Chronic hypoxia upregulates connexin43 expression in rat carotid body and petrosal ganglion

J. CHEN, L. HE, B. DINGER, L. STENSAAS, AND S. FIDONE
Department of Physiology, University of Utah, Salt Lake City, Utah 84108

Received 5 December 2001; accepted in final form 6 December 2001

Chronic hypoxia upregulates connexin43 expression in rat carotid body and petrosal ganglion. J Appl Physiol 92: 1480–1486, 2002; 10.1152/japplphysiol.00077.2001.—Recent studies have demonstrated that oxygen-sensitive type I cells in the carotid body express the gap junction-forming protein connexin43 (Cx43). In the present study, we examined the hypothesis that chronic exposure to hypoxia increases Cx43 expression in type I cells as well as in chemosensitive neurons in the petrosal ganglion. Immunocytochemical studies in tissues from normal rats revealed diffuse and granular Cx43-like immunoreactivity in the cytoplasm of type I cells and dense punctate spots of immunoreactive product at the margins of type I cells and near the borders of chemosensory cell lobules. Cx43-like immunoreactivity was not detectable in petrosal ganglion neurons from normal animals. After a 2-wk exposure to hypobaric (380 Torr) hypoxia, Cx43 immunostaining was substantially enhanced in and around type I cells. Moreover, chronic hypoxia elicited the expression of Cx43-like immunoreactivity in the cytoplasm of afferent neurons throughout the petrosal ganglion. Quantitative RT-PCR studies indicate that chronic hypoxia evokes a substantial increase in Cx43 mRNA levels in the carotid body, along with a marked elevation of Cx43 expression in the petrosal ganglion. Increased Cx43 expression and gap junction formation in type I cells and sensory neurons may contribute to carotid body adaptation during sustained stimulation in extreme physiological conditions.

immunocytochemistry; gap junctions; ventilatory acclimatization to hypoxia; quantitative RT-PCR
type I cells and chemosensory nerve terminals may involve parallel chemical and electrical signaling mechanisms. Furthermore, Abudara et al. (5) have suggested that sustained elevations of cAMP levels during CH may promote the upregulation of Cx in type I cells, which could contribute to cell synchronization, thereby enhancing the release of excitatory neurotransmitters.

In the present study, we used immunocytochemical and molecular biological techniques to examine the hypothesis that CH upregulates Cx43 expression in carotid body type I cells and petrosal ganglion sensory neurons. These assessments involve tissue harvested from rats exposed in a hypobaric (high-altitude) chamber for periods of up to 14 days. Our data indicate that CH substantially elevates Cx43 transcript and protein levels in type I cells. Moreover, CH induces increased expression of Cx43 in petrosal ganglion neurons, suggesting its participation in nerve-receptor cell adaptive changes that occur in the chemoreflex pathway during sustained stimulation.

METHODS

Animals and exposure to hypobaric hypoxia. All procedures involving animals were approved by the University of Utah Institutional Animal Care and Use Committee. Adult male albino rats (180–200 g) were housed in standard rodent cages with 24-h access to pellet food and water. Cages containing two to four rats were placed in a hypobaric chamber, and pressures were reduced decrementally from ambient levels over 24–36 h (~640 Torr at Salt Lake City, 1,400 m), and then maintained at 380 Torr, equivalent to 5,500 m. The chamber was continuously flushed with fresh room air, and the internal temperature was maintained at 20–22°C. The hypobaric chamber was opened every 2 days to replenish food and water. All animals survived exposures up to 14 days in the hypobaric environment without signs of discomfort. Age-matched control male rats were similarly housed outside the chamber.

Immunocytochemical localization of Cx43 in carotid body and petrosal ganglion. Four normal rats and four rats exposed to CH were anesthetized with ketamine (10 mg/kg im) and xylazine (0.9 mg/kg im) and perfused intracardially with ice-cold 4% paraformaldehyde in 0.1 M PBS. Carotid bodies, petrosal ganglia, and superior cervical sympathetic ganglia were removed, cleaned of surrounding connective tissue, and immersed in the same fixative for 1 h, rinsed in 15% sucrose-PBS for 2 h, and stored at 4°C in 30% sucrose-PBS overnight.

