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Distribution of mRNA Encoding Three α_2-Adrenergic Receptor Subtypes in the Developing Mouse Embryo Suggests a Role for the α_{2A} Subtype in Apoptosis

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SUMMARY

α_2-Adrenergic receptors (α_2-ARs) respond to norepinephrine and epinephrine to mediate diverse physiological effects. Using *in situ* hybridization, the expression pattern of the mRNA encoding the three α_2-AR subtypes (α_{2A}, α_{2B}, and α_{2C}) was examined in the mouse embryo. The mRNA encoding the three subtypes was first detected at stage 9.5 days postcoitus (d.p.c.) for the α_{2A}-AR (coincident with norepinephrine availability), 11.5 d.p.c. for the α_{2B}-AR, and 14.5 d.p.c. for the α_{2C}-AR subtype. The mRNA encoding the α_{2A}-AR subtype shows both the earliest and the most widespread expression pattern, including developing stomach and cecum, many craniofacial regions and areas in the central nervous system. Strikingly, the α_{2A}-AR mRNA is expressed in the interdigital mesenchyme between stage 12.5 and 14.5 d.p.c. in parallel with digit separation, raising the possibility that the α_{2A}-AR might contribute to the apoptotic events underlying this process. To test whether α_{2A}-AR can signal apoptotic events, the α_{2A}-AR subtype was introduced into two mouse mesenchymal cell lines, C3H/10t½ and NIH-3T3; expression of the α_{2A}-AR correlated with accelerated apoptosis, as detected both by the TUNEL assay and the loss of cell viability. In contrast to the wide distribution of mRNA encoding the α_{2A}-AR subtype, the α_{2B}-AR mRNA was detected only in the developing liver and was most readily detectable between 11.5 and 14.5 d.p.c., when the liver is the principal site of hematopoiesis. The α_{2C}-AR mRNA is detected in the nasal cavity and cerebellar primordium only at ≥14.5 d.p.c. These studies represent the first characterization of the temporal and spatial expressions of the α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR subtypes during embryogenesis and provide important insights concerning the loci and possible roles of α_2-AR-mediated regulation of physiological processes during the developmental program.

α_2-ARs in adult animals and humans respond to EPI and NE to modulate metabolic effects in adipose, transepithelial Na^+ and water transport in renal and intestinal epithelial cells, suppression of insulin release from β cells of the pancreas, and attenuation of neurotransmitter release in the central and peripheral nervous systems (1). Consequences of activation of α_2-AR in the central nervous system include lowering of blood pressure, sedation, enhanced anesthesia, suppression of pain perception, and suppression of epileptogenesis (2).

Recently, α_2-ARs have been demonstrated to represent a family of three subtypes based on pharmacological (3) and molecular cloning (4–7) strategies. Northern and *in situ* hybridization analyses have identified mRNA expression of all three α_2-AR subtypes in a wide variety of tissues and organs that correspond to loci of known α_{2A}-AR regulated physiological functions, such as the central nervous system (8, 9), adrenal gland, cardiovascular system, and intestine (3). In contrast, virtually nothing is known about the localization and physiological roles of the AR subtypes in early development.

Recent findings from Thomas *et al*. (10) and Zhou *et al*. (11) indicate that the enzymes responsible for NE synthesis are moderately detectable at 8.5 d.p.c. (the earliest time point examined). The catecholamines dopamine and NE are first detectable at days 9.5 and 10.5 d.p.c., respectively, and EPI is consistently detected in fetuses older than 13.5 d.p.c. (10). NE and EPI activate α_2-ARs. Because the lack of NE synthesis leads to embryonic lethality (10), the possibility arises that particular α_2-AR subtypes may play important roles during development.

As a first step in exploring potential functions for α_2-AR in early embryonic development, we identified the distribution of the mRNA encoding α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR subtypes in the early developing mouse embryo through the use of *in*

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ABBREVIATIONS: d.p.c., days postcoitus; AR, adrenergic receptor; EPI, epinephrine; NE, norepinephrine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling; TH, tyrosine hydroxylase; DβH, dopamine β-hydroxylase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; BMP, bone morphogenetic protein.

situ hybridization techniques. In addition, when we observed that the distribution of mRNA encoding the α_{2A}-AR subtype coordinated temporally and spatially with the programmed cell death of mesenchymal cells during the digit formation, we tested the hypothesis that the α_{2A}-AR is capable of inducing or accelerating apoptosis in mesenchymal cells. The ability of the α_{2A}-AR to accelerate apoptosis in these cells reveals a heretofore unappreciated regulatory consequence of α_{2A}-AR activation in mammalian systems.

Materials and Methods

Embryo collection and fixation. The hybrid offspring between 129/SvEv and C57Black mice were used as mating pairs. Other embryos, derived from mating of 129/SvEv purebred mice, B6D2 F1 hybrids, and ICR mice, also were evaluated for comparison of mRNA expression patterns. The pattern of the α_{2A}-AR subtype expression was indistinguishable in these varying genetic backgrounds. Embryos harvested at 8.5 and 9.5 d.p.c. were fixed with 4% paraformaldehyde at 4° for 2 hr. Embryos harvested at 12.5, 13.5, 14.5, 15.5, and 16.5 d.p.c. were perfused with cold 4% paraformaldehyde, fixed overnight, transferred to 100% methanol, and stored at −20°.

