Expression and Distribution of the Type 1 and Type 3 Inositol 1,4,5-Trisphosphate Receptor in Developing Vascular Smooth Muscle

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Abstract—The recent discoveries of inositol 1,4,5-trisphosphate (IP_3) receptor subtypes with different affinities for IP_3 and their potential involvement in development has important consequences for vascular smooth muscle. This study has examined the expression and distribution of the type 1 and type 3 IP_3 receptor subtypes in developing rat vascular smooth muscles. Immunoblotting of portal vein and aorta from neonatal (2 to 4 days) and fully developed (6 weeks) rats revealed significantly higher levels of the type 3 IP_3 receptor expression in neonatal, compared with developed, vascular smooth muscles. In contrast, expression of the type 1 IP_3 receptor in neonates was lower compared with developed vascular smooth muscles. Immunolocalization of the type 3 IP_3 receptors in neonatal tissues revealed that staining corresponded to the distribution of the sarcoplasmic reticulum (visualized by osmium ferricyanide staining of thin tissue sections), which suggested localization of the type 3 IP_3 receptor throughout the sarcoplasmic reticulum network. We conclude that type 3 IP_3 receptors are the predominant subtype in the development of vascular smooth muscle and are distributed throughout the sarcoplasmic reticulum in these cells. The switch in isoforms of the IP_3 receptor during development from the type 3 with low affinity for IP_3 to the higher-affinity type 1 receptor may play a role in calcium-mediated regulation of developing vascular smooth muscle. (Circ Res. 1999;84:536-542.)

Key Words: receptor ■ muscle, smooth, vascular ■ Ins(1,4,5)P_3 ■ development

An important step in the vascular smooth muscle contractile process is an increase in the intracellular Ca^{2+} concentration. It is now well established that a major pathway for increasing intracellular Ca^{2+} in smooth muscle is the activation of phospholipase C via activation of a plasma membrane receptor, which leads to the production of inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to specific IP_3 receptors in the smooth muscle cell, which produces a release of Ca^{2+} from the intracellular stores. Several investigators have isolated full-length cDNA clones that encode at least 3 distinct IP_3 receptors: type 1, type 2, and type 3. Type 1 IP_3 receptor is expressed in many cell types, type 2 IP_3 receptor is expressed in brain and heart, and type 3 IP_3 receptor is expressed predominantly in nonneural tissues. Messenger RNA for type 1, 2, and 3 IP_3 receptors has been detected in nonvascular smooth muscle, and in a vascular smooth muscle cell line, only mRNA for type 1 and type 3 receptors was detected. In smooth muscle, the type 1 IP_3 receptor has been localized to the sarcoplasmic reticulum throughout the cell.

The role of the different IP_3 receptor subtypes in smooth muscle remains to be established, although distinct functions of the type 1 and type 3 IP_3 receptors are suggested by their different binding affinities for IP_3; type 3 receptor has a 10-fold lower affinity than type 1 receptor. In vascular smooth muscle cells, alterations in the IP_3 receptor subtype expression and/or localization could have functional implications for Ca^{2+} homeostasis in blood vessels. To date, no studies have investigated the IP_3 receptor subtypes expressed in vascular smooth muscle or examined possible circumstances in which these may be altered. There is evidence that the expression of IP_3 receptor subtypes changes during differentiation in some cell types, which suggests a possible involvement in cell development. Developmentally associated alterations in messenger RNA levels for IP_3 receptors have also been observed in the mouse cerebellum. These developmental changes may occur in vascular smooth muscle, which given the difference in IP_3 affinities of the different subtypes, could potentially have functional implications for the regulation of blood vessel development.

This study examined the expression and distribution of the type 1 and type 3 IP_3 receptor in vascular smooth muscle from neonatal and fully developed rats. We reveal a significant
increase in the expression of type 3 IP$_3$ receptors in neonatal vascular smooth muscle distributed throughout the sarcoplasmic reticulum.