Cryostat sections (4 μm) were thaw-mounted onto gelatin-subbed slides. Sections were first exposed to 100% cold (4°C) acetone for 10 min, followed by 3% H2O2 (10 min) at room temperature and 2% normal goat serum for 30–45 min (room temperature), and were then treated with avidin-biotin pre-blocking reagents (20 min, Vector). Sections were incubated at 4°C overnight in anti-Cx43 antibody (Santa Cruz Biotechnology) diluted 1:1,000 or 1:2,000 in PBS containing 2% normal goat serum. Sections were then rinsed in PBS at room temperature and incubated for 1 h in biotinylated goat anti-rabbit IgG (Vector) in PBS containing 2% goat serum, rinsed in PBS for 20 min, incubated in avidin-biotinylated horseradish peroxidase complex (1 h, Vector elite kit), and treated with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. In randomly selected sections, the primary Cx antibody was replaced with primary antibody preabsorbed with blocking peptide (Santa Cruz Biotechnology) or normal rabbit serum; no specific immunoreactivity was found in these specimens.

RNA extraction and cDNA synthesis. In accord with the kit instructions (Totally RNA, Ambion, Austin, TX), total RNA was extracted from four to six carotid bodies or two to three petrosal ganglia pooled from three groups of normal vs. CH rats (i.e., 5–7 normal and 4–5 CH animals for each experiment). The final RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (2 min), dissolved in water, and used immediately for PCR or stored at −20°C. Protein in the extract was measured by a modified Lowry method. After removal of contaminating DNA (MessageClean Kit, Gene Hunter, Nashville, TN), first-strand complementary DNA was synthesized by use of 2 μl of the total RNA incubated at 37°C for 60 min in 20 μl of 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 2 mM MgCl2, RNase inhibitor (20 units), 2.5 μM oligo(dT)16, and 50 units of Moloney murine leukemia virus reverse transcriptase, plus 1 mM of each 2-deoxynucleotide 5'-triphosphate. The reaction mixture was denatured at 99°C for 5 min and chilled at 5°C for 5 min.

Amplification of Cx43 and β-actin gene sequences. Aliquots of cDNA (0.5–3 μl; the size of each aliquot was inversely proportional to protein content in CH vs. normal samples) were introduced into a PCR reaction mix (25 μl) that contained 1.5 mM MgCl2, AmpliTaq DNA polymerase (2 units/25 μl), and primers for Cx43 (upstream: 5'-TGT AAC ACT CAATCAAC CCT GCC GC3'; and downstream: 5'-GGT TTT CTC CGTGGG ACG TGA-3') or β-actin (upstream: 5'-TTG TAA CCA ACT AGG ACG ATAGTG-3' and downstream: 5'-GAT CCT GAT CTG AGT AGG-3'). PCR was initiated at 94°C for 2 min followed by 30–40 cycles consisting of 40 s at 94°C, 40 s at 60°C, and 60 s at 72°C, with the final cycle extended to 5 min at 72°C, followed by termination at 4°C. Amplified products were electrophoresed on an agarose gel and stained with ethidium bromide. Photographed images of gels were digitized, and differences between normal and CH were approximately by quantifying the peak gray-scale (0–255) values, minus background areas located adjacent to gel bands of interest.