Whole-mount *in situ* hybridization. Digoxigenin-labeled cRNA probes encoding antisense (signal) and sense (control) templates were synthesized from templates representing the nearly full-length coding sequence of α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR genes, as described previously (9). Whole-mount *in situ* hybridization was performed as described previously (12) with minor modifications of hybridization and washing temperatures as outlined briefly below. After overnight hybridization at 70°, the hybridization temperature was slowly reduced to 55° and maintained at 55° for 2 hr. The washing was performed at 55° before RNase A treatment and 50° after RNase treatment. After whole-mount hybridization, the embryos were washed with PBS (1× = 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.9 mM NaCl, 8.06 mM $Na_2HPO_4 \cdot 7\ H_2O$, and 5.6 mM glucose, pH 7.4) and transferred into PBS containing 20% sucrose. Some embryos were sectioned after the hybridization and wash steps.

In come cases, cultured cells expressing α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR subtypes were injected into the brain cavity of early embryos to confirm the ability of the mRNA probes to penetrate and identify the relevant mRNA in a receptor subtype-specific manner. These control studies both validated the selectivity of the probes and ensured us that the lack of detectable endogenous mRNA expression for a given α_2-AR subtype at certain embryonic stages was not a result of technical limitations in mRNA detection.

Sectioning and evaluation of apoptosis in the embryo. After whole-mount *in situ* hybridization, the tissue of interest was post-fixed with 4% paraformaldehyde for 1 hr and sectioned with a frozen microtome to the thickness of 60 μm. A hallmark of apoptotic cells is DNA fragmentation, leading to the appearance of high concentrations of 3′-OH ends of single- and double-stranded DNA; these ends are detected by terminal deoxynucleotidyl transferase-catalyzed digoxigenin-dUTP end labeling of these 3′-OH ends (TUNEL assay). Digoxigenin labeling is revealed using a fluorescein-conjugated anti-digoxigenin antibody fragment that lacks the Fc portion of the antibody (Apop Tag Plus Kit; Oncor, Gaithersburg, MD). The TUNEL assay was performed according to the instructions of the manufacturer, except for the following modifications: (a) sections were rinsed twice with 0.2% Triton X-100 in PBS for 15 min before the reaction, (b) the reaction was performed in 54 μl of working-strength terminal deoxynucleotidyl transferase enzyme at 37° overnight, (c) sections were rinsed twice with STOP/WASH buffer at 37° for 20 min twice and with 2× standard saline citrate for 30 min three times, and (d) the anti-digoxigenin-peroxidase incubation was prolonged to 2–4 hr and washed four times for 30 min each.

Heterologous receptor expression and evaluation of apoptotic signals in cultured cells. To test the hypothesis that α_{2A}-AR are capable of inducing or accelerating apoptosis in mesenchymal cells, we introduced the α_{2A}-AR into two cultured cell lines derived from the mouse embryonic mesenchyme (C3H/10t½ and NIH 3T3) through electroporation of a cDNA encoding the epitope-tagged α_{2A}-AR subtype (13). Cells were cultured in Eagle's modified medium plus 10% fetal calf serum. For electroporation, 1.5×10^7 cells in 0.4 ml of OPTI-MEM (GIBCO BRL, Gaithersburg, MD) were incubated with 10 μg of α_{2A}-AR cDNA and/or 10 μg of pGREEN LANTERN cDNA (GIBCO BRL) and exposed to 280 V/975 μF in a Gene Pulser II (BioRad, Hercules, CA). Cells were then incubated in Eagle's modified medium plus 10% fetal calf serum for various times (see figure legends) in the absence (control) or presence of 10 μM UK 14304, an α_2-AR agonist, or yohimbine, an α_2-AR antagonist. Drug-containing and control media were changed every 12 hr. Control experiments comparing the expression of epitope-tagged α_{2A}AR, identified immunochemically, and GFP, detected by microscopy with 610-nm illumination, confirmed that there is a 100% coincidence of coexpression of these two proteins when cotransfected via the procedures used in this study. Thus, after cotransfection of cDNAs encoding GFP and α_{2A}AR, detection of expression of GFP can be used as a marker for cells expressing the cDNA encoding the α_{2A}AR.

Cells were harvested in PBS and fixed in 1% paraformaldehyde for 15 min at room temperature. Cells were washed by centrifugation at 800 rpm for 5 min and then resuspended in 70% ethanol. The cell sample was stored at −20°. The expression of epitope-tagged α_{2A}-AR was demonstrated by immunofluorescence microscopy after incubation with the 12CA5 monoclonal antibody directed against the hemagglutinin epitope engineered into the amino terminus of the α_{2A}-AR and a Cy3-conjugated donkey anti-mouse secondary antibody, as described in detail previously (14).

Apoptotic events were measured in control versus α_{2A}-AR-expressing cells using the TUNEL assay as described above. Two alternative strategies for cell handling were used, with comparable findings obtained. Cells were either plated onto coverslips and examined immunochemically for α_{2A}-AR expression and then via the TUNEL assay for apoptosis or they were harvested, fixed, and then examined in suspension for α_{2A}-AR expression and apoptosis, after which the cells were applied to microscope slides and sealed under coverslips using Permamount.

