Mechanism of the Enhanced Epidermal Growth Factor–Induced Growth Response of Genetically Hypertensive Vascular Myocytes

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Although enhanced growth of the vascular myocyte is believed to play a role in hypertensive cardiovascular disease, the cellular basis of altered growth regulation is not completely understood. The present study demonstrates that in the presence of 10% fetal calf serum, the logarithmic growth rate of cultured mesenteric artery myocytes of the spontaneously hypertensive rat (SHR) is similar to that of the normotensive Wistar-Kyoto (WKY) control rat. However, in the presence of low levels of fetal calf serum, SHR myocytes respond to epidermal growth factor (EGF) with increased growth, whereas WKY cells do not. This difference does not result from different numbers or affinities of EGF receptors in these cell lines. Examination of EGF-induced growth responses of SHR and WKY myocytes in the presence of varying levels of insulin or fetal calf serum indicates that, compared with WKY myocytes, SHR myocytes have a lower requirement for factors that confer competence to respond to EGF. Another property of the SHR myocytes is an elevation of free intracellular Ca^{2+}. To determine whether a difference in cellular Ca^{2+} metabolism might play a role in the differential growth response, growth of myocytes in medium containing 0.25, 0.75, or 1.25 mM extracellular Ca^{2+} and 5% fetal calf serum was examined. Myocytes of SHR showed enhanced growth in the presence of 5% fetal calf serum at all levels of extracellular Ca^{2+}. It is concluded that, although vascular myocytes of SHR and WKY rats have the capacity to grow at similar rates, under limiting conditions, the SHR myocyte growth response is enhanced. It is proposed that basal activation of a second messenger pathway, possibly Ca^{2+} related, underlies the apparent differential EGF response. (Circulation Research 1991;69:757–764)

Hypertension of unexplained origin is characterized by elevated peripheral resistance to blood flow, and one contributing factor to the elevated resistance is a narrowing of the arterial lumen. At the present time, the cellular events responsible for this architectural change are not well understood, although it is recognized that encroachment of the lumen can occur as a result of either medial hypertrophy, resulting from changes in vascular myocyte size or number, or remodeling of the vessel wall, which can occur independent of alterations in vascular myocyte growth.

Evidence obtained using in vivo animal models suggests that both hypertrophic and hyperplastic vascular myocyte growth occur in the arterial tree during hypertension. Furthermore, it has been proposed that alterations in vascular myocyte growth regulation may be intrinsically related to the hypertensive process. Supportive evidence comes primarily from studies that have examined growth characteristics of cultured aortic myocytes isolated from hypertensive and normotensive rats.

In the early 1980s, Yamori and colleagues demonstrated that vascular myocytes cultured from aortas of a genetic model of human essential hypertension, the spontaneously hypertensive rat (SHR), grow faster than myocytes isolated from the normotensive Wistar-Kyoto (WKY) control rat. These results have been confirmed by a number of laboratories, and the results have widely been interpreted to indicate that SHR myocytes have an intrinsic capacity to grow faster in culture than WKY myocytes. Furthermore, it has been postulated that the accelerated growth rate may account for or contribute to the arterial wall thickening that occurs during the development of hypertension. Given the potential relevance of these findings to this major human disease
entity, it is important that the mechanism of differential growth rate be delineated.

Recent reports indicate that aortic smooth muscle cells of the SHR have enhanced growth responses to peptide growth factors including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). It has been suggested that the differential responses are the result of either altered EGF receptor number, a derangement of second messenger pathways responsible for translating the growth factor signal into a cellular event, or a failure of growing SHR cells to be inhibited by cell-to-cell contact. To assess this problem further, we have examined the growth response of SHR and WKY mesenteric artery myocytes to EGF in the presence of conditions that confer different degrees of second messenger activity. The results show that cultured mesenteric artery myocytes of SHR and WKY rats respond differentially to varied growth conditions and are consistent with the hypothesis that a difference in second messenger signaling is responsible for the differential growth responses.

