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Ins(1,4,5)P₃ receptors in cerebral arteries: changes with development and high-altitude hypoxia

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CEREBRAL BLOOD VESSELS are richly innervated, and their vascular tone is mediated by adrenergic, serotoninergic, and other mechanisms that involve receptor coupling with the second messenger inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃]. Ins(1,4,5)P₃ released into the cytoplasm binds its specific receptor Ins(1,4,5)P₃ receptor [Ins(1,4,5)P₃-R] in sarcoplasmic reticulum. The Ins(1,4,5)P₃-R acts as a gated calcium channel to release Ca²⁺, an increase in concentration of which binds with calmodulin to effect contraction of vascular smooth muscle (2, 11, 14).

Recently, we have reported that common carotid artery (Com) and main branch cerebral blood vessels (MBC) of the adult sheep show marked heterogeneity in terms of norepinephrine (NE)-induced contractility, α₁-adrenergic receptor (α₁-AR) density, and adrenergically-induced Ins(1,4,5)P₃ response (8). Fetal and newborn cerebral vessels also show marked differences in NE-induced contraction, α₁-AR density, and Ins(1,4,5)P₃ responses as compared with the adult (9). We thus performed the following study to test the hypothesis that differences in the contractile responses in the cerebral vasculature, as well as differences as a function of development result, in part, from differences in Ins(1,4,5)P₃-R density and/or affinity. In addition, cerebral arteries of high-altitude-acclimatized adult and fetal sheep have altered contractility (7) and dramatically decreased α₁-AR densities and NE-stimulated Ins(1,4,5)P₃ responses as compared with normoxic controls (15). Thus we also tested the hypothesis that the long-term, hypoxemia-induced decrease in cerebrovascular contractility is associated with decreased Ins(1,4,5)P₃-R density and/or affinity.

METHODS

Tissue preparation. We used cerebral blood vessels from near-term fetal (140–143 days), newborn (3–5 days), and nonpregnant and pregnant adult sheep that had been maintained near sea level (300 m) or at high altitude (3,820 m, 12,470 ft; Barcroft Laboratory, White Mountain Research Station, Bishop, CA) for ~110 days. The animals from high altitude were transported to our laboratory immediately before the studies. All hypoxemic fetuses were singletons, and, when normoxic twin fetuses were obtained, we used only one fetus of the pair for the present studies. Nonpregnant and pregnant ewes were obtained from Neberker Ranch (Lancaster, CA). Animals were killed with 100 mg/kg intravenous pentobarbital sodium. We removed the brain, placed it in a pan of iced saline, and dissected out and cleaned the several cerebral vessels. We obtained segments from Com and two cerebral vessel groups, namely, circle of Willis vessels (including basilar artery) (Wil) and the MBC. We also obtained a 10-cm segment from the thoracic aorta. The vessels were rapidly frozen in liquid nitrogen and kept at ~80°C until use (usually within 1 wk). For the Wil and MBC cerebral arteries, six to eight brains for fetus and newborn and four brains for adult were required to obtain enough membrane protein for a single binding assay. In RESULTS, n refers to the number of assays performed, not vessels used.

Materials. The materials and their sources were as follows: [³H]Ins(1,4,5)P₃ (21 Ci/mmol; DuPont-New England Nuclear, Boston, MA), Ins(1,4,5)P₃, and bovine serum albumin (BSA) (Calbiochem, La Jolla, CA), Lubrol-Px (ICN, Costa Mesa,
CA), Spectra/Mesh nylon filters (10 µm pore size) (Spectrum, Los Angeles, CA), Whatman no. 1 paper filters (Whatman, Hillsboro, OR), and Dowex AG 1-X2 resin (chloride form, 200–400 mesh) (Bio-Rad, Hercules, CA). All other biochemical reagents were from Sigma Chemical (St. Louis, MO).

