Differential Muscarinic Receptor mRNA Expression by Freshly Isolated and Cultured Bovine Aortic Endothelial Cells

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Endothelial cells, either in vivo or freshly isolated, respond when exposed to muscarinic agonists with an increase in cytosolic free calcium concentration ([Ca\(^{2+}\)]) and release of endothelium-derived relaxing factor (EDRF). When placed in culture, however, endothelial cells rapidly lose these responses, which may be related to changes in muscarinic receptor expression. Northern blot analysis of poly(A) + RNA from freshly isolated or cultured bovine aortic endothelial cells was used to address this problem. Through the use of specific cDNA probes complementary to the nonconserved regions of the m1, m2, m3, m4, and m5 muscarinic receptors, mRNA transcripts for the m1 (3.9 kb), m2 (3.8 kb), and m3 (3.1 kb) receptor subtypes were identified in freshly isolated endothelial cells, whereas m1 and m3 transcripts were identified in aortic smooth muscle. In contrast, cultured endothelial cells contained mRNA for only the m2 receptor subtype. Transcripts for the m4 or m5 receptors were not detected in either freshly isolated or cultured endothelial cells. Since m1 and m3 receptor subtypes are coupled to phospholipase C, activation of which is required for EDRF release, these observations may explain the failure of muscarinic agonists to elicit a rise in [Ca\(^{2+}\)], and EDRF release from cultured endothelial cells. (Circulation Research 1992;70:234–240)

Furchgott and Zawadzki\(^1\) were the first to demonstrate the phenomenon of endothelium-dependent relaxation of blood vessels while using acetylcholine. These investigators,\(^1\) as well as others,\(^2-5\) established that the ability of acetylcholine to elicit the release of endothelium-derived relaxing factor (EDRF) was mediated through a muscarinic receptor–linked pathway.

Even though many investigators have assumed that the muscarinic receptors responsible for acetylcholine-induced endothelium-dependent relaxation are located on the endothelium, the localization of these receptors is unclear. When studied in culture, endothelial cells fail to respond to muscarinic agonist administration with either an increase in cytosolic free calcium concentration ([Ca\(^{2+}\)]) or release of EDRF, although they continue to elevate [Ca\(^{2+}\)], and produce EDRF in response to other receptor-coupled agonists.\(^5,7\) In addition, previous autoradiographic investigations have not detected the presence of endothelial muscarinic receptors.\(^8,9\) Other studies in which classic pharmacological approaches were used have classified the receptor responsible for mediating endothelium-dependent relaxation as M\(_2\),\(^4,5\) or M\(_3\),\(^3,10,11\) whereas a study that examined isolated endothelial cells identified a muscarinic receptor of the M\(_3\) subtype.\(^12\)

In the present study, we chose to use a different approach, that of Northern blot analysis, to answer the questions regarding the existence of endothelial muscarinic receptors and the identity of the receptor subtypes present on the endothelium. We addressed the hypothesis that muscarinic receptors are expressed by endothelial cells, but the subtype(s) responsible for coupling acetylcholine to EDRF production is no longer expressed once the cells are placed in culture.

Materials and Methods

Muscarinic Receptor Subtype–Specific cDNA Probes

Muscarinic receptor subtype–specific partial cDNA probes for the nonconserved third intracellular (i3) loop of the m1–m5 receptor subtypes were a gift from T. Bonner (Laboratory of Cell Biology, National Institute of Mental Health). The m1 (390-bp) and m4 (520-bp) probes were Bal I/Xma I fragments of the i3 loop of the rat m1 and m4 clones.\(^13\) The m2 probe was a 500-bp Ava I fragment of the i3 loop of the human...
m2 clone; the m3 probe was a 700-bp Stu I/Nhe I fragment of the i3 loop of the rat m3 clone. The m5 probe was a 500-bp Bgl II/BamHI fragment of the i3 loop of the rat m5 clone.

Harvesting and Culture of Endothelium

Endothelial cells were harvested from bovine aortas as previously described, with minor modifications. Harvested cells were initially plated in six-well culture plates and grown to confluence in Waymouth’s MB media (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 0.1 mg/ml l-glutamine, and 1% penicillin/streptomycin, pH 7.4. Endothelial cell cultures were maintained at 37°C in 95% room air–5% CO2. The cell cultures were split and amplified in 25-cm2 culture flasks, followed by fluorescent labeling with Dil-acetylated low density lipoprotein and sorting using a fluorescence-activated cell sorter (EPICS Dye Laser System, Coulter Corp., Hialeah, Fla.). Subsequent characterization of endothelial cells was performed as previously described.

