
<tr>
<td>VT, ml</td>
<td>0.237 ± 0.026</td>
<td>0.455 ± 0.043</td>
<td>0.191 ± 0.018</td>
<td>0.376 ± 0.041</td>
</tr>
<tr>
<td>Ti, s</td>
<td>0.103 ± 0.005</td>
<td>0.094 ± 0.005</td>
<td>0.091 ± 0.003</td>
<td>0.103 ± 0.003</td>
</tr>
<tr>
<td>TE, s</td>
<td>0.281 ± 0.024</td>
<td>0.174 ± 0.012</td>
<td>0.203 ± 0.011</td>
<td>0.190 ± 0.010</td>
</tr>
<tr>
<td>TT, s</td>
<td>0.383 ± 0.027</td>
<td>0.366 ± 0.087</td>
<td>0.294 ± 0.013</td>
<td>0.301 ± 0.011</td>
</tr>
<tr>
<td>VE, ml/min</td>
<td>45.6 ± 6.8</td>
<td>109.4 ± 8.6</td>
<td>42.0 ± 3.2</td>
<td>79.7 ± 9.9</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 wild-type and 7 neutral endopeptidase knockout (NEP -/-) mice. f, breathing frequency; VT, tidal volume; Ti, TE, TT: inspiratory, expiratory, and total cycle times, respectively; VE, minute ventilation. Intergroup comparisons were assessed by a Wilcoxon/Kruskal-Wallis test for nonparametric data and a Tukey Kramer honestly significant difference test for parametric data (*P < 0.01, †P < 0.05).
specifically, exposure to 8% O2 in N2 failed to decrease differences in VT between the NEP -/- and Wt mice (P = 0.01) but not in the Wt group. An intergroup comparison of f at 8% O2 in N2 revealed significantly greater f (P < 0.0001) in the NEP -/- mice; this was due to a shortening of both Ti (P = 0.04) and TE (P = 0.002). An analysis of VE in the NEP -/- and Wt mice with 8% O2 in N2 revealed a significantly greater response in the NEP -/- group (P = 0.015). There were no significant differences in VT between the NEP -/- and Wt mice at 8% O2 in N2. Interestingly, whereas there were significant hypoxia-induced decreases in both Ti (P = 0.04) and TE (P = 0.002) in the NEP -/- group, the Wt mice did not increase their frequency response to 8% O2 in N2. Specifically, exposure to 8% O2 in N2 failed to decrease either Ti or TE significantly in the Wt group. The changes in Ti, TE, and VT are graphically depicted in the average spirogram shown in Fig. 1.

Respiratory response to hypoxia after treatment with thiorphan. Mean ± SE values of respiratory parameters for this set of experiments are listed in Table 2. Exposure to 8% O2 in N2 significantly increased both VT (P = 0.0007) and VE (P = 0.002) in the vehicle-treated group; however, there was no hypoxia-induced increase in f in this group. Comparison of all ventilatory parameters between vehicle and thiorphan (100 mg/kg ip)-treated animals at 8% O2 revealed significant thiorphan-induced changes in the ventilatory responses to this acute hypoxic challenge, similar in pattern to those observed for the comparison of the Wt and NEP -/- mice. Specifically, f (P = 0.0038) and VE (P = 0.04) were significantly increased; TE (P = 0.0298) and TT (P = 0.0029) were significantly shortened by the thiorphan treatment in the C57BL/6 mice. There were no significant differences in Ti between the vehicle-treated mice with 8% O2 in N2 and after thiorphan treatment under similar conditions of hypoxia. The average spirogram for Ti, TE, and VT under normoxic conditions and at 8% O2 in N2 hypoxia after treatment with vehicle or 100 mg/kg thiorphan is shown in Fig. 2.

**DISCUSSION**

The present study confirms our hypothesis that NEP is an important regulator of the ventilatory response of the peripheral and/or central chemoreceptors to acute hypoxia. These studies are the first to demonstrate that the targeted deletion of the NEP gene by homologous recombination alters both the magnitude and temporal components of the ventilatory response to acute hypoxia. Ventilatory responses in the NEP -/- mice were compared with Wt mice bred on the same genetic background (C57BL/6). The significantly greater ventilatory response to hypoxia (8 and 12% O2 in N2) in the NEP -/- mice is shown in the average spirogram and as differences in VT. There were no significant differences in ventilatory measurements (normoxia) compared with the Wt group (68 ± 10.1 vs. 42 ± 3.2 ml/min; P = 0.02). Exposure to 8% O2 resulted in a significant increase in VT in both the Wt and NEP -/- mice (P = 0.001 and 0.004, respectively). Additionally, exposure to 8% O2 in N2 resulted in a significant increase in f in the NEP -/- mice (P = 0.01) but not in the Wt group. An intergroup comparison of f at 8% O2 in N2 revealed significantly greater f (P < 0.0001) in the NEP -/- mice; this was due to a shortening of both Ti (P = 0.04) and TE (P = 0.002). An analysis of VE in the NEP -/- and Wt mice with 8% O2 in N2 revealed a significantly greater response in the NEP -/- group (P = 0.015). There were no significant differences in VT between the NEP -/- and Wt mice at 8% O2 in N2. Interestingly, whereas there were significant hypoxia-induced decreases in both Ti (P = 0.04) and TE (P = 0.002) in the NEP -/- group, the Wt mice did not increase their frequency response to 8% O2 in N2. Specifically, exposure to 8% O2 in N2 failed to decrease either Ti or TE significantly in the Wt group. The changes in Ti, TE, and VT are graphically depicted in the average spirogram shown in Fig. 1.