Solubilized membrane preparations. Membranes were solubilized as previously described (17). Arteries (0.5–0.8 g wet wt) were minced and suspended in 10 volumes of buffer A [composition in mM: 20 tris(hydroxymethyl)aminomethane (Tris)-HCl, 20 NaCl, 100 KCl, and 1 EDTA plus 1 mg/ml BSA, 0.02% NaN3, pH 7.7]. Tissue was then homogenized in a Polytron tissue homogenizer (Brinkman, Westbury, NY) at setting 3.5 for bursts of 15 s each. The homogenate was centrifuged at 100,000g for 30 min (model XL-70 ultracentrifuge, Beckman). Pellets were rehomogenized in one-half the original volume of buffer A at setting 3.5 for 15 s and recentrifuged using the same conditions. The resulting supernatant was discarded, and the pellet was resuspended in buffer B (composition in mM: 20 Tris·HCl, 20 NaCl, 100 KCl, and 1 EDTA, 0.02% NaN3, pH 8.5) containing 2% (wt/vol) Lubrol-PX. The suspension was gently homogenized with 20 strokes in a glass-glass homogenizer on ice, followed by incubation on ice with stirring for 20 min. The detergent extract was then centrifuged at 80,000g for 40 min. The resultant supernatant [1.6 mg protein/ml by the method of Bradford (1)] was kept on ice until use.

Radioligand binding studies. For the Ins(1,4,5)P3-R binding study, we used the syringe assay of Hingorani and Agnew (8), as modified by Zhang et al. (17). The syringe ion-exchange column was prepared with 0.5–0.6 ml of Dowex AG 1-X2 resin (Bio-Rad), equilibrated in 2 volumes of buffer C (composition: 50 mM Tris·HCl, 1 mM EDTA, 0.02% NaN3, 10 mg/ml BSA, pH 8.5) for 2 h. A small Spectra/Mesh nylon disk (10 µm pore size) was inserted into the barrel of a 3-ml plastic syringe, followed by a disk of Whatman no. 1 paper filter and then Dowex AG 1-X2 resin. Saturation binding experiments employed concentrations of [3H]Ins(1,4,5)P3 from 0.5 to 40 nM, and nonspecific binding was determined by the addition of unlabeled Ins(1,4,5)P3. The assay was carried out in a final volume of 200 µl, consisting of 150 µl solubilized membrane supernatant (soluble protein concentration equaled ~1.6 mg/ml), 40 µl radioligand [3H]Ins(1,4,5)P3, specific activity 21 Ci/mmol; DuPont-New England Nuclear], and 10 µl buffer or unlabeled Ins(1,4,5)P3 (final concentration 12 µM, Calbiochem). The assay tubes were mixed and incubated for 15 min at 4°C. To perform the assay, an equilibrated sample (0.2 ml) was delivered to the wall of the syringe, held horizontally, away from the resin. The plunger was rapidly inserted, the column turned vertically, and the sample, followed by the head of air, forced through the resin. The separation could be completed reliably within ~1 s. Bound and free radioligand were separated by rapidly pushing equilibrated samples through the syringe columns. The labeled receptor-ligand complex was collected directly into a vial for counting at 45% efficiency in a liquid scintillation counter (model 1900 CA TriCarb, Packard Instruments, Downer's Grove, IL). Saturation curves were analyzed using a computer program (Graphpad Inplot V. 3.0; Graphpad Software, San Diego, CA).

Statistics. For normoxic controls, we used cerebral vessels from 52 near-term fetal, 32 newborn, and 40 adult sheep (20 nonpregnant and 20 pregnant). For the studies of long-term hypoxia, we used vessels from 26 fetuses and 20 adults. As noted above, for the receptor binding studies in cerebral arteries we pooled vessels from four to eight brains. In these cases, n refers to the number of receptor assays. All values were calculated as the mean ± SE. For each vessel type, differences among the several age groups were analyzed by analysis of variance. To compare differences between normoxic and hypoxic vessels, we used Student's t-test. Unless otherwise indicated, statistical significance implies P < 0.05.

RESULTS

Ins(1,4,5)P3-R binding in normoxic vessels. Figure 1 shows representative Ins(1,4,5)P3 binding curves for Com of normoxic control, fetal (Fig. 1A), and adult (Fig. 1B) sheep. Saturable Ins(1,4,5)P3 binding was observed for each vessel type in these two age groups, as well as in the newborn. Nonspecific binding was linear in all preparations and accounted for 40% total binding at low Ins(1,4,5)P3 concentrations and 20% of total binding at higher concentrations (median value for all studies, 32%). Scatchard plots were linear in all studies, with Pearson r coefficients of 0.88–0.99, suggesting the presence of a single class of Ins(1,4,5)P3 binding sites in vascular smooth muscle of each of the several age groups.