Photography. Photography for the whole-mount embryos was performed under an Olympus SZH10 dissecting microscope. Photog-

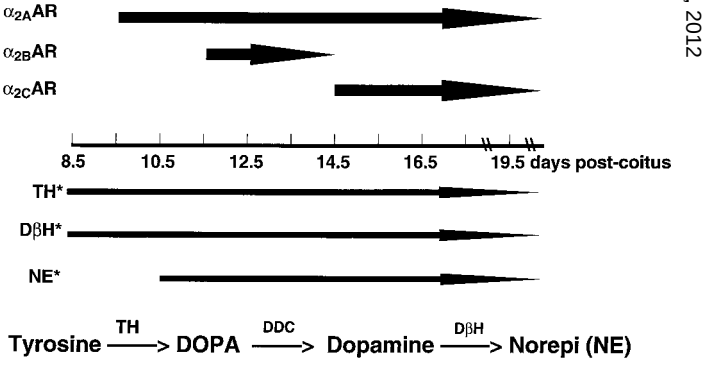

Fig. 1. Schematic of the relative onset of expression of mRNA encoding α_2-AR subtypes revealed by *in situ* hybridization in comparison with the expression of mRNA encoding TH and DβH in the developing mouse embryo. Synthesis of the catecholamine NE occurs via the hydroxylation of tyrosine, via TH, to L-3,4-hydroxyphenylalanine (*L-DOPA*); aromatic L-amino acid decarboxylase [or DOPA decarboxylase (*DCC*)] modifies this intermediate to dopamine, which, via dopamine β-hydroxylase, is converted to NE. EPI can be synthesized from NE by the enzyme phenylethanolamine-*N*-methyltransferase. ∗Data shown for TH and DβH summarize findings reported in Thomas *et al.* (10) and Zhou *et al.* (11).

Fig. 2. Endogenous α_{2A}-AR mRNA distribution in different mouse embryonic stages as detected through *in situ* hybridization. A, 9.5 d.p.c. B, 10.5 d.p.c. C, 12.5 d.p.c. D, 14.5 d.p.c., lateral view. E, 14.5 d.p.c., medial view after midline bisection of the embryo to create left and right halves. F, 14.5 d.p.c. embryo probed with sense strand cRNA synthesized from the α_{2A}-AR gene as a template, serving as a control for labeling. *Tel*, telencephalon; *Md*, mandible; *H*, hindlimb; *F*, forelimb; *2*, hyoid arch.

raphy for cultured cells and sectioned embryos was performed under a Zeiss Axioplan microscope. All photographs were made using Kodak Ektachrome 160 Tungsten film.

Results

Using whole-mount *in situ* hybridization strategies with digoxigenin-labeled cRNA probes, the expression pattern of the distinct mRNAs encoding the α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR subtypes was examined in mouse embryonic stages 8.5–16.5 d.p.c. At later stages of development, some of the hybridized embryos also were sectioned using a cryostat to permit more detailed evaluation of the pattern of expression of mRNA encoding the α_{2A}-AR subtypes.

Temporal Expression

Fig. 1 summarizes, via a schematic time-line, the onset of the expression of the mRNA for each α_2-AR subtype during embryogenesis. For comparison, the temporal expression of mRNAs encoding TH and DβH, enzymes responsible for the synthesis of NE, an endogenous ligand for α_2-AR, also are shown (10, 11). The mRNA encoding the α_{2A}-AR subtype was detected earliest and could be identified readily in the 9.5 d.p.c. embryo of the mouse, a time that parallels the detection of catecholamines in the brain cavity of embryonic mice (10). The mRNA encoding the α_{2B}-AR subtype was detected only over a narrow time frame during development (11.5–14.5 d.p.c.). The mRNA encoding the α_{2C}-AR subtype

Fig. 3. Endogenous α_{2A}-AR mRNA distribution in craniofacial areas of the mouse embryo as detected through *in situ* hybridization. A, 10.5 d.p.c. embryo showing α_{2A}-AR mRNA distribution in the maxillary arch (*Mx*) and hyoid arch (*HA*) [mandibula (*Md*)]. *F*, forelimb. B, 12.5 d.p.c., medial view of the head region after midline bisection of the embryo; shown is the left half. *Arrowhead*, expression of α_{2A}-AR mRNA in the nasal septum. C, 13.5 d.p.c., front view of the head showing expression of α_{2A}-AR mRNA in tissue surrounding nasal septum (*S*) [falx cerebri (*FC*)]. D, 14.5 d.p.c., medial view after midline bisection of the embryo; shown is the left side. *Arrowhead*, expression of α_{2A}-AR mRNA in the nasal septum (*S*); E, 14.5 d.p.c., lateral view showing expression of α_{2A}-AR mRNA in the external auditory meatus (*EAM*) and condensation of perioptic mesenchyme (ectomeninx). F, 13.5 d.p.c., bottom view of the head after lower jaw and tongue were removed, showing expression of α_{2A}-AR mRNA in tissue around the nasal septum at position of the posterior naris (*P*).

was not detected until 14.5 d.p.c. and was still evident at 16.5 d.p.c.

