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β-ADRENERGIC RESPONSIVENESS IN CULTURED AORTA SMOOTH MUSCLE CELLS

EFFECTS OF SUBCULTURE AND AGING

MAURICE F. CRASS, III,*† STEPHEN E. BORST‡ and PHILIP J. SCARPACE‡

* Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430; and ‡ Geriatric Research, Education and Clinical Center, Department of Veterans Affairs Medical Center and Department of Pharmacology, University of Florida, Gainesville, FL 32602, U.S.A.

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Abstract—β-Adrenoceptor-mediated vasorelaxation is diminished in vessels from a variety of aged species including humans. This phenomenon was studied for the first time in cultured aorta smooth muscle cells (ASMC) from young (4- to 6-month) and old (24- to 26-month) F-344 rats. Cyclic AMP (cAMP) accumulation was assessed following isoproterenol and forskolin stimulations in primary cultures and after 1–4 passages of aorta smooth muscle cells. Isoproterenol and forskolin increased cAMP accumulation 6- and 10-fold, respectively, in primary cultures from young rats. Isoproterenol stimulation was reduced markedly in passaged cells. Forskolin stimulation was unaffected, indicating passage-related phenotypic changes in receptor-mediated stimulation, but not in post-receptor adenylate cyclase activation. The response to isoproterenol was diminished in old animals, but that to forksolin was unaltered. Thus, cultured ASMC from F-344 rats are highly responsive to β-adrenoceptor stimulation and demonstrate age-related changes, but undergo phenotypic modulation during passage.

One consequence of normal aging, in particular in the cardiovascular system, is a decline in β-adrenergic function [1]. This loss of responsiveness occurs in a variety of cell types, including arteries and veins from rats and humans [2–7]. The increased incidence of hypertension in the elderly may be due, in part, to the diminished β_2-adrenoceptor activity in vascular tissue, and thus a decreased vasodilatory potential. Indeed, β-adrenoceptor-induced vasorelaxation is reduced markedly in old as compared with young rats [2, 4, 6, 8]. Furthermore, since β-adrenergic receptor number for the most part is unchanged with age, the decline in β-adrenergic responsiveness may be specific to the post-receptor generation of cyclic AMP (cAMP§) [1]. In contrast, cAMP-independent mechanisms of vasorelaxation are not reduced in aged animals [4, 9]. Smooth muscle cells in culture are a useful tool to study vascular hormone/receptor and signal transduction interactions. Successful application of this tool to studies of aging in blood vessels remains to be accomplished, as this would require cultures of vascular smooth muscle cells from old animals using an accepted aging model. The F-344 rat, a widely used model of aging, was used in the present studies for the culture of aorta smooth muscle cells (ASMC), specifically to assess the relationship of aging and vascular β-adrenergic reactivity. In addition, the effects of passage or subculture on β-adrenoceptor activity were examined in the context of possible changes in functional characteristics or phenotypic modulation [10] of the cells. As noted elsewhere [11], the usefulness of passaged cells as a model capable of predicting *in vivo* events is dependent upon the maintenance of phenotypic characteristics.

† Corresponding author. Tel. (806) 743-2533; FAX (806) 743-2218.

§ Abbreviations: ASMC, cultured aorta smooth muscle cells; cAMP, cyclic AMP; HBSS, Hanks' Balanced Salt Solution; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; PAP, antibody peroxidase–antiperoxidase method; [^{125}I]ICYP, [^{125}I]iodocyanopindolol; and ANOVA, analysis of variance.