Figure 2A shows the Ins(1,4,5)P3-R density values (Bmax), as measured with saturation binding of [3H]Ins(1,4,5)P3, for Com of near-term fetus, newborn, and adult. These values in Com (fmol/mg protein) were 85 ± 3 (n = 4), 150 ± 18 (n = 4), and 357 ± 21 (n = 11), respectively (P < 0.01 for both fetus and newborn.
all vessels in the three age groups, the mean value of
8) for fetus, newborn, and adult, respectively. For
10
were 109
Ins(l,4,5)P3-R density values (fmol/mg protein) for Com of fetus and adult in response to high-altitude, long-term hypoxia. In fetal Com, Ins(l,4,5)P3-R density fell 32% from normoxic control (to 5 ± 8, n = 4, P < 0.01). In adult Com, Ins(l,4,5)P3-R density decreased 70% from control value (to 109 ± 12, n = 5, P < 0.01). Figure 3A shows the hypoxic-associated decreases in Ins(l,4,5)P3-R density for fetal and adult MBC. For the fetus, Ins(l,4,5)P3-R density fell 80% from control value (to 23 ± 3, n = 3, P < 0.01). For the adult MBC, long-term hypoxia was associated with a decrease of 47% from control (to 53 ± 7, n = 4, P < 0.01).

In hypoxic fetal and adult Wil vessels, the Ins(l,4,5)P3-R density (fmol/mg) did not change significantly from normoxic control values (46 ± 11 and 22 ± 2, respectively). For comparison, in the fetal and adult aorta, Ins(l,4,5)P3-R density fell 46% (to 59 ± 7) and 66% (to 60 ± 8), respectively, from normoxic control values. Again, the Ins(l,4,5)P3-R affinity did not change significantly in the hypoxic vessels (Kp = 11.2 ± 0.7 nM; range = 9.8–13.8 nM).

**DISCUSSION**

In the present study, we report that the vascular Ins(l,4,5)P3-R density increased significantly as a function of developmental age in Com, and to a lesser extent

![Figure 2A](image)

**Fig. 2. A:** Ins(1,4,5)P3 receptor density (Bmax) values (fmol/mg protein) as determined with [3H]Ins(1,4,5)P3 in common carotid artery and main branch cerebral arteries of near-term fetal (hatched bars), newborn (open bars), and adult (filled bars) sheep. Data are mean values ± SE. **P < 0.01. B:** relationship of maximum norepinephrine (NE)-induced contraction (expressed as %K+ maximum response) to Ins(1,4,5)P3 receptor density values for fetal (●), newborn (♦), and adult (▲) common carotid arteries and fetal (○), newborn (▲), and adult (△) main branch cerebral arteries. For common carotid artery, r = −0.99, P = 0.052. For main branch cerebral arteries, r = 0.51, P = 0.66.

versus adult). Figure 2A also presents the Ins(1,4,5)P3-receptor density values for the MBC arteries of fetus, newborn, and adult. These values were 115 ± 15 (n = 4), 105 ± 9 (n = 4), and 99 ± 5 (n = 5), respectively.

Figure 2B shows the relationship of NE-induced contraction (expressed as %K+ maximum) to Ins(1,4,5)P3-R density for both Com and MBC. In neither Com nor MBC of three age groups did Ins(1,4,5)P3-R density correlate with the NE-induced maximal contraction as percent of K+ induced maximum (r = −0.99, P = 0.052 and r = 0.51, P = 0.66, respectively). Nonetheless, the higher Ins(1,4,5)P3-R values in Com compared with MBC correlated with the much greater NE-induced contraction of this vessel.

Ins(1,4,5)P3-R density values (fmol/mg protein) for Wil of the three age groups were 54 ± 4 (n = 3), 65 ± 10 (n = 3), and 20 ± 2 (n = 4), respectively. For comparison, we also quantified Ins(1,4,5)P3-R density values in the thoracic aorta of the three age groups. These values were 109 ± 10 (n = 5), 112 ± 14 (n = 4), and 181 ± 18 (n = 8) for fetus, newborn, and adult, respectively. For all vessels in the three age groups, the mean value of

![Figure 3A](image)

**Fig. 3. A:** Ins(1,4,5)P3-R density values (fmol/mg protein) in common carotid artery of long-term hypoxic (filled bars) as compared with normoxic, control (open bars) fetuses and adults. Data are mean values ± SE. *P < 0.01 for each pair, hypoxic vs. normoxic. **B:** Ins(1,4,5)P3 receptor density values (fmol/mg protein) in main branch cerebral arteries of long-term hypoxic (filled bars) as compared with normoxic, control (open bars) fetuses and adults. Data are mean values ± SE. *P < 0.01 for each pair, hypoxic vs. normoxic.
in the aorta. In contrast, in MBC and in Wil vessels there was no significant change in Ins(1,4,5)P₃-R density with development. In addition, the present study demonstrates that high-altitude, long-term hypoxia was associated with a significant decrease in Ins(1,4,5)P₃-R density in the fetal and adult Com and MBC, as well as in the aorta.