Spatial Expression

α_{2A}-AR subtype. The expression of mRNA encoding the α_{2A}-AR, first detected at 9.5 d.p.c., was distributed in the developing somites and in scattered cells covering the neural tube. The segmental expression of the α_{2A}-AR in the somites lasts until 14.5 d.p.c. but is no longer detected in ≥15.5 d.p.c. embryos (Fig. 2). However, after 10.5 d.p.c., the α_{2A}-AR mRNA can be detected in many regions, including a variety of craniofacial areas and developing limbs. The regional distribution of the α_{2A}-AR through development is given (see Figs. 2–5). Fig. 2 provides a whole-embryo view at days 9.5 (Fig. 2A) and 10.5 (Fig. 2B) d.p.c. and a view of bisected embryos at 14.5 d.p.c. (Fig. 2, D– F). Also shown is the regional expression of mRNAs encoding the α_{2A}-AR subtype at 11.5–14.5 d.p.c. (see Figs. 3–5).

In craniofacial areas of 10.5–13.5 d.p.c. embryos, α_{2A}-AR mRNA is prominently expressed in the maxillary arch, hyoid arch (Figs. 2B and 3A), mesenchyme cells covering the teleocephalon, brain mesoderm (Figs. 2A and 4A), and mesenchyme condensation forming future falx cerebri (Figs. 3C and 4B). In embryos harvested from 10.5–14.5 d.p.c. embryos, α_{2A}-AR mRNA is detected in mesenchyme of the nasal septum next to the posterior naris (Fig. 3, B, C, and F). From 13.5 to 16.5 d.p.c., this expression of α_{2A}-AR mRNA in the mesenchymal cells spreads to all areas of the nasal cavity, which parallels, temporally, the folding and expansion of this cavity. At the lower and central sides of the anterior naris, expression of α_{2A}-AR mRNA is first detected at 11.5 d.p.c. (Fig. 3, C and F) and then becomes very prominent in embryos harvested at 12.5 and 13.5 d.p.c. (Fig. 3, B–E).

The mRNA encoding the α_{2A}-AR subtype also is detected in other craniofacial areas, such as the external auditory meatus and the condensation of perioptic mesenchyme (ectomeninx), which subsequently differentiates to form the sclera in 11.5 and 12.5 d.p.c. embryos (Fig. 3E). Expression of α_{2A}-AR

Fig. 4. Endogenous α_{2A}-AR mRNA distribution in the 11.5 d.p.c. mouse embryo as detected through *in situ* hybridization. A, Back view showing α_{2A}-AR mRNA distribution in mesoderm on both sides of the neural tube [hindlimb (*H*), forelimb (*F*), rhombencephalon (*Rho*), nasal cavity (*NC*), midbrain (*MB*)]. B, Front view showing α_{2A}-AR mRNA distribution in the intersegmental area and in areas around the telencephalon (*Tel*) [falx cerebri (*FC*)]. C, Lateral view showing expression of α_{2A}-AR mRNA in some craniofacial and abdominal areas. *F*, Forelimb; *St*, stomach; *Lv*, liver; *Ce*, cecum; *Md*, mandibula; *Mx*, maxillary arch; *NLG*, nasolateral groove.

mRNA also is noted in the tissues covering the olfactory bulb from 10.5 and 16.5 d.p.c. (Fig. 3) and in the submandibular gland in embryos between 14.5 to 16.5 d.p.c. (data not shown).

In addition to its craniofacial distribution, mRNA encoding the α_{2A}-AR subtype is detected in mesoderm of the back of the cervical region in embryos harvested between 10.5 and 12.5 d.p.c. (Fig. 4A). The expression of α_{2A}-AR mRNA also is noted between 10.5 and 14.5 d.p.c. in the developing cecum (a limited area of midgut loop within the physiological umbilical hernia; Figs. 4C and 5), in the stomach between 11.5 and 12.5 d.p.c. (Fig. 4C), and in the genital tubercle in embryos harvested at 14.5 and 15.5 d.p.c. (data not shown).

A striking and unexpected finding of this study was the detection of α_{2A}-AR mRNA in the mesenchyma of the interdigital areas in the developing limbs between 12.5 and 14.5 d.p.c. of embryonic development (Fig. 6), closely paralleling the apoptotic regression leading to digit separation. This interdigital expression of α_{2A}-AR mRNA is significantly intensified at the developmental stage of 13.5 d.p.c. (Fig. 6B) in comparison to 12.5 d.p.c. (Fig. 6A). At 14.5 d.p.c., α_{2A}-AR mRNA is mainly detected at the base and remaining web of the nearly separated digits, as well as at newly forming finger joints (Fig. 6C). The α_{2A}-AR mRNA is no longer detected in the 15.5 d.p.c. limb, a time when digit separation is fully completed (data not shown). We also observed that α_{2A}-AR expression in the forelimb (Fig. 6, A and C) is always slightly more advanced than that of the hindlimb, as can be

Fig. 5. Endogenous α_{2A}-AR mRNA distribution in the developing cecum (*Ce*). A, Cecum of 11.5 d.p.c. embryo. B, Section of 11.5 d.p.c. cecum. C, Cecum of 13.5 d.p.c. embryo. *S*, Stomach; *L*, liver; *VM*, visceral mesoderm.