Quantitative assessment of transcript levels in carotid body and petrosal ganglion. In these experiments, cDNA from a separate batch of RNA was amplified with target gene primers in a competitive reaction that also included primers for an internal standard mimic molecule. Target molecules included Cx43 and tyrosine hydroxylase (TH), a transcript that is known to be upregulated in the carotid body by sustained exposure to hypoxia (14, 23). Primers for TH were upstream: 5’-CCCAAGCGCGCCCTTCGTGCCAGCG TGA-3’ and downstream: 5’-GCA TTC CCA TCC TTC CTC TCCA AA3’. Mimic molecules were constructed in accord with instructions provided in a kit (Clontech, Palo Alto, CA), and contained upstream and downstream primer sites identical to the target cDNA, but with a different intervening sequence and slightly different base pair size, which allowed coamplification in the same tube and subsequent gel separation from the target product. PCR reactions occurred in 20–80 μl buffer solution containing MgCl2 (final concentration 1.5 mM), AmpliTaq DNA polymerase (final concentration 5.0 units/100 μl), and 32P-end-labeled primers (final concentration 0.5 μM). PCR was initiated at 95°C for 2 min followed by 35 cycles consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with the final cycle extended to 5 min at 72°C, followed by termination at 4°C. Quantification of the target sequence was obtained by amplifying a dilution series of the mimic molecule in a constant amount of target cDNA derived from the tissue. A plot
of the target-to-mimic ratio on the y-axis (quantified from the radiolabeled products separated on an agarose gel) vs. the reciprocal of the concentration of added mimic on the x-axis is used to estimate the target concentration, which is the point on the graph where the target and mimic products are equal (i.e., ratio = 1). The corresponding x-intercept is the theoretical equivalent of the original target concentration. Comparison of levels of gene expression between normal and CH preparations were made after the data were normalized to the concentration of protein in tissue extracts (see RESULTS, Figs. 3 and 4).

RESULTS

Effect of CH on Cx43 immunoreactivity in carotid body and petrosal ganglion. Figure 1A shows that in normal carotid body Cx43-like immunoreactivity is localized exclusively in type I cells. The immunoreaction product occurs diffusely throughout the cell cytoplasm and as darker granules surrounding the unstained ovoid nucleus. In some instances, densely stained crescentic accumulations were located at the perimeter of type I cells, along with nearby dark, punctate spots (arrows and arrowheads in Fig. 1A). Other tissue elements in the carotid body, including type II cells, nerve fibers, fibroblasts, capillary endothelial cells, and smooth muscle cells, were immunonegative for Cx43.

After a 14-day exposure to hypoxia, Cx43 immunostaining in the carotid body was noticeably increased (Fig. 1B). The cytoplasm of all type I cells contained a dark granular reaction product interspersed with regions of intensely dark, diffuse staining. The nuclei of type I cells were not stained, nor was there evidence of immunoreactivity either in type II cells or in nerve fibers coursing through the tissue. Other tissue elements, including fibroblasts and vascular elements, were also immunonegative.

In the petrosal ganglion of normoxic animals, the incidence of immunopositive staining was extremely low.
low and appeared to be limited to a few lightly stained Schwann cells encircling primary sensory neurons (arrows in Fig. 1C). However, after 2 wk of CH, robust staining appeared in a subset of neurons scattered throughout the petrosal ganglion (Fig. 1D). Reaction product in these immunopositive cells was dispersed uniformly within the cytoplasm, and it was not present in the nucleus. In some instances, reaction product was noted in processes emerging from the neuron soma (Fig. 1D, arrowheads). CH did not appear to alter the expression of Cx43 in Schwann cells or other nonneuronal cells in the petrosal ganglion. Immunostaining was absent in postganglionic sympathetic neurons and Schwann cells in the superior cervical ganglion. However, after CH a few groups of cells resembling small intensely fluorescent cells were immunopositive for Cx43 (not shown). Previous studies have shown that small intensely fluorescent cells share multiple cytological features with carotid body type I cells (15).

