Laboratory Studies

Epidermal Growth Factor Responsiveness in Smooth Muscle Cells From Hypertensive and Normotensive Rats

Timothy Scott-Burden, Thérèse J. Resink, Ursula Baur, Maria Bürgin, and Fritz R. Bühler

Aortic smooth muscle cells from spontaneously hypertensive rats (SHR) exhibit inappropriate proliferation characteristics in culture that suggest a modified response to serum mitogens or growth factors. The present study compares vascular smooth muscle cells from SHR and normotensive Wistar-Kyoto (WKY) rats with respect to their proliferative and functional response to growth factors. Specific attention was focused on the interaction of these vascular smooth muscle cells with epidermal growth factor. An increased growth rate of vascular smooth muscle cells from SHR (vs. WKY rats) was observed when cells were cultured in the presence of serum (10% and 0.5%), but not under serum-free conditions. The additional presence of low serum concentrations (0.5%) was required for epidermal growth factor to elicit a proliferative response, whereupon smooth muscle cells from SHR displayed an increased (vs. WKY rats) growth rate. Saturation binding of [125I]epidermal growth factor to intact smooth muscle cells indicated a twofold increase in receptor density in SHR-derived cells (p<0.001 vs. WKY rats) without an alteration in affinity for the growth factor. Cells derived from SHR also exhibited greater functional responsiveness to epidermal growth factor when compared with smooth muscle cells from WKY rats as evidenced by amplifications of both S6 kinase activation, phosphoinositide catabolism, elevation of intracellular pH, and DNA synthesis (nuclear labeling). We conclude that increased responsiveness of SHR-derived smooth muscle cells to epidermal growth factor could contribute to alterations in vascular smooth muscle growth and tone that may be fundamental to the pathogenesis of hypertension and atherosclerosis.

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The inappropriate in vivo proliferation of vascular smooth muscle cells has long been recognized as a characteristic of vascular pathology. Although the control of vascular smooth muscle cell growth in vivo is still unclear, a number of studies have indicated some of the factors that may influence proliferative behavior both positively and negatively. Furthermore, the study of vascular smooth muscle cells in culture, using cells from many different animal sources including humans, has contributed to our understanding of how proliferative responsiveness and its control may be mediated. The culture of vascular smooth muscle cells from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) normotensive rats has yielded a model for the study of pathological vascular proliferative responsiveness. Although there are some contradictions, the general finding has been that vascular smooth muscle cells from the thoracic aortas of SHR grow faster in culture than those from WKY rats. Because these studies have been performed under culture conditions where both cell types are exposed to 10% fetal calf serum, which contains several growth factors or mitogens, the differences in growth rates observed between SHR and WKY rat cell isolates possibly reflect differences in their responsiveness to one or a combination of such growth promoting substances.

In an attempt to elucidate the mechanism of this responsive difference, we made several isolates of aortic vascular smooth muscle cells from SHR and WKY rats and compared them with respect to both proliferative and functional response to growth factors. We report here our findings on the interaction of epidermal growth factor (EGF) with vascular smooth muscle cells from the two rat strains.
compound, described as a "progressive factor" for cells of mesenchymal origin, has been shown to interact with extracellular, matrix-associated macromolecules, which leads to the activation of protein kinase, which leads to the activation of protein synthesis and later initiation of deoxynucleotide acid (DNA) synthesis. More recently EGF has been found to both promote growth of rat vascular smooth muscle cells and induce contraction in helical strips from rat aortas. Our findings with vascular smooth muscle cells from SHR and WKY rats, showing that there are marked differences in the responsiveness to EGF, contribute to these overall findings and underline the important role of EGF in the pathophysiology of vasoactivity.