METHODS

Segments of thoracic aorta measuring 5–7 cm from arch to diaphragm were removed from pentobarbital-anesthetized rats. For studies of passage and aging effects on cAMP accumulation. 4- or 26-month barrier-raised NNia F-344 virgin females were obtained from Harlan Industries (Indianapolis, IN) under contract to the National Institutes of Aging. F-344 rats of 6-, 18- and 24-months of age from the same source were used for studies of β-adrenoceptor density and affinity. The animals were maintained on a 12-hr light–dark cycle and fed rat chow and water *ad lib.*

After excision, aortas were placed in Hanks' Balanced Salt Solution (HBSS), pH 7.4, containing 25 mM HEPES and 1.3 mM Ca^{2+}. Vessel segments were cleaned by dissection under magnification and treated with a series of enzyme dispersion steps according to the method of Ramos and Cox [11] with minor modifications. Briefly, three to six segments were pooled for eventual seeding of primary cultures. Initially, intact segments were filled and flushed with plain HBSS and then refilled with HBSS containing collagenase (1 mg/mL) and incubated to remove endothelial cells. After washout of endothelial cells with plain HBSS, the segments were transferred to petri dishes and incubated with HBSS containing trypsin (1.25 mg/mL). The

segments were minced during incubation with HBSS containing a mixture of collagenase (1 mg/mL) and elastase (0.5 mg/mL). Lastly, the mince was subjected to three consecutive incubations with trypsin. The cell-containing fluid fraction of each of the above incubations, with the exception of that of the initial collagenase step which was discarded, was centrifuged and washed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. The washed cell-containing fractions were pooled and filtered through sterile gauze. Cells were counted on a Coulter counter, and viability was assessed by dye exclusion using trypan blue. The cells were seeded and grown at 37° under 95% room air–5% CO_2 in plastic flasks or multiwell tissue culture plates (Falcon, Primaria, Becton Dickinson Co., Lincoln Park, NJ) in DMEM containing 10% fetal bovine serum, 2 mM glutamine, gentamycin sulfate (100 μg/mL), and amphotericin B (5 μg/mL). The cells were seeded at a density of 2–11 × 10^4 cells/mL. The medium was changed and the cells were fed 48 hr post-seeding, and then every 3 days thereafter. Confluency of primary cultures occurred after 7–10 days. For passage (subculture), the cells were lifted with phosphate-buffered saline (PBS) containing trypsin (0.05%) and EDTA (0.02%) followed after 2 min by addition of serum-containing DMEM. After lifting, the cells were counted and viability was assessed as before. Confluency of cells after one to four passages occurred after 5–8 days.

Growth of the ASMC in culture was assessed by phase-contrast microscopy. Twenty-four to forty-eight hours post-seeding, the cells attached, flattened out and began to grow and proliferate. By 3–5 days, the cells assumed a typical spindle or ribbon-shape, with a phase dense cytosol. By 5–7 days, the cells were dedifferentiating into a synthetic state [10] as evidenced by electron microscopic examination, which showed few contractile myofilaments but numerous ribosomes and extensive rough endoplasmic reticulum. This transition was expected as seeding density was below 10^6 cells/mL. Five to ten days post-seeding, the cells had assumed a confluent monolayer with "hillock and valley" appearance. In addition to phase-contrast and electron microscopic examination of the ASMC, both thin contractile (muscle-specific actin) and noncontractile intermediate filament proteins (vimentin and desmin) were quantitated immunocytochemically using the unlabeled antibody peroxidase–antiperoxidase (PAP) method coupled with monoclonal antibodies for these filament types. ASMC from confluent primary cultures showed strong staining reactions for actin and vimentin with moderate staining for desmin. This profile of vimentin and desmin reactivity was typical of ASMC which, unlike small resistance arteries, have been shown to react with antivimentin antibodies, whereas only 50% of ASMC react positively with antidesmin antibodies [10].

Assays for cAMP accumulation in confluent monolayers of ASMC in multiwell plates were begun by aspirating off the serum-containing DMEM. This step was followed by a 15-min incubation at room temperature with serum-free HBSS containing 0.8 mM ascorbic acid without, and subsequently for 20 min at 37° with 0.5 mM isobutylmethylxanthine.