Materials and Methods

Antibody Specificity and Immunoblotting

Polyclonal anti-type 1 IP$_3$ receptor antibody was, as described previously,$^{18}$ raised in guinea pigs against the COOH-terminal 16 amino acids (residues 2733 to 2749) of the rat type 1 IP$_3$ receptor protein. Polyclonal anti-type 3 IP$_3$ receptor antibody was prepared, as described by Blondel et al.$^7$ in rabbits against the COOH-terminal 15 amino acids (residues 2656 to 2670) of the rat type 3 IP$_3$ receptor protein. Characterization of antibody specificity for the IP$_3$ receptor subtypes has been previously performed in other studies.$^7,18$

Adult male Sprague-Dawley rats (6 weeks old, 300 to 350 g) were killed by cervical dislocation followed by exsanguination, neonatal male Sprague-Dawley rats (2 to 4 days old, 6 to 10 g) were killed by destruction of the brain, and the aorta and portal vein were quickly removed from all rats. Blood vessels were carefully cleaned of connective tissue with the use of a dissecting microscope, and the endothelium was removed by gentle rubbing of the lumen. In the case of portal vein, the outer layer of smooth muscle was removed. All procedures performed on animals were in accordance with institutional guidelines. Tissues were homogenized at 4°C in a Braun homogenizing vessel in buffer containing 0.25 mol/L sucrose, 10 mmol/L Tris/HCl (pH 7.4), 1 mmol/L EDTA, 0.1 mmol/L PMSF, and 50 μmol/L leupeptin. In the case of neonatal rats, tissues were pooled from 6 rats to obtain sufficient material. Whole-cell homogenates were used for immunoblotting with anti-calponin and anti–smooth muscle actin antibodies (Dako Ltd) and routinely prepared from the same pooled whole-cell homogenate. For IP$_3$ receptor immunoblots, homogenates were centrifuged twice at 130 000g for 20 minutes and the supernatant was discarded. Forty micrograms of each sample (membrane protein or whole-cell homogenate) was loaded and electrophoresed on 6% SDS-polyacrylamide gel.$^{19}$ Proteins were transferred onto activated Immobilon-P transfer membrane (Millipore). The membranes were blocked with 5% skim milk and incubated with primary antibody (either anti-type 1 or anti-type 3 IP$_3$ receptor antibody for membrane preparations or either anti-calponin or anti–actin antibodies for whole-cell homogenates) followed by horseradish peroxidase–conjugated secondary antibody. Specific protein bands were visualized with enhanced chemiluminescence (Amersham Life Science). The relative density of protein bands was analyzed with an imaging densitometer (Biorad GS-690). Some gels were not transferred but stained with Coomasie blue to show protein loading between samples (not shown).

Sample Preparation for Confocal Laser Scanning Microscopy

Portal vein and aorta from neonatal rats (2 to 4 days old) and 6-week-old rats were dissected and treated as previously described.$^{19}$ The muscle was fixed in freshly made 3% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4), encased in 5% gelatin, and infused with sucrose in phosphate buffer. Tissue blocks were rapidly frozen by plunging them into liquid N$_2$–cooled Freon-22. Cryosectioning was performed on a Reichert Jung cryostat E microtome at −25°C. Sections 8 μm thick were covered with 1% PBS/BSA before incubation with 5% donkey serum. Sections were incubated with either anti-type 3 IP$_3$ receptor antibody or anti–type 1 IP$_3$ receptor antibody in PBS/BSA overnight at 4°C. Control sections were not exposed to primary antibody but incubated with either PBS/BSA or nonimmune serum in PBS/BSA. Sections were incubated with a TRITC-conjugated affinity-purified F(ab$^\prime$)$_2$ fragment species specific IgG (Jackson ImmunoResearch Laboratories, Inc) secondary antibody at a dilution of 1:400 in PBS/BSA for 2 hours. Confocal images were obtained with a Bio-Rad MRC-1024 laser scanning microscope equipped with a krypton-argon laser and a ×40 oil-immersion lens. The laser was fitted with either a blue (excitation, 488 nm) or a yellow (excitation, 568 nm) filter block.