Materials and Methods

SHR and WKY rats (male, age 20 weeks, and weighing 200–250 g) were obtained from Madörin AG, Füllinsdorf, Switzerland. Blood pressures were measured (tail-cuff procedure) before decapitation, and typical values (mean ± SD, n = 6) obtained were 215 ± 6 mm Hg for SHR and 130 ± 5 mm Hg for WKY rats. The chemicals and media for tissue culture were obtained from Gibco AG, Basel, Switzerland with the exception of fetal calf serum, which was purchased from Pabko AG, Basel, Switzerland. Platelet-derived growth factor (PDGF) and EGF were supplied by Collaborative Research Inc., Bedford, Massachusetts. Somatomedin C, insulin-like growth factor (IGF), was obtained through Rahn und Co. (Amersham), Zurich, Switzerland as was all the radiotopes used in this study. These included myo-[2-3H]inositol (16–20 Ci/mmol), 3-[(125I)iodotyrosyl EGF (–10 μCi/μg), [γ-32P]adenosine triphosphate (ATP) (3,000 Ci/mmol), 5,5-dimethyl-[2-14C]oxazolidine-2,4-dione (50 mCi/mmol), [14C]urea (56 mCi/mmol), and [methyl-3H]thymidine (92 Ci/mmol). Other chemicals and reagents were purchased from E. Merck, Darmstadt, FRG or Sigma Chemical Co., St. Louis, Missouri. Lab.Tek eight-chamber slides were supplied through Bayer AG (Miles), Zürich, Switzerland.

Methods

Isolation and culture of smooth muscle cells. This study used vascular smooth muscle cell isolates made from eight separate pairs (SHR and WKY rat) of age-matched (20 weeks old) male rats. In total, 18 pairs of isolates were made, and for any given pair, the areas from which the tissue was obtained were carefully matched for length and location. Procedures were used as described previously by Jones et al and Chamley-Campbell et al with minor modifications. After decapitation of animals, thoracic or abdominal aorta vessel sections (from the ventricular origin to the branching of the renal arteries) were rapidly removed and washed in phosphate-buffered saline (4°C) that contained 200 units/ml of both penicillin and streptomycin. Once stripped of adventitia, matched lengths and zones (WKY rats vs. SHR) of vessels were taken and opened longitudinally before cutting into fine pieces for preparation of cell suspensions by enzymatic disaggregation. This was carried out by digestion of tissue pieces with elastase (0.05%, wt/vol) for 45 minutes at 37°C followed by collagenase (0.3%, wt/vol) for 3–4 hours at 37°C. Digestions were performed in minimal essential medium that contained Earles salts, 20 mM glutamine, 20 mM HEPES-NaOH, 20 mM HEPES-NaOH (both at pH 7.3), and 100 units/ml penicillin and 100 units/ml streptomycin as bacteriostatic agents; heat-inactivated fetal calf serum (10%) was present for collagenase digests. Just before centrifugation of cell suspensions obtained by enzymatic digestion, deoxynucleotide (500 units/ml) was added to reduce the viscosity of suspensions (due to DNA release). This latter procedure facilitated cell pelleting and resuspension. Cells were plated into tissue culture flasks (T35, 10⁴ cells/flask). Primary cultures were routinely passaged for use in experimental regimes, and phenotypic characterization was performed as described by Jones et al. For the present study, we used smooth muscle cells from the 3rd–14th passages, during which the investigated parameters remained steady. The large number of experiments performed necessitated use of different pairs of isolates at differing passage numbers, but for each single experiment the cells within any pair were used at a matched passage number. Cell cultures were grown and maintained in minimal essential medium supplemented as above and containing 10% fetal calf serum with routine medium changes every 3 days. To obtain quiescent nondividing cells, normal medium was substituted with minimal essential medium that contained 0.1% wt/vol bovine serum albumin (in place of 10% fetal calf serum), and all other ingredients were as described above. Experiments with cultures at quiescence were performed after 48 hours incubation with serum-free medium, and for some experimental procedures (nuclear labeling), serum-free medium was replaced once during this 48-hour period. Cell numbers were routinely obtained by counting aliquots of cell suspensions in Isoton with a Coulter Counter after enzymatic disaggregation of cell layers as described already.

Preparation of extracellular matrix–coated dishes. Tissue culture plasticware coated with extracellular matrix material were prepared as previously described. Briefly, vascular smooth muscle cells grown in the presence of sodium ascorbate (50 μg/ml, replenished daily) were lysed with 25 mM NH₄OH at the end of culture periods. Cell-free matrix material, which remains firmly associated with the plastic surface of culture dishes, was washed three times with phosphate-buffered solution and stored under phosphate-buffered solution.
until use. Before plating of fresh vascular smooth muscle cells on matrix substrata, phosphate-buffered solution was removed, and wells were washed once with complete medium. The same washing procedures were followed for control uncoated plasticware. Vascular smooth muscle cells used for matrix-related growth experiments were the same as those used for matrix preparation.