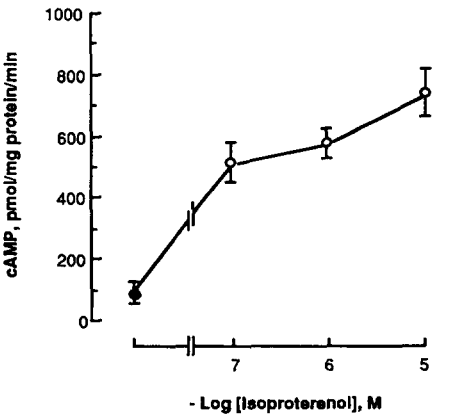

Fig. 1. Isoproterenol stimulation of cAMP accumulation in ASMC from 4-month-old rats. Basal or unstimulated cAMP accumulation is shown by the solid circle. Each value represents the mean ± SEM (N = 4–10).

Stimulators were added individually and incubated for 5 min. The reaction was stopped by removal of the supernate and the addition of 500 μL of 0.1 N HCl. The supernate and lysate were combined and then neutralized with NaOH, and cAMP was quantitated by radioimmunoassay. Protein was assayed by the method of Bradford [12].

β-Adrenergic receptors were assessed by multipoint Scatchard analysis of [^{125}I]iodocyanopindolol ([^{125}I]ICYP) binding data as described previously [13].

Data are expressed as means ± SEM of the number of duplicates of culture wells. Statistical analyses were performed using one-way analysis of variance (ANOVA) with post hoc multiple comparison using Duncan's Multiple Range Test. Student's *t*-test for independent samples was used where appropriate.

RESULTS

The β-adrenoceptor response of ASMC primary cultures from 4-month-old rats is shown in Fig. 1. Isoproterenol stimulated a concentration-dependent increase in ASMC cAMP accumulation. At 10^{-5} M, isoproterenol increased cAMP 6-fold over basal values (132.28 ± 34.28 vs 783.68 ± 78.23 pmol/mg protein/min). Receptor-independent stimulation of adenylate cyclase was determined by use of forskolin which acts directly on the catalytic unit. In ASMC from the same 4-month primary cultures, forskolin increased cAMP accumulation in concentration-related fashion (Fig. 2). At 10^{-4} M, forskolin increased cAMP production 10.3-fold over basal values (140.47 ± 27.55 vs 1448.55 ± 331.04 pmol/mg protein/min).

β-Adrenoceptor responsivenses was determined in primary cultures and after each of four successive passages of ASMC from 4-month old rats (Fig. 3). In terms of basal (unstimulated) cAMP accumulation, no differences were observed between primary

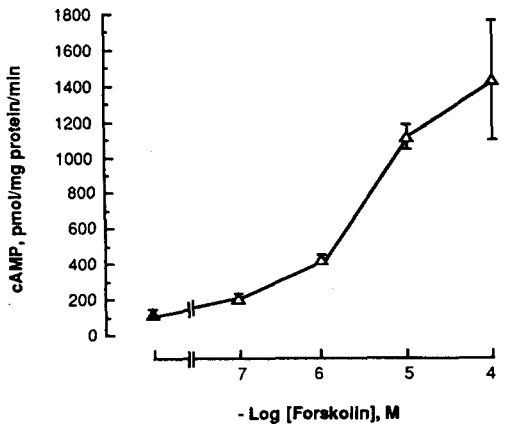

Fig. 2. Forskolin stimulation of cAMP accumulation in ASMC from 4-month-old rats. Basal or unstimulated cAMP accumulation is shown by the solid triangle. Each value represents the mean ± SEM (N = 4–14).

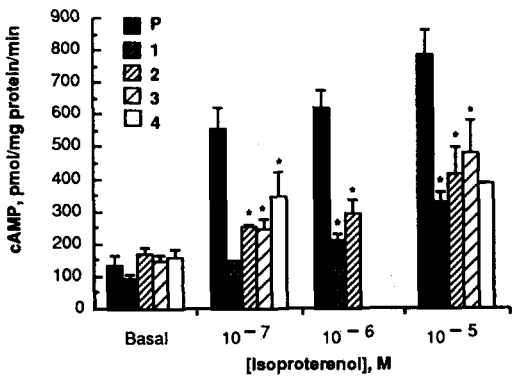

Fig. 3. Effects of passage on isoproterenol stimulation of cAMP accumulation in ASMC of 4-month-old rats. P represents values from ASMC in primary culture; 1–4 represent values from ASMC in culture after the indicated number of passages. "Basal" represents cAMP accumulation in the absence of isoproterenol. Each column represents the mean ± SEM (N = 3–10, except those without error bars which represent single observations). Key: (*) significant difference ($P < 0.01$) between means of primary culture and means of respective passages as determined by one-way ANOVA followed by Duncan's Multiple Range Test.