**Fig. 2.** Standard RT-PCR assessment of gene expression in normoxic and chronically hypoxic carotid body and petrosal ganglion. An analysis of competitive PCR reactions using internal standard mimic molecules provided a more exacting analysis of CH-induced changes in transcript levels (Fig. 3). Data for the carotid body indicate that the Cx43 tran-

Quantitative assessment of CH on Cx43 transcript levels in carotid body and petrosal ganglion. An analysis of competitive PCR reactions using internal standard mimic molecules provided a more exacting analysis of CH-induced changes in transcript levels (Fig. 3). Data for the carotid body indicate that the Cx43 tran-

---

**Fig. 3.** Effect of CH on Cx43 gene expression in rat carotid body (A) and petrosal ganglion (B). Data are ratios of target-to-mimic product amounts (y-axis) generated in multiple “competitive” PCR reactions. ○, normoxia; ●, CH. Values are normalized to protein concentration in tissue homogenates; cpm, counts/min. Plots according to least-square best-fit derivations. The x-axis is the reciprocal of mimic molecule concentration (see detailed METHODS and RESULTS).
responding to target-to-mimic ratio \( = 1 \) is \( 5.6 \times 10^{-6} \) amol/mg protein in normal (normoxic) tissue (Fig. 3A). For comparative purposes, separate experiments established the concentration of the transcript for TH, an abundant enzyme in the carotid body that controls the rate of catecholamine synthesis in this and other catecholaminergic tissues. These assays (Fig. 4) yielded TH mRNA levels of \( 1.2 \pm 0.39 \) amol/mg protein, some 214,000-fold higher than the concentration of Cx43 mRNA, suggesting that the constitutive expression of this gap junction-forming protein is relatively low in normal chemosensory tissue. Even lower levels of Cx43 transcript were found in petrosal ganglion, where target Cx43 PCR products were less than the lowest concentration of competing mimic molecules (see Fig. 3B). This finding is in accord with the paucity of Cx43 immunopositive cells in the normal petrosal ganglion (Fig. 1C). Also consistent with the immunocytochemical findings were changes in Cx43 transcript expression after exposure to CH. In tissues harvested after a 15-day exposure at 380 Torr, the levels of Cx43 transcript in the carotid body were elevated more than sixfold (Fig. 3A, ratio of CH vs. normoxic data), and, in the petrosal ganglion, CH induced increased expression of Cx43 transcript to levels that were within the range of the mimic molecule (Fig. 3B, CH data). TH transcript levels were increased some 23-fold in the carotid body after a similar 14-day exposure to CH (Fig. 4), in accord with previous studies that demonstrated elevated TH enzyme activity and gene expression in this tissue after CH (14, 23).

**DISCUSSION**

Our data demonstrate for the first time that a gap junction-forming protein, namely Cx43, is constitutively expressed in chemoreceptor type I cells in the intact carotid body. These findings are in accord with a recent report by Abudara et al. (5), who used immunocytochemical and biochemical techniques to show that type I cells maintained in a culture environment express Cx43. In numerous other tissues known to possess gap junctions, Cx immunocytochemistry reveals a punctate staining pattern outlining points of cell-to-cell gap junction contact (28, 31). Although we noted regions of punctate staining near the perimeter of type I cells in the carotid body, our results, and those of Abudara et al., additionally indicate a cytoplasmic pattern of Cx43 distribution varying from diffuse to granular in the chemosensory cells. It is noteworthy that both patterns of Cx43 distribution occur in the pituitary. In that tissue, light and electron microscopy reveal punctate immunostaining primarily in the posterior lobe and predominately granular reaction product in secretory vesicles of gonadotrophs of the anterior lobe (34). These findings not only implicate Cx-like proteins in secretory events (34) but also suggest that the diverse staining patterns are related to differences in the synthesis, processing, and membrane insertion of Cx proteins in different types of cells. We regard the presence of Cx43 in the carotid body as a logical correlate of electrical coupling between intact and cultured type I cells (2, 5, 29).

In the normal petrosal ganglion, Cx43 appears to be present in only a few Schwann cells enveloping sensory neurons, a pattern consistent with the low levels of transcript detected in this tissue with both the standard and quantitative RT-PCR assays. However, a 2-wk exposure to CH resulted in substantially elevated transcript levels and induced the expression of Cx43 immunoreactivity in neurons throughout the ganglion. The upregulation of Cx43 in both type I cells and petrosal ganglion neurons suggests that gap junction-forming proteins may play an important role in the physiological adaptation of the chemoreflex pathway during CH. Previous studies have demonstrated that CH elicits elevated chemosensitivity in the carotid body, but mechanisms underlying the increased chemoreceptor discharge are poorly understood (11). Our data suggest possible Cx43 involvement in altered type I cell secretory activity during CH (20) and are consistent with the hypothesis of Abudara et al. (5) that CH may not only increase electrical coupling between type I cells but may also favor increased activity via cell synchronization.