S6 kinase activation and phosphorylation assays. The activation of S6 kinase in quiescent cultures of SHR or WKY rat–derived cells was performed as described previously. For each activation experiment, cells were stimulated by addition of fetal calf serum (10%) as a positive control; cell numbers were also determined on at least three culture chambers. Cells were plated into six-well multiwell plates at 2×10^5 cells/well and grown to confluency in normal media. Thereafter, medium was replaced with serum-free media and appropriate additions as indicated in Results. After 48 hours, the latter culture medium was replaced with fresh serum-free media that contained growth factors or agonists (Results), and then cells were incubated at 37°C for the required periods. At the end of activation, incubation plates were washed with 3×2 ml aliquots of cold extraction buffer (20 mM HEPES, 15 mM MgCl2, 20 mM EGTA, 1 mM dithiothreitol [DTT], and 80 mM β-mercaptoethanol, pH 7.3). Cell layers were scraped into microcentrifuge tubes with 300 μl extraction buffer that contained 0.2% Triton X-100. Intracellular pH (pHi) was calculated after solubilization of cells in 0.2 NaOH/1.2 HCl for 15 minutes at 11,000 g at 5°C. Samples were stored at −70°C until required for S6 kinase phosphorylation assays. S6 kinase phosphorylation assays were performed with 40S ribosomal subunits as substrate as described previously (specific activity of [γ-^32P]ATP at ~2×10^6 dpm/μmol). Based on extractions of cell layers that have between 1.0 and 3.2×10^4 cells/well, assays were linear with respect to time and protein concentration and 40S subunits were used at saturation levels. Stimulation of cells with 10% fetal calf serum resulted in the incorporation of between 4 and 4.65 pmol PO4/10^4 cells into S6 polypeptide, and unstimulated cells (serum-free) exhibited values of 0.42–0.52 pmol PO4/10^4 cells. Neither of these values differed significantly between SHR-derived and WKY rat-derived vascular smooth muscle cells.

[^125]I[epidermal growth factor binding. Confluent, quiescent cultures were used for saturation binding studies that used radiolabeled EGF (0.035–10 ng/ml) and were performed (90 minutes at 4°C) exactly as described by Wasilenko et al., except that binding was performed in 0.5 ml of HEPES-buffered, serum-free medium made up of the components already described above. Nonspecific binding of[^125]I[EGF was determined by inclusion of excess (0.5 μg/ml) unlabeled growth factor to parallel wells and was always less than 10% of total cell-associated radioactivity. Binding parameters were obtained for each individual experiment by computerized weighted nonlinear curve-fitting analysis.24,25

Phosphoinositide metabolism. Confluent vascular smooth muscle cells were rendered quiescent and inositol lipids prelabeled to equilibrium by incubation in serum-free medium that contained myo-[2-^3H]inositol (5 μCi/ml) for 48 hours. Thereafter, cells were washed three times with phosphate-buffered saline solution, 1 ml of isotonic phosphate-buffered saline solution that contained 50 mM LiCl was added, and preincubation was carried out for 30 minutes at 37°C. After preincubation, cells were exposed to EGF (2 minutes at 37°C) at levels and times shown in the relevant figures (see Results). Incubation was terminated by rapid aspiration of buffer and addition of 1 ml chloroform/methanol/HCl (1:2:0.05, vol/vol/vol).26 Dishes were maintained at 4°C for 30 minutes before collection of extracts plus a 500 μl rinse. After phase separation,26 incorporation of[^3H]myo-inositol into inositol phosphates and phosphoinositides (after deacylation) was quantitated by liquid scintillation spectrophotometry subsequent to chromatographic resolution on Dowex 1-X4 ion exchange columns.27,28