Endothelial cells (fourth or fifth passage) were plated at a density of 1 x 10^4 cells/cm^2 in 1,700-cm2 plastic roller bottles (Corning Glass Inc., Corning, N.Y.) and grown at 37°C in Waymouth’s MB media (including 10% fetal bovine serum, 0.1 mg/ml l-glutamine, and 1% penicillin/streptomycin). After the cells reached confluency, the media was discarded, and the cells were rinsed once with 40 ml ice-cold PBS-A containing (mM) sodium chloride 137, sodium phosphate 8.1, potassium chloride 2.7, and potassium phosphate 1.5. The endothelial cells were scraped into 20 ml ice-cold guanidinium thiocyanate for extraction of mRNA. The cell suspension was triturated 10 times with a sterile 19-gauge needle and then sonicated (3 x 10-second bursts) on ice.

Harvesting of Fresh Isolates of Endothelium

Bovine aortas were obtained at a local slaughterhouse. Aortas were cut open and gently rinsed with ice-cold PBS-A to remove any blood. Endothelial cells were obtained by light scraping of the lumen with a scalpel blade and were immediately placed in ice-cold guanidinium thiocyanate. Endothelial cell suspensions were triturated five times with a sterile 19-gauge needle, followed by Dounce homogenization (40-ml Wheaton glass homogenizer and B pestle) on ice. The endothelial cell homogenates were frozen on dry ice and transported to the laboratory.

Preparation of Other Tissues for RNA Extraction

Bovine aortic smooth muscle was obtained at the slaughterhouse by separating the media longitudinally and using only the tissue from the lumen to the midline of the vascular wall. This technique avoided any possible contamination of the vascular smooth muscle by adventitial cells, and the endothelium had been removed previously by mechanical scraping. Vascular smooth muscle samples were snap-frozen in liquid nitrogen and transported to the laboratory.

Whole rat brains, both atria, or liver was obtained, and RNA was extracted to be used for positive and negative controls. All tissue samples were homogenized on ice in guanidinium thiocyanate using a polytron (Brinkmann Instruments, Inc., Westbury, N.Y.) on setting 5.

RNA Extraction and Preparation of Northern and Dot Blots

RNA was extracted by the method of Chirgwin et al., including pelleting of the RNA through a cesium chloride cushion. Poly(A)+ RNA was isolated by passage of total RNA over oligo (dT)-cellulose spin columns (5 Prime-3 Prime, Inc., West Chester, Pa.) per the supplier’s instructions. Before gel electrophoresis, 3 μg poly(A)+ RNA, 20 μg total RNA (liver), or 6 μg of a 0.24–9.5-kb RNA ladder (BRL, Gaithersburg, Md.) was heat-denatured (65°C, 10 minutes) in a 30-μl solution containing 50% (vol/vol) deionized formamide, 2.2 M formaldehyde, and 1× Northern buffer (0.02 M MOPS, 0.005 M sodium acetate, and 0.001 M EDTA, pH 7.0) and then rapidly cooled on ice. Poly(A)+ RNA from rat brain served as a positive control in experiments in which the m1, m3, m4, and m5 cDNA probes were used; poly(A)+ RNA from rat atria was used as a positive control during hybridization with the m2 cDNA probe. In each case, total RNA from rat liver served as a negative control.

The poly(A)+ RNA samples were subjected to electrophoresis through a 1.0% agarose gel containing 2.2 M formaldehyde and 1× Northern buffer. After removing the lane containing the RNA ladder from the remainder of the gel, molecular weight standards were visualized using ethidium bromide. The gel was rinsed in 20× SSPE (1× SSPE is 0.15 M sodium chloride, 0.017 M sodium phosphate, and 0.001 M EDTA, pH 7.4) for 30 minutes, followed by transfer of the RNA to a 0.45-μm nylon membrane (Fisher Scientific, Pittsburgh, Pa.) by Northern blotting (using 10× SSPE). The resulting blot was irradiated ultravioletly to cross-link the RNA to the membrane and then baked at 80°C, under vacuum, for 4 hours.

Dot blots were prepared by the method of Zeng and Lynch using the m1–m5 plasmid DNAs and a dot blot template (Schleicher & Schuell, Inc., Keene, N.H.). Thirty femtomoles (8–14 ng) of DNA was introduced into each well.