**Ins(1,4,5)P₃-R and development.** The responsiveness of cerebrovascular smooth muscle to NE and other agonists changes significantly as a function of development (9, 13). However, the mechanisms that underlie these age-dependent changes remain to be identified. In part, such maturational changes can be accounted for by changes in α₁-AR density and NE-induced Ins(1,4,5)P₃ release (9). Nonetheless, changes in Ins(1,4,5)P₃-R density and/or affinity may also play a role in these contractility changes.

In the present study in the adult, Ins(1,4,5)P₃-R density was fourfold greater in the Com and almost twofold greater in aorta compared with fetus. In contrast, in the MBC Ins(1,4,5)P₃-R density did not change significantly with developmental age. In Wil, Ins(1,4,5)P₃-R density values were significantly less than in the other vessels and decreased from newborn to adult. In contrast to these striking changes in Ins(1,4,5)P₃-R density, receptor affinity did not vary significantly as a function of vessel size, developmental age, or hypoxia. Our values of Ins(1,4,5)P₃-R affinity are comparable to those reported for smooth muscle of bovine aorta (2, 10), rabbit trachea (14), and dog colon (17).

We are unaware of other developmental studies of Ins(1,4,5)P₃-R in vascular smooth muscle. Nonetheless, several investigators have examined Ins(1,4,5)P₃-R density and affinity as a function of development in brain. For instance, in dog cerebellum, both Ins(1,4,5)P₃-binding and Ins(1,4,5)P₃-induced Ca²⁺ release increased as a function of age from the newborn to adult. This increase in Ins(1,4,5)P₃-R density paralleled both the growth of Purkinje neurons and synaptogenesis (16). In cat visual cortex, Ins(1,4,5)P₃ binding also increased from the day of birth to adulthood (5). In rabbit tracheal smooth muscle, Ins(1,4,5)P₃-R density and affinity were similar in immature and adult animals (14).

**Correlation of Ins(1,4,5)P₃-R density with contractility.** The relation between a given agonist concentration and its smooth muscle contractile response involves multiple factors, all of which are potentially subject to physiological regulation, e.g., plasma membrane receptor, the second messenger response, intracellular Ca²⁺ release, and so forth. We have previously reported that, in regard to noradrenergic-mediated cerebral vascular contraction, both the α₁-AR density and Ins(1,4,5)P₃ response vary significantly as a function of developmental age (9).

In the present study, in both newborn and adult sheep, the Ins(1,4,5)P₃-R density values of Com were considerably higher than in MBC and other vessels. This is compatible with the finding that the Com showed greater NE-induced contraction (%K⁺ maximum) than MBC vessels (9). Also, in MBC neither Ins(1,4,5)P₃-R density nor the NE-induced maximal response (%K⁺ maximum) changed with development. Thus, in these vessels, the two variables correlated well. Nonetheless, the idea that in cerebral arteries different components of the α₁-AR-mediated excitation-contraction pathway are independently regulated is supported because the Ins(1,4,5)P₃-R densities did not correlate tightly with NE-induced contractile responses in all vessels (9). For Com of normoxic fetus, newborn, and adult, r = 0.98 and P = 0.12, and for MBC r = −0.11 and P = 0.93.

As we have previously noted, not only can coupling efficiency of α₁-AR to its second messenger change as a function of developmental age and/or vessel order, but tissue sensitivity to Ins(1,4,5)P₃ itself also may vary. The lack of strong correlation of Ins(1,4,5)P₃-R density with NE-induced contraction or Ins(1,4,5)P₃ increase for either Com or MBC suggests that variations in agonist potency may be due to variations in other factors in these two vessel groups. For instance, despite robust contraction to NE, fetal and newborn Com showed essentially no NE-induced Ins(1,4,5)P₃ response (9), suggesting that, in this vessel, Ca²⁺-mediated contraction occurs by non-Ins(1,4,5)P₃ calcium release mechanisms, e.g., receptor-operated or voltage-gated calcium channels, and/or other mechanisms.