Measurement of intracellular pH. Intracellular pH was measured with the weak acid 5,5-dimethyl-[2-^14C]oxazolidine-2,4-dione (DMO) using procedures exactly as described by Mendoza and Rozengurt.29 The electrolyte solution contained (mM): NaCl 50, KCl 5, CaCl2 1.8, MgCl2 0.9, glucose 25, choline chloride 90, and HEPES-Tris 30 (pH 7.2). Confluent, quiescent vascular smooth muscle cells were exposed to EGF (5 ng/ml) or fetal calf serum (10%) for 45 minutes at 37°C before addition of[^14C]DMO (final concentration 150 μM, 1.5–2.0×10^6 dpm/well). Values for “trapped” extracellular[^14C]DMO were routinely subtracted from the experimental points. Intracellular volumes (μl/mg protein) were determined with[^14C]urea and were 7.19±1.06 (n=4) and 7.52±0.67 (n=4) for cells derived from SHR and WKY rats, respectively. Protein concentrations were measured after solubilization of cells in 0.2 NaOH/0.2% Triton X-100. Intracellular pH (pHi) was calculated by using the formula of Waddell and Butler.31

Mitogenesis assays and nuclear labeling. These were performed on cultures plated into Lab.Tek eight-chamber slides that had previously been washed with 1% gelatine to promote cell attachment to glass.31 Sparsely confluent cultures were maintained at 37°C on serum-free medium for 48 hours with the replacement of such medium once during this period. Cells were exposed to EGF at levels described under the relevant figure (see Results) for 24 hours at 37°C in the presence of 1 μCi/ml [methyl-^3H]thymidine exactly as described previously.10 After fixation and drying, cells were coated with nuclear emulsion (Kodak NTB2, Rochester, New York), exposed, and then developed. Slides were stained with Mayer’s hemalum solution to facilitate the observation of total nuclei and the
number of radiolabeled (black) nuclei scored as a percentage of the total nuclei for randomly selected microscopic fields (a minimum of 150 nuclei were counted per well).

**Statistical analysis.** Unless otherwise stated, all values in the text are given as mean±SD, where n is the number of separate experiments. Statistical analysis was performed by using Student's t test for unpaired data.

**Results**

The growth rates of vascular smooth muscle cells isolated from the thoracic aortas of SHR were faster than those isolated from matched-vessel regions of WKY rats. The data in Figure 1 is representative of a single paired (SHR vs. WKY rat) isolation but such differences were routinely found for all matched isolates. Typically, the initial growth rates of vascular smooth muscle cells from both rat sources were slower than rates at later times after plating (Figure 1), but the proliferation rate of SHR-derived cells was faster throughout all culture periods relative to that of WKY rat-derived cells. The initial doubling time (in the presence of 10% fetal calf serum) for SHR-derived cells was between 24 and 32 hours, whereas for WKY rat-derived cells it was between 48 and 70 hours even when the latter were plated at higher densities (10^5 cells/well vs. 7.5x10^4 cells/well for SHR) to compensate for their lower plating efficiency (data not shown). Vascular smooth muscle cells from both rat sources could be maintained on serum-free medium for several days with no apparent loss in cell number (Figure 1). Low levels (0.5%) of serum stimulated the growth of SHR-derived cells to a greater extent than those derived from WKY rats (Figure 1). Also, plating of cells on extracellular matrix-coated plasticware (see Materials and Methods), which resulted in a marked stimulation in proliferation of cells from the two rat sources, did not result in any diminution in the differential rates of growth observed for SHR versus WKY rat vascular smooth muscle cells (Figure 7). Cells from the former still grew significantly (p<0.01) faster than cells from normotensive rats on such substrata (see Discussion). Such data is in accord with findings of other laboratories and prompted our further study on factors present in serum that could be responsible for the different proliferative responsiveness of vascular smooth muscle cells from the two sources.

It has been shown that S6 kinase activation is an integral part of the process leading to transition of quiescent cells to the proliferative state. Therefore, using an assay system that permits quantitation of the in situ extent of S6 kinase activation, quiescent vascular smooth muscle cells from SHR and WKY rats were screened for their ability to respond to stimulation by IGF1, EGF, and PDGF. Vascular smooth muscle cells from SHR and WKY rats exhibited differential S6 kinase activation responses to all the growth factors (each at 5 ng/ml) mentioned. In particular, however, SHR-derived cells exhibited a significantly greater degree of S6 kinase activation in response to EGF (5 ng/ml) as compared with that exhibited by WKY rat-derived VSMC at the fourth-, sixth-, and eighth-day culture periods shown. Data points represent mean±SD and were obtained from three separately performed growth experiments in each of which cell number determinations were performed in quadruplicate. All our paired isolates (n=18) exhibited essentially the same differences illustrated here.
Increased Vascular Responsiveness to EGF in Hypertension