Labeling of cDNA Probes and Hybridization Conditions

cDNA probes for the m1–m5 receptor subtypes or a near full-length (i.e., coding region– and noncoding region–containing) smooth muscle α-actin cDNA from a rat stomach cDNA library (provided by K. McHugh and J. Lessard, Children’s Hospital Research Foundation, Cincinnati, Ohio) was labeled with [α-32P]dCTP by random priming (Prime-A-Gene, Promega Corp., Madison, Wis.). The cDNA probes were hybridized with the Northern or dot blots by the
method of Church and Gilbert\textsuperscript{20} at 60°C (muscarinic receptor probes) or 65°C (smooth muscle \( \alpha \)-actin probe) for 2 days. Washing of the blots was done at the same temperatures at which the hybridizations were performed. Blots probed with muscarinic receptor cDNAs were washed twice with wash buffer A (0.5% bovine serum albumin, 1 mM EDTA, 40 mM sodium phosphate [pH 7.0], and 5% sodium lauryl sulfate) and once with wash buffer B (1 mM EDTA, 40 mM sodium phosphate [pH 7.0], and 1% sodium lauryl sulfate) (10 minutes per wash); blots probed with the smooth muscle \( \alpha \)-actin cDNA were washed twice with wash buffer A and four to six times with wash buffer B. All blots were rinsed with 25 mM sodium phosphate buffer (pH 7.0) before being air-dried. Membranes were exposed to Kodak X-Omat AR film at \(-70\)°C in the presence of intensifying screens.

**Results**

**Specificity of Muscarinic Receptor cDNA Probes**

Under the defined hybridization conditions used in the present study, the five different muscarinic receptor i3 loop cDNA probes were demonstrated to be subtype specific (i.e., each hybridized only to a single cDNA) (Figure 1).

**Muscarinic Receptor mRNA Expression in Endothelial Cells and Vascular Smooth Muscle**

mRNA transcripts for \( m1 \), \( m2 \), and \( m3 \) receptor subtypes were detected in freshly isolated endothelial cells (Figure 2). The transcripts were approximately 3.9 kb (\( m1 \)), 3.8 kb (\( m2 \)), and 3.1 kb (\( m3 \)) in size. \( m1 \) and \( m3 \) receptor transcripts were detected also in bovine aortic vascular smooth muscle (Figure 2). In
the cultured endothelial cells, mRNA for the m2 receptor was observed (Figure 2). In contrast to the results obtained with freshly isolated endothelial cells, transcripts for the m1 or m3 subtypes were not present in the cultured endothelial cells (Figure 2). Complementary mRNA sequences for the m4 and m5 muscarinic receptor cDNA probes were not detected in either the freshly isolated or cultured endothelial cells (Figure 3).

Although the hybridization signals for the m1 and m3 receptor transcripts in the brain appear somewhat diffuse (Figure 2), this reflects the exposure time necessary to detect the endothelial cell mRNA transcripts. Since the lanes were loaded with equivalent quantities of poly(A)+ RNA, as demonstrated by the comparable β-actin hybridization signals for each tissue (Figure 4), the m1 and m3 receptor mRNAs appear to be significantly less abundant in the endothelial cells than in the brain (Figure 2). Shorter exposure times yielded a single well-defined band in each control tissue, except in the case of the m1 cDNA-labeled blots, where a second minor band was evident in the brain at a position corresponding to that of the 28S ribosomal subunit. It is unclear whether this band represented an additional m1 receptor transcript or nonspecific binding. The cDNA probes did not exhibit any hybridization to the negative control (liver).

**Contribution of Vascular Smooth Muscle Contamination to the m1 and m3 mRNA Hybridization Signals Detected in Freshly Isolated Endothelium**

To control for the possibility that the m1 and m3 hybridization signals detected in freshly isolated endothelial cells represented contamination of the preparations by vascular smooth muscle, the blots were rehybridized with a full-length cDNA probe for smooth muscle α-actin. Cross-hybridization of the cDNA probe to β-actin (2.1 kb) in each tissue demonstrated that equivalent quantities of mRNA were loaded into each lane of the gel (Figure 4). α-Actin mRNA (1.7 kb) was detected in the vascular smooth muscle, whereas only minor amounts were detected in the freshly isolated endothelial cell preparations; cultured endothelial cell preparations did not contain α-actin (Figure 4). Based on the relative densities of the respective α-actin bands, a conservative estimate is that <1% of the m1 and m3 transcripts detected in the freshly isolated endothelial cells can be attributed to the presence of vascular smooth muscle in the preparations.

**Discussion**

Endothelial cells, when either present on the intimal surface of an isolated blood vessel or when freshly isolated, have been demonstrated to release EDRF in response to stimulation with muscarinic agonists. Similarly, freshly isolated endothelial cells respond to acetylcholine administration with a rise in 

\[ \text{[Ca}^{2+}]_{i} \text{]}.^{24,25} \] However, once grown in culture, endothelial cells rapidly lose their ability to respond to muscarinic agonists with either a rise in 

\[ \text{[Ca}^{2+}]_{i} \text{] or EDRF release.}^{6,7} \] This difference in responsiveness to muscarinic agonist stimulation between freshly isolated and cultured endothelial cells may be due to either the absence of a necessary regulatory cell type (i.e., the vascular smooth muscle) or a change in muscarinic receptor expression.
Previous studies have classified the receptor subtype responsible for mediating endothelium-dependent relaxation as either M2 or M3, whereas M1 receptors have been identified on isolated endothelial cells. However, there are other studies in which muscarinic receptors have not been detected on endothelial cells. To circumvent some of the difficulties associated with autoradiographic or pharmacological approaches to the identification of endothelial muscarinic receptors, we decided to use Northern blot analysis to establish clearly whether endothelial cells possess muscarinic receptors and, if so, the identity of the expressed receptor subtypes.