Staining of the Sarcoplasmic Reticulum for Electron Microscopy

Portal vein and aorta from neonatal rats (2 to 4 days old) and 6-week-old rats were stained as previously described by Nixon et al.$^{14}$ Briefly, strips of smooth muscle were fixed in a 0.1 mol/L sodium cacodylate buffer solution that contained 2% glutaraldehyde, 4.5% sucrose, and 50 mmol/L CaCl$_2$. The muscle strips were postfixed in 2% OsO$_4$ and 0.8% potassium ferricyanide for 2.5 hours at room temperature followed by a buffer wash. Samples were incubated for 90 minutes in a saturated uranyl acetate and dehydrated in a graded series of ethanol concentrations up to 100%. The pieces were placed in a Spurr resin overnight, embedded in resin, and polymerized at 70°C. Tissue blocks were sectioned on a Jung SuperCut. Sections were cut at <100-nm thickness, mounted on 200-mesh copper grids, and examined on a Jeol electron microscope at 60 kV.

All chemicals and reagents were obtained from Sigma-Aldrich Co unless otherwise stated.

Results

Expression of Calponin and Smooth Muscle Actin in Developing and Fully Developed Vascular Smooth Muscle

Calponin, a marker of differentiated contractile smooth muscle, was expressed in neonatal and fully developed tissues (Figure 1A). Immunoblotting for calponin revealed a decreased expression in neonatal portal vein and aorta compared with developed smooth muscles based on equal protein loading of whole-cell homogenates. Similarly, smooth muscle actin, which also increases in expression with postnatal development in vascular smooth muscle, was lower in neo-
natal aorta and portal vein than in fully developed tissues (Figure 1B).

Expression of Type 1 and Type 3 IP$_3$ Receptor in Developing and Fully Developed Smooth Muscle

Immunoblotting with anti–type 3 IP$_3$ receptor antibody revealed a substantially increased expression of the type 3 IP$_3$ receptor in neonatal aorta compared with fully developed aorta based on equal membrane-protein loading (Figure 2A). Similarly, neonatal portal vein showed an increased expression of the type 3 IP$_3$ receptor compared with developed portal vein preparations.

Analysis of type 1 IP$_3$ receptor expression showed a decrease in the neonatal vascular smooth muscle (both portal vein and aorta; Figure 2B) compared with fully developed smooth muscles which was at, or below, the limits of detection for equal membrane-protein loading. Other bands observed with this antibody represent breakdown products and some nonspecific binding.\textsuperscript{18} Gels with the same pooled membrane preparations for both type 1 and type 3 IP$_3$ receptor immunoblotting showed similar quantifiable changes in both subtypes, which suggested minimal cross-reactivity of subtype-specific antibodies.

Localization of the Type 3 and Type 1 IP$_3$ Receptor in Neonatal and Developed Vascular Smooth Muscle

Neonatal rat aorta sections displayed a patchy, bright staining pattern throughout the cell cytoplasm (Figure 3A). Nuclei were mostly devoid of any signal. The staining of sections from fully developed rat aorta was similar in distribution to neonatal aorta although different in appearance. Fully developed rat aorta sections showed intense staining of distinct cytoplasmic structures, which were observed throughout the cytoplasm (Figure 3B) and extended along the axes of the cell. These stained structures reached from adjacent to the nuclear membrane to the plasma membrane and did not appreciably stain the nucleus itself. Immunostaining of neonatal–portal vein smooth muscle sections for the type 3 IP$_3$ receptor produced a staining pattern that extended throughout the length of the cell (Figure 4), with higher-magnification views revealing an apparently continuous network structure (Figure 4 inset). The fully developed portal vein showed no staining for type 3 IP$_3$ receptor above background levels (not shown), which was in agreement with results from immunoblots. The type 1 IP$_3$ receptor antibody showed no staining in neonatal tissues as predicted by immunoblots. Both the fully developed aorta and portal vein showed staining of the type 1 IP$_3$ receptor (Figure 5). Staining in both cell types was observed throughout the cytoplasm. Notably, the localization for the type 1 IP$_3$ receptor in developed aorta is similar in distribution but different in staining pattern than the type 3 IP$_3$ receptor in developed aorta. Although control sections incubated with only TRITC-conjugated secondary antibody or with nonimmune serum and secondary antibody occasionally showed autofluorescence of elastin, no significant labeling was detected when imaged at pinhole and laser conditions similar to those used in Figures 3, 4, and 5. Immunostaining was completely blocked by the preincubation of primary antibody with peptides that corresponded to the amino acid sequence of the antibody.
Distribution of \([\text{Ca}^{2+}]\) Stores in Neonatal and Developed Smooth Muscle