Fig. 6. Endogenous α_{2A}-AR mRNA distribution in developing limbs at different stages as detected through *in situ* hybridization. A, Limbs of 12.5 d.p.c. embryo. B, Limbs of 13.5 d.p.c. embryo. C, Limbs of 14.5 d.p.c. embryo. D, Section of 13.5 d.p.c. forelimb. E, Transverse section of 12.5 d.p.c. forelimb. F, Forelimb. H, Hindlimb.

seen in a comparison of α_{2A}-AR mRNA expression at stages 12.5 d.p.c. (Fig. 6A) and 14.5 d.p.c. (Fig. 6C). This temporal pattern is, again, consistent with the time course of limb morphogenesis (15–17). Sectioning of the limb after *in situ* hybridization reveals that only interdigital mesenchyme expresses α_{2A}-AR mRNA (Fig. 6, D and E). After sectioning, a hollowed indentation is detected between mesenchymal cells that have expanded as the embryo gets older, presumably as a result of mesenchyme regression after apoptosis (Fig. 6, D and E). Evaluation of the apoptotic zone in limb sections via the TUNEL assay demonstrates a close spatial correlation between cells undergoing apoptosis and cells expressing α_{2A}-AR mRNA (Fig. 7).

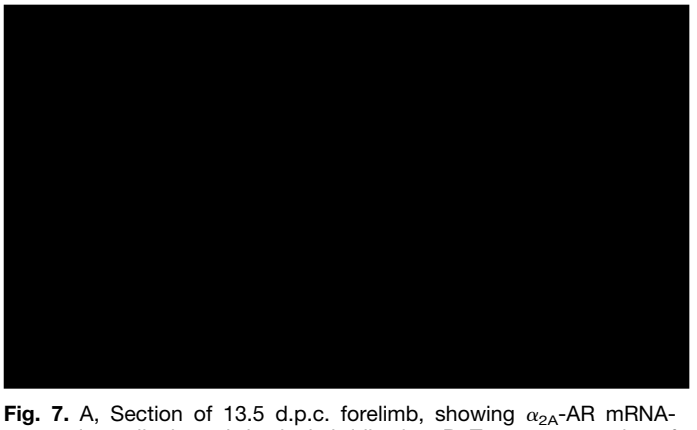

Fig. 7. A, Section of 13.5 d.p.c. forelimb, showing α_{2A}-AR mRNA-expressing cells through *in situ* hybridization. B, Transverse section of 13.5 d.p.c. forelimb, showing double-labeling of α_{2A}-AR mRNA-expressing cells and apoptotic nuclei.

The correlation between expression of α_{2A}-AR mRNA and mesenchymal regression in digit separation suggested the possibility that the α_{2A}-AR induces apoptosis in embryonic mesenchymal cells. To evaluate whether α_{2A}-ARs are capable of evoking apoptotic events, two mouse embryonic mesenchymal cell lines, C3H/10t½ and NIH-3T3 cells, were used as model systems to determine whether activation of α_{2A}-AR can induce apoptosis. A cDNA encoding an epitope-tagged α_{2A}-AR (13) was cotransfected via electroporation into cells with a cDNA encoding GFP Lantern. Control studies confirmed that immunodetection of heterologously expressed α_{2A}-AR always coincided with GFP expression, so monitoring of GFP expression permitted detection of α_{2A}-AR-expressing cells after electroporation. We observed (Fig. 8) that cells expressing α_{2A}-AR disappeared more rapidly in response to the α_2-agonist UK 14304 compared with control cells (non-GFP-expressing cells) cultured in the same dish, implying that the α_{2A}-AR-expressing cells were excluded from the cell population, likely via apoptosis, due to activation of the transfected α_{2A}-AR receptor. To test the correlation between apoptotic events and α_{2A}-AR expression more directly, C3H/10t½ (Fig. 9) or NIH-3T3 (not shown) cells transiently expressing α_{2A}-AR cells were incubated in the presence of an α_{2A}-AR agonist, UK14304 (10 μM) or, alternatively, an α_{2A}-AR antagonist, yohimbine, and α_{2A}-AR expression, as detected immunochemically, was correlated with apoptotic events detected in the TUNEL assay (see Materials and Methods). As shown in Fig. 9, apoptotic nuclei were detected in α_{2A}-AR-expressing cells, identified via the 12CA5 antibody directed against the amino-terminal hemaglutinin epitope

Fig. 8. Mouse embryonic C3H/10t½ cells were cotransfected with cDNA encoding the α_{2A}-AR and GFP (A–C) or the pCMV4 vector backbone (vector, control) and GFP (D–F), as described in Materials and Methods. At various time points after transfection, the relative density of GFP-expressing cells was examined after culture in minimal essential medium plus 10% fetal calf serum (no drugs added; A and D), medium plus α_{2A}-AR agonist (10 μM UK 14301; B and E), or α_2-AR antagonist (10 μM yohimbine; C and F). Data demonstrate that the α_{2A}-AR agonist UK 14304 causes an accelerated loss of GFP-expressing cells and, hence, α_{2A}-AR - expressing cells; this accelerated decline in α_{2A}-AR -expressing cells can be delayed, but not eliminated, by culture in the presence of the α_{2A}-AR antagonist yohimbine. These findings are consistent with the known agonist-independent activation of the α_{2A}-AR, presumably due to agonist-independent conformational changes toward the active state of the receptor (R*) known to occur in a variety of cell types for this receptor subtype (see Ref. 18 and text for discussion).