Our finding that CH induces the expression of Cx43 in the petrosal ganglion, in addition to the significant upregulation of Cx in type I cells, suggests that physiological adaptation in the chemoreflex pathway involves important adjustments in the chemoafferent neurons as well as in the carotid body. Adaptation to chronic stimulation by primary sensory neurons has been documented elsewhere. In chronic pain models, inflammation induces significant changes in neuropeptide synthesis in the somata of sensory neurons and favors release at their peripheral and central sites of axon termination (13, 19). Similarly, exposure of afferents of the vagus nerve to an inhaled allergen results within 24 h in a three- to fivefold elevation in the levels of substance P, neurokinin A, and calcitonin gene-related peptide immunoreactivity in the guinea pig lung. In these experiments, a 10-fold increase in the incidence of nodose ganglion neurons expressing SP

![Fig. 4. Effect of CH on tyrosine hydroxylase (TH) gene expression in rat carotid body. For details, see Fig. 2 legend and text.](image-url)
was also observed (32). In this context, the present findings provide further support for the hypothesis that dynamic phenotypic adjustments in sensory neurons are an important component of an adaptive response to chronically altered physiological conditions. It is important to note that the experimental condition used in the present study, namely hypobaric hypoxia, is known to elicit significant reductions in blood P\textsubscript{CO\textsubscript{2}} and bicarbonate (30). The effect of these changes on Cx43 expression is unknown; however, it is well established that CSN activity evoked from the carotid body by hypoxia is decreased as P\textsubscript{CO\textsubscript{2}} falls (18). Thus, if Cx43 upregulation is related to increased chemosensory activity, even higher levels of expression may occur in sensory neurons and type I cells during sustained isocapnic hypoxia.

Although the precise role of Cx-like protein in the carotid body is presently unknown, its presence in both type I cells and chemosensory neurons is consistent with the proposal of Eyzaguirre and Abudara (17) that type I cells may be electrically coupled to afferent nerve terminals. The development of electrical coupling at synapses during CH could potentiate chemotransmission, thereby ensuring a higher level of chemoreceptor input to the central nervous system. In CH preparations, Cx43 immunostaining was evident in neuronal somata in the petrosal ganglion, but immunostaining in afferent nerve fibers could not clearly be distinguished from the darkly immunoreactive type I cells in the carotid body. On the other hand, the absence of observable staining in nerve fibers is not a conclusive finding because, as was noted earlier, immunostaining is frequently limited to sites of dense Cx accumulation in the tissues, namely at gap junctions (28, 31). Although dense labeling of the cytoplasm of the type I cells (~10 μm) would tend to mask the presence of Cx43 in the much smaller calyciform and bouton nerve endings apposed to their surface, the existence of dark punctate regions of immunoreaction product near the periphery of type I cells may portend the existence of immunostained nerve terminals. The resolution of these issues will require detailed ultrastructural studies of the distribution of gap junctions and connexin proteins in the carotid body.

REFERENCES
25. Kondo H and Iwasa H. Effects of chronic hypoxia on peptide and neurotransmitter levels in the tissues, namely at gap junctions (28, 31). Although dense labeling of the cytoplasm of the type I cells (~10 μm) would tend to mask the presence of Cx43 in the much smaller calyciform and bouton nerve endings apposed to their surface, the existence of dark punctate regions of immunoreaction product near the periphery of type I cells may portend the existence of immunostained nerve terminals. The resolution of these issues will require detailed ultrastructural studies of the distribution of gap junctions and connexin proteins in the carotid body.