Electron microscopy of vascular smooth muscle treated with osmium ferricyanide revealed a network staining throughout the cytoplasm, with an interconnecting reticulum that is continuous with the outer nuclear membrane in all smooth muscle tissues examined (Figure 6). In neonatal rat aorta, the reticulum network was well developed and abundant, especially around the nucleus. This is expected in developing smooth muscle cells in which not all of the intracellular \([\text{Ca}^{2+}]\) stores are sarcoplasmic reticulum and in which there is an increased volume of the rough endoplasmic reticulum network. Therefore, in this study we have used the term “endoplasmic/sarcoplasmic reticulum” to describe the reticulum in developing smooth muscle cells. Fully developed rat aorta showed a sarcoplasmic reticulum distribution proportionately more in central areas of the cytoplasm than in areas adjacent to the plasma membrane. In neonatal portal vein, osmium ferricyanide–stained tissue showed the endoplasmic/sarcoplasmic reticulum network to be concentrated predominantly at the center of the cell similar to neonatal aorta, with large regions around the nuclear poles, and a proportionately smaller volume at the periphery of the cell. In contrast, developed rat portal vein contained sarcoplasmic reticulum that was located predominantly at the periphery of the cell, in close apposition to the plasma membrane, with occasional elements in the central cytoplasm.

Discussion

Morphological studies of development have revealed that at birth, the portal vein in the rat is unusual with respect to structure because the smooth muscle cells are in an undifferentiated state. Two to 4 days after birth, the smooth muscle cells are myoblasts, and development is essentially complete at ~28 days. Morphological studies of aortic development have shown that at birth, the aorta is still undergoing structural development, including hypertrophy and hyperplasia. This also includes a doubling in vessel wall thickness produced primarily by the growth of the extracellular matrix. The developmental time points used in this study were chosen on the basis of these previous studies. Confirmation of a developing vascular smooth muscle cell phenotype in 2 to 4 day postnatal portal

![Figure 3](Image)

**Figure 3.** Confocal photomicrograph of rat aorta tissue sections stained with anti-type 3 IP3 receptor antibody followed by TRITC-conjugated secondary antibody. A, Neonatal rat aorta section (longitudinal) that showed continuous staining throughout the cell at central areas surrounding the nucleus and at more peripheral areas in the cytoplasm close to the plasma membrane. N indicates nucleus; scale bar=5 \(\mu\)m. B, Fully developed rat aorta section (longitudinal) that showed staining of cytoplasmic structures both close to the nucleus (arrowheads) and at areas adjacent to the plasma membrane (arrows). Asterisk denotes autofluorescence from elastin. There is a single layer of smooth muscle cells between each layer of elastin. Fully developed aorta cells were 2 to 3 times larger than neonatal cells. N indicates nucleus; scale bar=5 \(\mu\)m.

![Figure 4](Image)

**Figure 4.** Confocal photomicrograph of rat neonatal portal vein section (longitudinal) stained with anti-type 3 IP3 receptor antibody followed by TRITC-conjugated secondary antibody. Staining of a punctate nature was observed throughout the cytoplasm. A higher-magnification image (inset) that showed a single cell highlighted (asterisk denotes extracellular space) revealed staining of a continuous network throughout the cell (long arrows). Short arrows denote axes of the cell. Scale bar=5 \(\mu\)m.
vein and aorta was verified by the relative expression of calponin, a thin filament–associated protein, and smooth muscle actin. Calponin is expressed late in the development of vascular smooth muscle cells and is indicative of a contractile phenotype. In both the developing vascular smooth muscles studied, the calponin expression was lower (~3-fold) than in the fully developed tissue, which would be expected in a developing contractile smooth muscle cell. Previous studies have shown that smooth muscle–specific actin increases throughout postnatal development of vascular smooth muscle. The neonatal aorta and portal vein both show decreased expression of smooth muscle–specific actin compared with fully developed smooth muscles. This is further evidence that the neonatal tissues used in this study are developing vascular smooth muscles.