**Long-term hypoxia and the Ins(1,4,5)P₃-R.** In previous studies we have reported that, in both adult and fetal sheep, high-altitude, long-term hypoxia is associated with a significant decrease in α₁-AR density and NE-stimulated Ins(1,4,5)P₃ responses in the Com (adult only) and MBC (15). In companion studies in uterine vasculature of the pregnant ewe, acclimatization to high altitude was associated with a 32–38% decrease in α₁-AR density in the main uterine artery and its fourth-order branch, as well as a significant decrease in sensitivity of both vessels to NE (i.e., higher half-maximal effective concentration) (4). In other related studies, long-term hypoxemia was associated with significantly decreased potassium-induced tension in both fetus and adult Com and cerebral arteries and, in the fetus, depressed potassium-induced stress and amine (serotonin plus histamine)-induced tensions (7). In addition, Pearce (12) has reported that long-term hypoxemia is associated with depressed norepinephrine sensitivity of adult Com and cerebral arteries, as well as in fetal middle cerebral artery. The present studies demonstrate significant (30–80%) decreases in Ins(1,4,5)P₃-R density in adult Com and MBC and fetal cerebral arteries in response to long-term hypoxia (Fig. 3). These decreases correlate well with similar decreased NE-induced tone in these vessels (12). The hypoxia-mediated downregulation of Ins(1,4,5)P₃-R density in Com and MBC (and aorta) suggests a role in the maintenance of normal cerebral blood flow or blood pressure during long-term hypoxia. These decreases in Ins(1,4,5)P₃-R density could act to avoid excessive intracellular calcium concentrations in response to Ins(1,4,5)P₃ or may reflect the combination of impaired...
receptor synthesis and relatively rapid receptor turnover.

Perspectives

The cerebral circulation is richly innervated, and its vascular contractility is mediated by adrenergic, serotonergic, and other mechanisms. The signal transduction cascades of several of these systems utilize the second messenger Ins(1,4,5)P$_3$ and its receptor to effect Ca$^{2+}$ release and vascular contraction. In the adult (and newborn), the relatively high Ins(1,4,5)P$_3$-R density in Com as compared with MBC correlates with their NE-induced contractility, suggesting the functional importance of Ins(1,4,5)P$_3$-R in these vessels.

In the adult, high-altitude, long-term hypoxia has served as a useful model of how the body's physiological mechanisms acclimatize over a period of days or weeks or genetically adapt over the course of generations. Because under normoxic control conditions mean intracellular O$_2$ tension values are relatively low (3–5 Torr), it may be surprising to discover that such a moderate elevation as employed in the present studies (3,820 m) was associated with cellular hypoxia (or other stimulus) to effect such a major reduction in Ins(1,4,5)P$_3$-R density. Nonetheless, the significant decrease in cerebrovascular Ins(1,4,5)P$_3$-R density correlates with the decreased vessel contractility under these circumstances.

For the fetus under normoxic control conditions, despite its arterial O$_2$ content being similar to that of an adult, because of its relatively low Po$_2$ values simulating “Mt. Everest in utero,” the question of how it can thrive in a mother at high altitude has posed a dilemma (6). Although it is often assumed that the fetus in utero is “buffered” from the external environment by the maternal organism, the present results indicate that even relatively mild chronic hypoxemia can significantly alter an element such as the Ins(1,4,5)P$_3$-R.

Hopefully, the present studies will help to illuminate a rather obscure aspect of regulatory-integrative physiology. That different elements of the adrenergic-mediated signal transduction cascade are independently regulated, both in the adult and in the fetus, should come as no surprise. Ins(1,4,5)P$_3$-R changes may modify vascular tone and regulatory response to ensure that vessel contractility is decreased, so that cerebral oxygenation is not compromised. Nonetheless, the manner in which these changes serve to regulate cerebrovascular tone under these circumstances in vivo must await further studies.

Summary and conclusions. In normoxic Com (and aorta), Ins(1,4,5)P$_3$-R density increased dramatically as a function of developmental age from fetus to adult. In contrast, in the MBC, Ins(1,4,5)P$_3$-R density did not change significantly with age. In Wil vessels, Ins(1,4,5)P$_3$ density decreased somewhat from the newborn to adult. In general, the higher values of Ins(1,4,5)P$_3$-R density in Com correlated with its higher NE-induced contraction as compared with MBC.

In the adult Com and MBC, acclimatization to long-term hypoxia was associated with significant reduc-

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