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FIGURE 2. Graphs showing activation of S6 kinase platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), or epidermal growth factor (EGF), all at 5 ng/ml (panel A), and by EGF (panel B) at levels shown in the figure. Activation of S6 kinase was performed as described under Materials and Methods on quiescent vascular smooth muscle cells (VSMC). The data in panel A (mean±SD) was obtained from duplicate experiments on four separately paired VSMC isolates from spontaneously hypertensive rats (SHR) (hatched bars) and Wistar-Kyoto (WKY) rats (open bars), whereas the data in panel B (mean±SD) represent data from experiments repeated three times on one such pair (●, SHR; ○, WKY rat) and was typical of such isolates. *p<0.01 and **p<0.001 indicate significant differences between SHR and WKY rats.

Such data could be accounted for either by increased EGF receptor density on SHR-derived vascular smooth muscle cells or by such receptors having an increased affinity for the growth factor. We tested these possibilities by performing radiolabeled ligand binding assays on intact vascular smooth muscle cells, and the results are presented in Figure 4. Scatchard analysis of data obtained from [125I]EGF saturation binding profiles for SHR-derived and WKY rat-derived vascular smooth muscle cells revealed single-receptor populations of comparable affinity (Kd [nM] 0.80±0.02 and 0.86±0.04, respectively). However, the maximum number of specific EGF receptors on vascular smooth muscle cells from SHR (3,824±146 sites/cell) was nearly twice (p<0.01) that on vascular smooth muscle cells from WKY rats (2,120±94 sites/cell).

The interaction of growth factors with their membrane receptors can result not only in the activation of cellular kinases but also in a stimulated catabolic phosphoinositides response as well as an elevation of intracellular pH. We compared the metabolism of [3H]inositol prelabeled phosphoinositides in response to EGF in vascular smooth muscle cells from the two rat strains (Figure 5). EGF induced a time-dependent (data not shown) activation of the phosphoinositide metabolic cascade (i.e., accumulation of inositol phosphates and breakdown of phosphatidylinositols). To compare the efficacy of EGF in promoting this response in vascular smooth muscle cells from SHR and WKY rats, a 2-minute exposure period was selected as this was found to be optimal for quantitation of changes in both phospholipids and inositol phosphates. SHR-derived cells exhibited an enhanced phosphoinositide breakdown response after the 2-minute exposure to EGF as evidenced by their elevated accumulated levels (~threefold greater than WKY rats at saturating EGF concentrations) of triphosphorylated, diphosphorylated, and monophosphorylated inositol phosphates (InsP3, InsP2, and InsP, respectively) (Figure 5). This amplified phospho-
The alkalinization response of both cell types to EGF from SHR was significantly greater than for WKY rat-derived vascular smooth muscle cells (Table 2). The intracellular lar pH (A pHj) for vascular smooth muscle cells more alkaline. However, the increase in intracellular pH was not different between quiescent vascular smooth muscle cells from SHR and WKY rats (Figure 5), which indicated an absence of phospholipase A2-mediated phosphatidylinositol breakdown (data not shown). Glycerol phosphoinositol (Gro-PIns) did not accumulate in response to EGF for vascular smooth muscle cells from either origin (Figure 5), which exhibited evidence for elevated pressure. 10, and this finding was substantiated by our nuclear labeling experiments (inset Figure 6). Nevertheless, the mitogenic response of SHR-derived vascular smooth muscle cells to EGF was markedly greater than their normotensive counterpart (inset Figure 6). However, when vascular smooth muscle cells from SHR and WKY rats were cultured in the combined presence of low levels of serum (0.5%) and EGF, their rates of proliferation increased, which in the case of SHR approached that found in the presence of 10% fetal calf serum (compare Figures 1 and 6).

Discussion

The response of vascular smooth muscle cells in culture to growth factors and mitogens is multifaceted.11 Several groups have shown that the numerous extracellular and intracellular events elicited by such compounds can interact in a bewildering number of combinations. We have shown that clear differences in EGF-receptor densities between SHR- and WKY rat-derived vascular smooth muscle cells may be associated with differences both in proliferation rates and intracellular metabolic responses. We do not know if the differences found in our cultured cells truly reflect the in vivo situation as our studies were performed on passaged cells from adult rats. Furthermore, the changes we have observed may be of a secondary nature as all of our vascular smooth muscle cell sources (SHR) already exhibited clear evidence for elevated pressure.