In the present study, mRNA for the m1, m2, and m3 muscarinic receptor subtypes was identified in freshly isolated bovine aortic endothelial cells. However, once placed in culture, the endothelial cells (by fourth passage) failed to contain detectable mRNA for the m1 and m3 receptors, whereas they retained mRNA for the m2 receptor subtype. Similar results were obtained in a recent pharmacological study by Brunner and Kukovetz, although these investigators detected only the presence of M1 receptors on bovine aortic endothelial cells. Thus, Northern blot analysis provides an independent and perhaps more sensitive method with which to examine endothelial muscarinic receptor expression.

Bovine muscarinic receptor mRNAs have not been characterized previously. Our data indicate that the sizes of the various muscarinic receptor transcripts detected in the bovine endothelial cells and vascular smooth muscle differ from the sizes of those detected in the rat brain and atria. The sizes of the muscarinic receptor transcripts detected in the rat brain and atria corresponded to those reported previously.

The mRNA transcripts for the m1 and m3 receptors detected in the freshly isolated endothelial cells were not due to contamination by the vascular smooth muscle. This was demonstrated by the relative amounts of the muscarinic receptor mRNA compared with the amount of α-actin mRNA present in each preparation. It should be noted that the films from blots that were hybridized with the smooth muscle α-actin cDNA probe required extensive overexposure to detect any evidence of smooth muscle contamination in the freshly isolated endothelial cell preparations. These experiments also demonstrated the purity of the cultured endothelial cell preparations. Therefore, our data indicate that freshly isolated endothelial cells, in contrast to cultured endothelial cells, do in fact express m1 and m3 receptor mRNA transcripts. Additional studies are required to distinguish between failure of transcription and destabilization of mRNA for the m1 and m3 receptors during culture.

Our observations provide a possible explanation for the inability of muscarinic agonists to elicit EDRF release from cultured endothelial cells. Although the signal transduction pathways in the endothelium through which m1 and m3 receptors are coupled have not been elucidated, agonist binding to these receptors in various cell types leads to the activation of phospholipase C.
sodilators stimulate phospholipase C and inositol 1,4,5-trisphosphate production in intracellular stores, followed by inositol 1,4,5-trisphosphate–dependent release of calcium from intracellular stores. Therefore, it would seem reasonable to suggest that cultured endothelial cells no longer respond to muscarinic agonist stimulation with either a rise in [Ca\(^{2+}\)]\(_i\) or EDRF release because of the loss of the m1 and/or m3 muscarinic receptor.

Based on conceptual problems associated with the hypothesis that the endothelium expresses muscarinic receptors (i.e., very little acetylcholine is present in the blood, or little direct evidence for innervation of the endothelium) and the outcome of several studies that have failed to demonstrate the presence of endothelial muscarinic receptors, a theory has been advanced that proposes that muscarinic agonists act on vascular smooth muscle to release a mediator that is then responsible for stimulating the endothelium to release EDRF. Although not designed to test this hypothesis directly, our data do not support such a postulate. We have observed, however, that when endothelial cells and vascular smooth muscle cells are grown together in mixed culture (from four to six passages), muscarinic agonists continue to elicit EDRF release from the endothelium.

It is possible that a diffusible factor(s) or matrix produced by the vascular smooth muscle is responsible for maintaining the expression of m1 and/or m3 receptors by the endothelium, thus allowing continued release of EDRF in response to muscarinic agonist stimulation. We are investigating this hypothesis currently.

In conclusion, we have demonstrated the presence of mRNA encoding the m1, m2, and m3 muscarinic receptors in freshly isolated endothelial cells. Once placed into culture, however, endothelial cells no longer contain detectable amounts of mRNA for the m1 and m3 receptors, whereas they continue to express mRNA for the m2 receptor. These observations provide a possible explanation for why cultured endothelial cells no longer respond to muscarinic agonist stimulation with either a rise in [Ca\(^{2+}\)]\(_i\), or release of EDRF. Assuming that translation of these mRNAs occurs, bovine aortic endothelium contains m1, m2, and m3 muscarinic cholinergic receptors.

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