cultures and passaged ASMC. Cells from primary cultures and from each passage of ASMC demonstrated concentration-dependent responses to isoproterenol. However, responses to isoproterenol in the passaged cells were markedly less than those of primary cultures. Mean percent decrements in cAMP accumulation in passaged cells relative to those of primary culture were 55 ± 7, 59 ± 6, and 49 ± 4% after stimulation with 10^{-7}, 10^{-6} and 10^{-5} M isoproterenol, respectively.

Forskolin stimulation of adenylate cyclase was

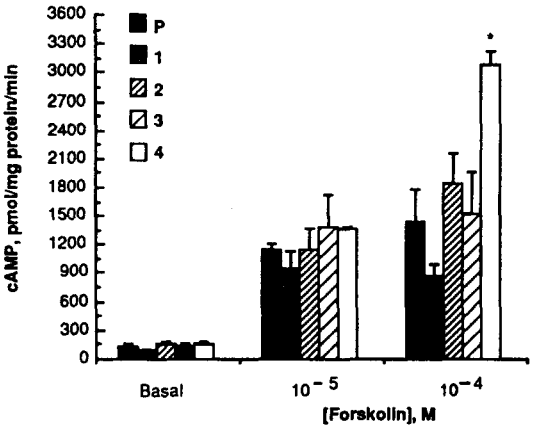

Fig. 4. Effects of passage on forskolin stimulation of cAMP accumulation in ASMC of 4-month-old rats. P represents values from ASMC in primary culture; 1–4 represent values from ASMC in culture after the indicated number of passages. "Basal" represents cAMP accumulation in the absence of forskolin. Each column represents the mean ± SEM (N = 3–10). Key: (*) significant difference ($P < 0.01$) from mean of primary culture as determined by one-way ANOVA followed by Duncan's Multiple Range Test.

assessed in primary and passaged ASMC from 4-month-old rats (Fig. 4). ASMC were from the same cultures as those used in obtaining the data in Fig. 3. As before, basal cAMP accumulation was similar in passaged and primary cultures of ASMC. Unlike the responses to isoproterenol, passaged ASMC were as responsive to forksolin stimulation as were primary culture cells. Although the responses were somewhat variable at 10^{-4} M, forksolin stimulation of cAMP accumulation in passaged cells was similar to, or greater (fourth passage) than, that of primary ASMC.

The central aim of this study was to assess possible differences in β-adrenoceptor sensitivity in ASMC from young (4-months) versus old (26-month) F-344 rats. The results from primary cultures and, for comparison, after each of two passages are shown in Fig. 5. The response to isoproterenol was diminished markedly in ASMC from 26-month-old relative to 4-month-old animals. Phenotypic modulation during passage was again evident in that β-adrenoceptor sensitivity in the old rats was negligible after only two passages. In contrast to isoproterenol-stimulated activity, receptor-independent adenylate cyclase activation by forskolin was similar in young and old rats whether from primary or passaged ASMC. The apparent age-related decrease observed in 4- versus 26-month second passage cells was not statistically significant ($P > 0.15$).

Finally, β-adrenoceptor densities (B_{max}) and antagonist affinities (dissociation constants) were assessed by Scatchard analysis of [^{125}I]ICYP binding in ASMC of 6-, 18- and 24-month old rats (Table 1). As can be seen, no differences in ASMC β-adrenergic receptor densities or affinities for ICYP were observed in the three age groups.