A variety of mitogens have been shown to activate the turnover of phosphoinositides in different cellular systems.32,35 The resultant generation from phosphatidyl (Ptd)-InsP3 of diacylglycerol (which activates protein kinase C, a cellular target of tumor-promoting phorbol diesters) and InsP3 (which releases Ca2+ to facilitate cell cycle progression) together with observations of synergism between peptide mitogens and phorbol diesters36 have led to the proposal that degradation of phosphoinositides may be a primary step whereby peptide mitogens or growth factors stimulate mitogenesis.32,35 In this study, the degradation of phosphoinositides and concurrent elevation of inositol phosphates evidence the ability of EGF to activate the turnover of and fetal calf serum was completely abolished in the presence of Na+/H' antiporter inhibitors such as amiloride (0.5 mM) and its analogues dimethylamiloride (10 μM) or ethylisopropylamiloride (1 μM) (data not shown).

To assess whether the observed differences (SHR vs. WKY rats) in EGF-receptor binding, EGF-stimulated phosphoinositide catabolism, and EGF-induced alkalinization might be relevant to the differential proliferation rates of these vascular smooth muscle cells, we tested the ability of EGF to stimulate growth of cells maintained on serum-free medium. The data we obtained from growth kinetics (Figure 6) supported the findings of other groups that EGF alone is only mildly mitogenic for vascular smooth muscle cells,10 and this finding was substantiated by our nuclear labeling experiments (inset Figure 6). The Scatchard plot inset was obtained by transformation of saturation binding data from one representative matched pair (●, SHR vs. ○, WKY rat). The x axis indicates femtomole EGF specifically bound per 10⁶ cells. Computed maximal bound (Bmax) values (mean±SD) obtained for SHR and WKY rats were 6.35±0.36 and 3.52±0.23 fmol/10⁶ cells, respectively (p<0.01).
phosphoinositides. However, although our observations agree with those for A-431 cells, a lack of activation by EGF was demonstrated for Swiss mouse 3T3 cells and BALB/c 3T3 fibroblasts. Since EGF is strongly mitogenic for the latter two cell types but only mildly mitogenic for vascular smooth muscle cells (this study and Reference 10), a dissociation of mitogenic capability from the capacity to stimulate phosphoinositide degradation is indicated.

Our observation of intracellular alkalinization after exposure of quiescent vascular smooth muscle cells to EGF (or fetal calf serum) is in accord with typical cellular responses to growth factors or mitogens. Blockade of this response by amiloride and amiloride analogues indicates that the elevation of intracellular pH occurred via activation of \( \text{Na}^+ / \text{H}^+ \) exchange. Since the alkalinization response was greater for SHR-derived vascular smooth muscle cells than for those from WKY rats, increased growth factor-stimulated \( \text{Na}^+ / \text{H}^+ \) exchange in the former is implied. Such an aberration in intracellular pH may contribute to the acquisition of altered growth properties and contractile tone and might also have important consequences in the signal transduction for agonist-stimulated noncontractile events. The intracellular pH values obtained herein are lower than those reported by Berk et al for vascular smooth muscle cells. This

### Table 1. Epidermal Growth Factor-Stimulated Hydrolysis of Phosphoinositides in Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>PtdIns</th>
<th>PtdInsP</th>
<th>PtdInsP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (( n=4 ))</td>
<td>94.6±3.5</td>
<td>81.6±3.7</td>
<td>86.4±6.4</td>
</tr>
<tr>
<td>SHR (( n=4 ))</td>
<td>89.4±7.9</td>
<td>71.0±6.0*</td>
<td>72.3±2.0*</td>
</tr>
</tbody>
</table>

Tritiated myo-inositol prelabeled quiescent vascular smooth muscle cells were incubated without or with EGF (5 ng/ml) for 2 minutes at 37°C. Phospholipids were extracted and tritium content of phosphatidylinositol (PtdIns), phosphatidylinositol-phosphate (PtdInsP), and phosphatidylinositolbisphosphate (PtdInsP2) determined after decylation and separation by ion-exchange chromatography. The deacylated derivatives of phosphoinositides have been referred to by their parent names in the table. Experimental details are described in Materials and Methods. Values express tritium content (% of unstimulated) for each phosphoinositide relative to their respective unstimulated levels (100%).