tag engineered into the α_{2A}-AR (13). When findings from 500-1000 cells examined in random fields by two investigators blinded to the cellular treatment were tabulated at each time point, it was observed that α_{2A}-AR-expressing cells manifest an increased appearance of apoptotic nuclei and fragmented cells compared with nonexpressing cells in the same culture dish (Fig. 9C) or control cells never transfected with the α_2-AR cDNA (not shown). Furthermore, treatment with the α_{2A}-AR agonist seemed to accelerate the apoptotic process. The fact that antagonist treatment delayed but did not entirely prevent the α_{2A}-AR-dependent increase in apoptotic events is likely due to the known capability of the α_{2A}-AR to undergo agonist-independent conformational changes that lead to productive receptor/G protein/effector interactions in target cells (18).

α_{2B}-AR and α_{2C}-AR subtypes. A moderate amount of mRNA encoding the α_{2B}-AR subtype was detected only in the liver and only in embryos harvested between 11.5 and 13.5 d.p.c.(Fig. 10).

Expression of mRNA encoding the α_{2C}-AR subtype is detected only very late during development (see Fig. 1). At 14.5 d.p.c., mRNA encoding α_{2C}-AR is weakly detectable in the nasal cavity and a part of cerebellar primordium; this distribution pattern remains the same until embryonic stage 16.5 d.p.c.

We are confident that the lack of detection of mRNA encoding the α_{2B}-AR and α_{2C}-AR subtypes during differing developmental stages is not due to technical limitations because we readily detected a strong signal for hybridization to mRNA encoding these two subtypes when α_{2B}-AR- or α_{2C}-AR-expressing cells were injected into the embryonic cavity before fixation and processing in parallel control experiments.

Discussion

The current results have revealed that the mRNAs encoding three α_{2A}-AR subtypes display distinctive temporal and spatial distribution patterns during embryonic development of the mouse. The temporal expression of the α_{2A}-AR mRNA

Fig. 9. Expression of α_{2A}-AR in mouse embryonic cell cultures induces or accelerates apoptosis. Mouse C3H/10t½ cells were transfected with cDNA encoding the α_{2A}-AR or with the pCMV4 vector backbone (control, not shown). At several time points after transfection, cells were harvested and sequentially treated for (A) immunochemical identification of α_{2A}-AR via Cy3-conjugated donkey anti-mouse secondary antibody (*red*) or (B) for apoptotic nuclei via fluorescein-conjugated anti-digoxigenin antibody (*green*). C, Comparison of many fields of cells at various times after transfection revealed that a markedly greater percentage of α_{2A}-AR-expressing cells manifest apoptotic nuclei compared with non-α_{2A}-AR-expressing cells on the coverslip and that incubation with the antagonist yohimbine delays apoptosis compared with incubation with the agonist UK14304. In the absence of α_{2A}-AR expression, UK14304 and yohimbine did not alter the fraction of cells undergoing apoptosis, as manifest by the fluorescein positive nuclei in the TUNEL assay.

coincides with the first detection of the catecholamines, NE and EPI, in the mouse central nervous system (10) and occurs 1 day later than expression of mRNA encoding two critical enzymes in catecholamine biosynthesis, TH and DβH (10, 11). The observation that regional distribution of α_{2A}-AR mRNA occurs in a variety of embryonic areas, including stomach and developing cecum (Figs. 4C and 5C), interdigital mesenchyme (Fig. 6) of developing limbs during the interim of digit separation, many craniofacial areas (Figs. 3–5), and limited regions in the brainstem (Fig. 5D) implies that effects of NE during development in these areas might be mediated by the α_{2A}-AR subtype. Interestingly, expression of α_{2A}-AR corresponds to a number of regions that undergo rapid cell proliferation, including stomach at 11.5 and 12.5 d.p.c. and cecum between 10.5 and 14.5. Because the α_{2A}-AR has been demonstrated to be capable of activating the mitogen-activated protein kinase pathway (19–22), it is possible the α_{2A}-AR modulates critical proliferation events during development.

Because it has been postulated that tissues with high proliferative activity may be more likely to undergo programmed cell death or apoptosis (23), it was of particular interest to note that α_{2A}-AR expression also occurs in some cells that undergo programmed cell death (Fig.6, A and C), such as the mesenchyme in the interdigital region of the limb bud from 12.5–14.5 d.p.c. (15–17, 23). Our results show that the onset and disappearance of α_{2A}-AR mRNA expression are slightly advanced in the forelimb compared with hindlimb buds (Fig. 6), a temporal expression pattern that matches the time frame of forelimb and hindlimb development (24). This temporal and spatial pattern of α_{2A}-AR mRNA expression in the interdigital areas suggested that α_{2A}-AR may be related to pattern formation of the limb (i.e., apoptotic events responsible for digit separation), perhaps in response to available norepinephrine. Our findings in C3H/10t½ and NIH-3T3 cells demonstrate that α_{2A}-AR can accelerate apoptotic events, as demonstrated by accelerated cell lysis and appearance of apoptotic nuclei (Figs. 9 and 10). These findings are the first demonstration that mRNA encoding α_{2A}-AR is expressed in apoptotic mesenchyme cells and that α_{2A}-AR can evoke apoptotic signals when examined in a mesenchymal cell preparation after heterologous expression.