Respiratory response to hypoxia after treatment with thiorphan. Mean ± SE values of respiratory parameters for this set of experiments are listed in Table 2. Exposure to 8% O2 in N2 significantly increased both VT (P = 0.0007) and VE (P = 0.002) in the vehicle-treated group; however, there was no hypoxia-induced increase in f in this group. Comparison of all ventilatory parameters between vehicle and thiorphan (100 mg/kg ip)-treated animals at 8% O2 revealed significant thiorphan-induced changes in the ventilatory responses to this acute hypoxic challenge, similar in pattern to those observed for the comparison of the Wt and NEP -/- mice. Specifically, f (P = 0.0038) and VE (P = 0.04) were significantly increased; TE (P = 0.0298) and TT (P = 0.0029) were significantly shortened by the thiorphan treatment in the C57BL/6 mice. There were no significant differences in Ti between the vehicle-treated mice with 8% O2 in N2 and after thiorphan treatment under similar conditions of hypoxia. The average spirogram for Ti, TE, and VT under normoxic conditions and at 8% O2 in N2 hypoxia after treatment with vehicle or 100 mg/kg thiorphan is shown in Fig. 2.

**Table 2.** Respiratory parameters measured in untreated (21% O2), vehicle-treated (8% O2), and thiorphan-treated (100 mg/kg) wild-type mice during 8% O2 hypoxia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (21% O2)</th>
<th>Vehicle (8% O2)</th>
<th>Thiorphan (8% O2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f, breaths/min</td>
<td>226 ± 2</td>
<td>236 ± 5</td>
<td>250 ± 7*</td>
</tr>
<tr>
<td>VE, ml/min</td>
<td>0.136 ± 0.019</td>
<td>0.302 ± 0.044‡</td>
<td>0.344 ± 0.062</td>
</tr>
<tr>
<td>Ti, s</td>
<td>0.090 ± 0.003</td>
<td>0.100 ± 0.004</td>
<td>0.097 ± 0.002</td>
</tr>
<tr>
<td>TE, s</td>
<td>0.204 ± 0.011</td>
<td>0.308 ± 0.005</td>
<td>0.208 ± 0.010‡</td>
</tr>
<tr>
<td>VT, ml</td>
<td>30.1 ± 3.5</td>
<td>70.7 ± 10.4t</td>
<td>84.8 ± 14.0t</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 wild-type mice. Within-group comparisons were made with paired t-tests, comparing vehicle with thiorphan treatment at 8% O2 (*P = 0.004, †P < 0.05) and comparing vehicle-treated (8% O2) with untreated (21% O2) baseline values (‡P < 0.01).
Our results clearly demonstrate an enhancement in the expression of the hypoxic ventilatory response in the NEP-/- mice. This enhancement in the ventilatory response to acute hypoxia may involve peripheral and/or central chemoreceptors. With regard to the possible role of peripheral chemoreceptors, the ventilatory response in the knockout mice resembles the enhanced hypoxic response of cat carotid bodies after inhibition of NEP activity (12). The greater increase in ventilatory response to acute hypoxia demonstrated in the NEP-/- mice is consistent with the results of in vivo data by Kumar et al. (12). In this study, they investigated NEP activity of the cat carotid body and assessed its significance in chemoreception. To assess the significance of NEP in chemoreception, close carotid body administration of phosphoramidon, a NEP inhibitor, significantly potentiated the carotid body response to hypoxia but not to hypercapnia (12). The results of their study are important, because they demonstrated for the first time that inhibition of NEP activity augments the hypoxic response of the carotid body, a finding similar to that observed in our NEP-/- mice under conditions of acute hypoxia. With regard to the possible role of NEP in degrading SP in the central nervous system and influencing the respiratory control apparatus, Matsas et al. (17) have demonstrated that a metalloendopeptidase that appeared to be similar to endopeptidase 24.11 is the principal enzyme hydrolyzing SP in various areas of the human brain and that the enzymatic activity was inhibited by phosphoramidon. Immunohistochemical staining in a later study by Back and Gorenstein (1) demonstrated significant NEP-like immunoreactivity throughout the medulla, a region where chemoreceptors are known to have their synaptic connections. The possibility that centrally located NEP may play a role in chemoreception is also bolstered by the findings in another study in which local application of NEP inhibitors increased the responses of tracheal tone and phrenic nerve activity to both hypercapnia and hypoxia in cats (10). Accordingly, because the findings of our study cannot distinguish whether the absence of NEP is exerting its effects peripherally and/or centrally, future studies will need to investigate the contribution of these two chemoreceptor foci in the NEP-/- mice.