*indicates significant difference (\( p<0.05 \)) between spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.

### Table 2. Epidermal Growth Factor-Stimulated Alkalinization in Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Control ( \Delta \text{pH}_i )</th>
<th>10% FCS ( \Delta \text{pH}_i )</th>
<th>5 ng/ml EGF ( \Delta \text{pH}_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>6.69±0.21</td>
<td>0.20±0.08</td>
<td>0.14±0.06</td>
</tr>
<tr>
<td>SHR</td>
<td>6.61±0.11</td>
<td>7.01±0.17*</td>
<td>6.92±0.10*</td>
</tr>
</tbody>
</table>

Intracellular pH was measured in quiescent (control) vascular smooth muscle cells and after exposure of quiescent cells to 10% fetal calf serum (FCS) or 5 ng/ml epidermal growth factor (EGF) as detailed under Materials and Methods. *\( p<0.01 \) significant difference between vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. †Compares actual intracellular pH values. ‡Compares changes in intracellular pH (\( \Delta \text{pH}_i \)) after stimulation. Values are given as mean±SD and were obtained from triplicate determinations on five separately matched (SHR and WKY) vascular smooth muscle cell isolates.
Another component proposed to be involved in the mechanism of stimulation of mitogenesis, and one that is dependent on alkalinization, is that of S6 kinase activation, which induces protein synthesis and a subsequent initiation of DNA synthesis.\textsuperscript{17,18,32} In the present study, the potent activation of S6 kinase by EGF was also contrary to the weak EGF-induced vascular smooth muscle cell mitogenesis. Coupled with our finding that exposure to EGF alone under serum-free conditions could not stimulate cell division and proliferation in vascular smooth muscle cells from WKY rats or SHR even in the face of amplified alkalinization, S6 kinase, and phosphoinositide turnover activation responses in SHR-derived vascular smooth muscle cells, such dissociative observations serve to remind us that a wide combination of events must occur to trigger proliferation.\textsuperscript{11,32} Thus, although elevation of intracellular pH, activation of S6 kinase, and stimulation of phosphoinositide turnover may well predispose cells toward the proliferative state, further actions or interactions are required before such cells undergo division.

The recent observations relating to the autocrine role of the extracellular glycoprotein thrombospondin\textsuperscript{10} make our findings more pertinent as the cooperative effects of thrombospondin and EGF

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The references are not provided in the given text.
result in a marked mitogenic response of cells exposed to both compounds. Furthermore, the levels of thrombospondin secreted in response to low levels of PDGF and its association with the extra-cellular matrix are subject to interactions with heparin and heparin-like compounds.9,10 Therefore, the differences in proliferative responsiveness of SHR-derived versus WKY rat-derived cells may arise from such matrix EGF or PDGF-coordinated events as opposed to simple changes in receptor density or postreceptor transduction processes. The fact that we consistently observed an increased rate of proliferation for both cell types (SHR and WKY rat) 4–5 days after plating (Figure 1), and at times when onset of full matrix production occurred as reported previously,8 suggested that the two events may be related. When cells were plated onto extracellular matrix-coated plasticware (Materials and Methods), they exhibited more rapid rates of proliferation as compared with those plated on the normal plastic substrata (Figure 7). This data lends support to the concept of a cell-matrix-component-interaction involvement in cellular responses such as proliferation.10,41

Moreover, EGF also stimulated a rapid increase in free cytosolic calcium concentrations in the human fibroblasts.8,42 Therefore, since we have shown that EGF clearly activates the Na+/H+ antiporter and phosphoinositide metabolic cascade in vascular smooth muscle cells, both of which can lead to elevation of intracellular calcium, it is possible that one consequence of an increased responsiveness to EGF in SHR-derived vascular smooth muscle cells is a latent enhanced contractility of such cells. Such an interpretation would be in agreement with the observations of increased basal tone and maximal degree of stimulated vasoconstriction in vascular tissue preparations from SHR as compared with WKY rats.

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