Recently, Zou and Niswander (25) demonstrated that introduction of a dominant negative form of BMP receptor, dnBMPR-1B, into developing chick limb resulted in a reduction in apoptosis, truncation of digits, and conversion of scales to feathers, suggesting that the BMP signaling pathway is involved in apoptosis of digit separation. Interestingly, BMP has been shown to be a critical factor in the development of sympathetic (catecholamine-synthesizing) neurons and neuronal regeneration (26, 27). Our findings that the α_{2A}-AR facilitates apoptosis in embryonic mesenchymal cells and that the expression of mRNA encoding the α_{2A}-AR directly parallels interdigital mesenchymal regression suggest that the sympathetic nervous system, regulated in its development by BMP, may play a role in programmed cell death

Fig. 10. A, Endogenous α_{2A}-AR mRNA distribution in developing liver at stage 12.5 d.p.c. of the embryo; midline view after bisection of the embryo. B, Embryo probed with sense strand cRNA as a control for A.

essential for digit formation. Even if α_{2A}-ARs do modulate the apoptotic events that contribute to digit formation, however, these receptor-mediated events cannot be solely responsible for digit formation because altered digits are not noted in mice expressing a mutant α_{2A}-AR (D79N) that behaves as a functional knockout for this receptor subtype (28). However, we do know that the spatial and temporal expression of mRNA encoding all three α_2-AR subtypes is indistinguishable in wild-type and D79N α_2-AR mice.[2] Thus, functional redundancy mediated by the α_{2B}-AR or α_{2C}-AR subtype in digit formation is unlikely; it is more likely is that the α_{2A}-AR, although modulatory, is not essential for this mesenchymal apoptotic event. The molecular signals modulated by α_{2A}-AR during this window of embryonic development have yet to be clarified.

The temporal and spatial expression of the mRNAs encoding the α_{2B}-AR and α_{2C}-AR also is of interest. For example, from the period of 11.5–14.5 d.p.c., the α_{2B}-AR mRNA is detected in the liver, which serves as a blood-forming organ in embryonic development. This profile of α_{2B}-AR mRNA expression suggests that the α_{2B}-AR subtype may play a role in embryonic hematopoiesis.

The current study represents the first comprehensive description of the pattern of expression of α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR subtypes in developing mouse embryo. The subtype-specific temporal and spatial patterns of expression suggest that the existence of α_2-AR subtypes, all of which couple to the same effectors and are comparable in their affinity for the endogenous agonist ligands EPI and NE, may serve as a means to provide diversity in the timing or localization of NE-responsive receptors (29); presumably, the 5′ and 3′ regulatory regions of the intronless genes for the α_2-AR subtypes define subtype-specific receptor patterning. Taken together, the correlation of α_{2A}-AR mRNA distribution with mesoderm regression, the observation that α_{2A}-AR can facilitate apoptosis in cultured mesenchymal cells, and the ability of particular G protein-coupled receptors to induce apoptosis in some settings (29, 30) suggest that α_{2A}-AR may control apoptotic events, such as those leading to digit separation, worthy of future exploration in other cellular settings. The patterns of expression noted for all three subtypes may reveal as-yet-unappreciated roles for all of these α_2-AR subtypes during embryonic development.

[2] R.-X. Wang and L. E. Limbird, unpublished observations.

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References

1. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2:**2686–2695 (1988)
2. Ruffolo, R. R., Jr., A. J. Nichols, J. M. Stadel, and J. P. Hieble. Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **32:**243–279 (1993).
3. Bylund, D. B. Subtypes of α_1- and α_2-adrenergic receptors. *FASEB J.* **6:**832–839 (1992).
4. Chruscinski, A. J., R. E. Link, D. A. Daunt, G. S. Barsh, and B. K. Kobilka. Cloning and expression of the mouse homolog of the human *alpha*$_2$-C2 adrenergic receptor. *Biochem. Biophys. Res. Commun.* **186:**1280–1287 (1992).
5. Fraser, C. M., S. Arakawa, W. R. McCombie, and J. C. Venter. Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells: evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *J. Biol. Chem.* **264:**11754–11761 (1989).
6. Guyer, C. A., D. A. Horstman, A. L. Wilson, J. D. Clark, E. J. Cragoe, Jr., and L. E. Limbird. Cloning, sequencing, and expression of the gene encoding the porcine α_2-adrenergic receptor: allosteric modulation by Na^+, H^+, and amiloride analogs. *J. Biol. Chem.* **265:**17307–17317 (1990).

7. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Geng, U. Franck, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Cloning, sequencing, and expression of the gene coding for the human platelet *alpha*$_2$-adrenergic receptor. *Science (Washington D. C.)* **238:**650–656 (1987).
8. Scheinin, M., J. W. Lomasney, D. M. Hayden-Hixson, U. B. Schambra, M. G. Caron, R. J. Lefkowitz, and R. T. Fremeau, Jr. Distribution of α_2-adrenergic receptor subtype gene expression in rat brain. *Mol. Brain Res.* **21:**133–149 (1994).
9. Wang, R.-X., L. B. MacMillan, R. T. Fremeau, Jr., M. A. Magnuson, J. Linder, and L. E. Limbird. Expression of α_2-adrenergic receptor subtypes in the mouse brain: evaluation of spatial and temporal information imparted by 3 kb of 5′ regulatory sequence for the α_{2A}AR gene in transgenic animals. *Neuroscience* **74:**199–218 (1996).
10. Thomas, S. A., A. M. Matsumoto, and R. D. Palmiter. Noradrenaline is essential for mouse fetal development. *Nature (Lond.)* **374:**643–646 (1995).
11. Zhou, Q. Y., C. J. Quaife, and R. D. Palmiter. Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. *Nature (Lond.)* **374:**640–643 (1995).
12. Wilkinson, D. G. Whole mount *in situ* hybridization of vertebrate embryos, in In Situ *Hybridization: A Practical Approach* (D. G. Wilkinson, ed.). Oxford University Press, Oxford, UK, 75–83 (1992).
13. Keefer, J., and L. E. Limbird. The α_{2A}-adrenergic receptor is targeted directly to the basolateral membrane domain of Madin-Darby canine kidney cells independent of coupling to pertussis toxin-sensitive GTP-binding proteins. *J. Biol. Chem.* **268:**11340–11347 (1993).
14. Wozniak, M., and L. E. Limbird. The three α_{2A}-adrenergic receptor subtypes achieve basolateral localization in Madin-Darby canine kidney II cells via different targeting mechanisms. *J. Biol. Chem.* **271:**5017–5024 (1996).
15. Zakeri, Z. F., D. Quaglino, T. Latham, and R. A. Lockshin. Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB J.* **7:**470–478 (1993).
16. Zakeri, Z., D. Quaglino, and H. S. Ahuja. Apoptotic cell death in the mouse limb and its suppression in the hammertoe mutant. *Dev. Biol.* **165:**294–297 (1994).
17. Mori, C., N. Nakamura, S. Kimura, H. Irie, T. Takigawa, and K. Shiota. Programmed cell death in the interdigital tissue of the fetal mouse limb is apoptosis with DNA fragmentation. *Anat. Record* **242:**103–110 (1995).
18. Neubig, R. R., R. D. Gantzos, and W. J. Thomsen. Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* **27:**2374–2384 (1988).
19. Ablas, J., E. J. van Corven, P. L. Hordiji, G. Milligan, and W. H. Moolenaar. Gi- mediated activation of the p21^{ras}-mitogen-activated protein kinase pathway by α_2- adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.* **268:**22235–22238 (1993).
20. Anderson, N. G., and G. Milligan. Regulation of p42 and p44MAP kinase isoforms in rat-1 fibroblasts stably transfected with *alpha*$_2$-C10 adrenoceptors. *Biochem. Biophys. Res. Commun.* **200:**1529–1535 (1994).
21. Bouloumie, A., V. Planat, J.-C. Devedjian, P. Valet, J.-S. Saulnier-Blache, M. Record, and M. Lafontan. α_2-Adrenergic stimulation promotes preadipocyte proliferation. *J. Biol. Chem.* **269:**30254–30259 (1994).
22. Faure, M., T. A. Voyno-Yasenetskaya, and H. R. Bourne. cAMP and subunits of $\beta\gamma$ heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269:**7851–7854 (1994).
23. Sulik, K. K., C. S. Cook, and W. S. Webster. Teratogen and craniofacial malformations: relationships to cell death. *Dev. Suppl.* **103:**213–232 (1988).
24. Kaufmen, M. H. *The Atlas of Mouse Development*. Academic Press, London (1991).
25. Zou, H., and L. Niswander. Requirement for BMP signaling an interdigital apoptosis and scale formation. *Science (Washington D. C.)* **272:**738–741 (1996).
26. Lein, P., M. Johnson, X. Guo, D. Rueger, and D. Higgins. Osteogenic bone morphogenic protein-1 induces dendritic growth in rat sympathetic neurons. *Neuron* **15:**597–605 (1995).
27. Lein, P., X. Guo, A. M. Hedges, D. Rueger, M. Johnson, and D. Higgins. The effects of extracellular matrix and osteogenic protein-1 on the morphological differentiation of rat sympathetic neurons. *Int. J. Dev. Neurosci.* **14:**203–215 (1996).
28. MacMillan, L. B., H. Lutz, M. S. Smith, M. T. Piascik, and L. E. Limbird. Central hypotensive effects of the α_{2a}-adrenergic receptor subtype. *Science (Washington D. C.)* **273:**801–803 (1996).
29. Yamada, T.,M. Horiuchi, and V. J. Dzau. Angiotensin II type 2 receptor mediates programmed cell death. *Proc. Natl. Acad. Sci. USA* **93:**156–160 (1996).
30. Yamatsuji, T., T. Matsui, T. Okamoto, K. Komatsuzaki, S. Takeda, H. Fukumoto, T. Iwatsubo, N. Suzuki, A. Asami-Odaka, S. Ireland, T. B. Kinane, U. Giambarella, and I. Nishimoto. G protein-mediated neuronal DNA fragmentation induced by familial Alzheimer's disease-associated mutants of APP. *Science (Washington D. C.)* **272:**1349–1352 (1996).

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