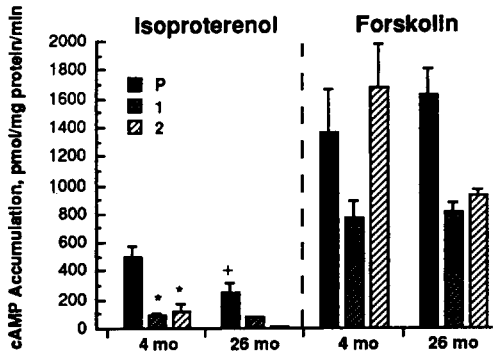

Fig. 5. Effects of age on cAMP accumulation (basal values subtracted) following isoproterenol (10^{-6} M) or forskolin (10^{-4} M) stimulation. P represents values from ASMC in primary culture; 1 and 2 represent values for ASMC in culture after the indicated number of passages. Each column represents the mean ± SEM (N = 4–6, except those without error bars which represent the average of 2 observations). Key: (*) significant difference ($P < 0.05$) between mean of primary culture and means of first and/or second passage cells as determined by one-way ANOVA followed by Duncan's Multiple Range Test; and (+) significant difference ($P < 0.05$) between 4-month and 26-month values as determined by Student's *t*-test for independent samples.

Table 1. β-Adrenergic receptor density in ASMC with age*

Age (months)	B_{max} (fmol/mg protein)	Dissociation constant (pM)
6	3.15	36
18	3.67	49
24	5.02	43

* Values are data from cultures derived from six rats of each age.

DISCUSSION

Cultured vascular smooth muscle cells provide an important tool to study hormone– or drug–receptor interactions and modes of signal transduction in vascular tissue. The present study indicated that cultured ASMC from the F-344 aging rat are useful to discern effects of age on vascular receptor function and signal transduction. However, major changes in efficacy of isoproterenol were discovered during passage of the cells beginning with the first subculture. Cultured myocytes represent a postsynaptic preparation and we would expect there to be postsynaptic β- as well as postsynaptic α-adrenergic receptors [14]. Isoproterenol stimulation thus represented activation of the β-adrenoceptor–adenylate cyclase complex and, as measured by cAMP accumulation, was decreased relative to that of primary cultures by as much as 55%. To identify the possible site(s) within the receptor complex involved in the decreased response, forskolin-induced activation of adenylate cyclase was studied. Forskolin activated adenylate cyclase directly at the catalytic unit and thus represents receptor-independent stimulation [15]. At maximal stimulatory concentrations of forksolin (10^{-5} and 10^{-4} M), cAMP accumulation was unaffected by passage of ASMC. This finding suggested that modulation of β-adrenoceptor activity during passage involved phenotypic alterations (decreases) in receptor density, impaired coupling to G protein, or altered G protein function. In view of this modulation(s) of phenotype during subculture, studies of vascular β-adrenoceptor function using ASMC should be interpreted with caution and be limited to primary cell cultures.

Isoproterenol-stimulated β-adrenoceptor responses were diminished markedly in ASMC from 26-month F-344 rats. This finding confirms the earlier observation of Volicer *et al.* [16] who found decreased cAMP accumulation in ASMC from Sprague–Dawley rats, and correlates with previously cited functional loss of vasorelaxant potential in old and senescent rats [2, 4, 6, 7]. In addition, we observed that the cAMP response to forskolin was not decreased, indicating that the decrement in receptor-stimulated cAMP accumulation with aging was probably not a result of impaired function of the catalytic unit of adenylate cyclase. β-Adrenoceptor density, for the most part, does not change with age in peripheral tissues [1]. The present study, using Scatchard analysis of [^{125}I]ICYP saturation binding in ASMC, also did not reveal differences in receptor densities among 6-, 18-, and 24-month-old F-344 rats. Others have reported similar findings in cultured mesenteric artery smooth muscle cells from F-344 rats [17] and particulate fractions of mesenteric artery from Sprague–Dawley rats [4]. Therefore, it is apparent that the age-induced deficit in β-adrenergic receptor-mediated cAMP production in ASMC, which appears to correlate with a decreased vasorelaxation potential in intact vessels, involves impairment of post-receptor signalling prior to adenylate cyclase stimulation. These findings permit speculation that the deficit involves receptor-G protein coupling and/or G protein–effector interaction [18].

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