The contribution of SP in hypoxia-induced ventilatory responses has been the focus of considerable interest. For example, the importance of SP as a chemo-transducer of hypoxia has been clearly demonstrated in a study in which the administration of a SP antagonist in vivo has been shown to block the response to infused SP and attenuate or completely abolish the carotid body excitation to hypoxia (24). An inhibition of SP degradation by NEP inhibitors (12) presumably increases the levels of this neuropeptide, both in the peripheral chemoreceptors and centrally in areas such as the nucleus tractus solitarius, a region rich in SP-like immunoreactivity (26) and an area demonstrated to receive afferent inputs from chemoreceptors (20). The importance of afferent nerve fibers in mediating the increase in the ventilatory response to hypoxia has been clearly demonstrated by Ledlie et al. (15), who

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**Fig. 2.** Average spirogram (means ± SE) for untreated Wt mice during normoxia (21% O₂) and hypoxia (8% O₂) 20 min after pretreatment with vehicle or 100 mg/kg ip thiorphan (n = 7).
demonstrated that the decrease in Te and increase in f with hypoxia were significantly less after carotid sinus denervation in anesthetized dogs. Furthermore, our laboratory has previously demonstrated that ablation of neuropeptide-containing C fibers by neonatal treatment with capsaicin significantly reduces the tachypnic component of the ventilatory response to hypoxia (6).

The contribution of SP to the ventilatory response to hypoxia has also been investigated in the respiratory centers of the brain. Microinjections of SP in the area of the nucleus tractus solitarius has been shown to produce an increase in f in parallel with an increase in VT (3). As this is an area known to receive chemoreceptor afferents, it is possible that an increase in SP levels may be responsible in part for the increase in f in parallel with the increase in VT so noted in the NEP -/- mice but not in the Wt mice. The increased ventilatory response in the NEP -/- mice may also be due to an increase in the levels of SP in the peripheral carotid body chemoreceptors and/or central chemoreceptors of the NEP -/- mice, possibly because of greater increases in the sensory discharge of these chemoreceptor foci. In support of this hypothesis, several studies have shown that local administration of SP increases the sensory discharge of carotid body chemoreceptors (18, 19). It is important to note that SP may elicit both an inhibitory and excitatory effect on chemoreceptor activity. McQueen (18) demonstrated in the cat that the effect of SP on carotid chemoreceptor activity appeared biphasic, where SP initially caused a slight inhibition of activity followed by a dose-dependent increase in discharge rate. In another study by Monti-Bloch and Eyzaguirre (19), SP either excited or depressed the carotid body discharge rate in the cat in a dose-dependent manner. Thus, whereas several studies have demonstrated an enhancement of chemoreceptor discharge rate with SP, the neuropeptide may also exert an inhibitory effect under certain circumstances.

In summary, the alteration in the ventilatory response to hypoxia in the NEP -/- mice suggests that NEP plays a critical role in regulating the expression of the ventilatory response to acute hypoxia via the peripheral and/or central chemoreceptors. Neurophysiological studies will be needed to delineate the contribution of NEP at these sites of chemoreception. The mechanism by which this increased ventilatory response occurs may be explained in part by an increase in the availability of SP, which in turn would cause an increase in the chemoreceptor discharge rate. This increase inafferent discharge would lead to an enhancement of the ventilatory response as observed in the NEP -/- and C57BL/6J mice treated with the NEP inhibitor. This mechanism is supported by a recent study by Kumar et al. (12), who demonstrated that close carotid body administration of a NEP inhibitor significantly potentiated the carotid body response to hypoxia in the cat. The lack of NEP or the inhibition of this enzyme leads to an enhanced ventilatory response to acute hypoxia, largely through an effect on the tachypnic response to hypoxia. Further work is required to evaluate the effects of NEP on the levels of neuroactive peptides, both peripherally and in the respiratory centers of the brain.

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REFERENCES