This study reveals that the IP$_3$ receptor subtype expression is altered during postnatal development of neonatal vascular smooth muscles. The developing smooth muscle has relatively higher expression of the type 3 IP$_3$ receptor, with low levels of the type 1 IP$_3$ receptor. In contrast, the fully developed smooth muscles.
developed vascular smooth muscles have low expression levels of type 3 compared with neonates and relatively higher levels of the type 1 IP3 receptor. The relative expression of these proteins as measured by immunoblotting is semiquantitative because, although the lanes were loaded with equal membrane protein, samples were prepared at different stages of development and are likely to have varying levels of expression of many membrane proteins. Data could be normalizing to the expression of other proteins, which would alter slightly the quantitative changes. For example, normalizing to smooth muscle–actin expression (Figure 1B) would increase the quantitative difference between neonatal and developed tissues with regard to the type 3 IP3 receptor and have no effect on the relative expression of type 1 IP3 receptor, because the type 1 receptor is not present in neonatal samples. We are therefore confident that this reflects a real change in cellular protein expression levels of IP3 receptors. Although this study has not examined a role for the type 2 IP3 receptor, a previous study has shown that an embryonic-aortic smooth muscle cell line contains no detectable amounts of mRNA for the type 2 receptor. Therefore, in neonatal vascular smooth muscle cells, the type 3 IP3 receptor has a predominant role in intracellular Ca2+ release. Evidence that neonatal portal vein has an IP3-releasable intracellular Ca2+ store was demonstrated in a recent study. The localization of the type 3 IP3 receptor in neonatal portal vein and aorta was consistent with the distribution of the sarcoplasmic reticulum, although immunostaining patterns were apparently more widespread than the sarcoplasmic reticulum distribution observed in electron micrographs. This discrepancy arises because electron micrographs are taken from ultrathin sections (<0.1 μm thick), whereas sections obtained for confocal microscopy are 8 μm thick. The thicker sections contain proportionately more sarcoplasmic reticulum than the 80 times thinner sections used for electron microscopy. This correlation is therefore a qualitative one and not quantitative. In adult aorta, the type 3 receptor also appeared to be localized throughout the sarcoplasmic reticulum, although the staining was of a different appearance compared with neonatal aorta. This difference is probably the result of a difference in distribution of the reticulum in neonatal aorta compared with developed aorta (see Figure 6). The type 1 IP3 receptor was also localized throughout the sarcoplasmic reticulum in developed aorta and portal vein, in agreement with the previous study, but was not detected in neonatal vascular smooth muscles. It is noteworthy that the distribution of the type 3 IP3 receptor was not only at discrete locations in the cell such as the extended poles of the cell or only at the plasma membrane. This may indicate a more general role in Ca2+ homeostasis rather than a specific function such as localized initiation of Ca2+ waves or a purely Ca2+ influx mechanism.

The observed changes in IP3 receptor subtypes presumably represents an important switch in expression during the normal development of vascular smooth muscle cells; however, the functional relevance of these findings and the role of type 3 receptor in development is still unclear. The type 3 receptor has an ≈10-fold lower affinity than the type 1 IP3 receptor. The increased expression of the type 3 IP3 receptor would presumably alter intracellular Ca2+ release profiles that may regulate some developmental processes. During development, the cells are in a proliferative state and have important synthetic functions, and the type 3 IP3 receptor may therefore play a role in proliferation or synthesis, although this remains to be determined.

The remodeling of the intima of blood vessels that occurs with restenosis and in atherosclerotic lesions involves a change in the phenotype of the vascular smooth muscle cell. If the type 3 IP3 receptor is involved in regulating the development of blood vessels, it seems likely that smooth muscle cells that are of a modulated synthetic phenotype will also show increased expression of the type 3 IP3 receptor. No studies to date have examined IP3 receptor subtype expression in vascular smooth muscle cells that have undergone a phenotypic modulation.

In conclusion, this study presents evidence that the predominant IP3 receptor subtype expressed in developing vascular smooth muscle is the type 3 IP3 receptor. Developed smooth muscle cells expressed proportionately more type 1 than type 3 IP3 receptor, which indicated a switch in subtype expression through development. The type 3 IP3 receptor expression in both developing and adult cells was located in areas consistent with the distribution of the endoplasmic/sarcoplasmic reticulum. The increased type 3 IP3 receptor expression and widespread subcellular distribution observed in developing cells indicates a general role in Ca2+ homeostasis during vascular smooth muscle development.

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