Materials and Methods

Cell Culture

Mesenteric artery myocytes were enzymatically dispersed from superior mesenteric artery segments taken from 12-week-old SHR and WKY rats obtained from Charles River, Wilmington, Mass., using recently described methods. Mesenteric arteries from six rats were pooled for each preparation, and two such preparations from each strain were used. The dispersed cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Grand Island, N.Y.) containing 10% fetal calf serum (Hyclone Laboratories Inc., Logan, Utah) and passed after achieving 70–80% confluence through five successive generations. After the fifth generation, the cells were frozen at a density of 2–3 × 10^6/ml in DMEM containing 20% fetal calf serum and 10% culture grade dimethyl sulfoxide (GIBCO) in liquid nitrogen and stored frozen. Before use in the experiments described below, the cells were rapidly thawed at 37°C and grown through one passage in T-75 culture flasks (Nalgene).

Although it is recognized that subcultured vascular smooth muscle cells may not faithfully represent the in vivo situation, passage 5–6 myocytes were used in this study. Cells at this passage were chosen because previous experiments in our laboratory indicated that at this time SHR cells grow faster and have elevated levels of free intracellular Ca^{2+} compared with WKY cells. In addition, the cells that were used in these experiments grow in the hill-and-valley morphology that is typical of vascular myocytes in culture and respond to angiotensin II with a rapid and transient mobilization of intracellular Ca^{2+}. These cells also stain positively, albeit to a lesser extent than freshly isolated tissue, to an antibody that specifically binds smooth muscle α-actin (Sigma Chemical Co., St. Louis, Mo.). These properties are typical of vascular myocytes in culture and support a vascular origin for the cells that were used in these studies.

Determination of Growth Rate

Cells were plated in DMEM plus 10% fetal bovine serum at densities of either 20,000 cells/well in 12-well plates or at 40,000 cells/35-mm dish and allowed to attach for 24 hours. Growth was then arrested by incubation in a serum-free medium (DMEM plus 0.1% bovine serum albumin) for a 48-hour period. The indicated additions were made, and cell number was determined at selected times thereafter by detaching the cells with a versene/trypsin solution (GIBCO) and counting them using a hemocytometer chamber or an automated cell counter (Coulter Electronics, Hialeah, Fla.).

When the effect of varying levels of extracellular Ca^{2+} on growth of myocytes was determined, a defined Ca^{2+} medium (DCM) was used. DCM consists of minimum essential medium (GIBCO) supplemented with amino acids, vitamins, sodium pyruvate, and NaHCO_3 to levels equivalent to those in DMEM.

Fetal calf serum was added (5%), and the amount of CaCl_2 needed to achieve final free Ca^{2+} concentrations of 0.25, 0.75, and 1.25 mM was empirically determined using an ionized Ca^{2+} electrode (Radiometer, Copenhagen). DCM was added to growth-arrested cells, and the cell number was determined after a 72-hour period.

Determination of EGF Receptors

Competitive [^{125}I]EGF binding was determined in cells plated at 40,000–60,000 cells/well in 12-well plates. After a 24-hour attachment period in DMEM plus 10% fetal calf serum, DMEM plus 0.1% bovine serum albumin was added for a 48-hour period. The cells were then washed two times with ice-cold DMEM plus 0.1% bovine serum albumin and exposed to 0.01–6 ng/ml [^{125}I]labeled EGF (100 μCi/μg, Amersham Corp., Arlington Heights, Ill.) at 4°C. Nonspecific binding was assessed by the addition of 1,000-fold excess unlabeled EGF. After a 90-minute period, the cells were washed four times with ice-cold HEPES-buffered salt solution and then lysed by the addition of 1N NaOH. Radioactivity was then determined using gamma counting techniques. Duplicate wells were run for each level of EGF, and duplicate plates that did not receive radioactivity were used to estimate cell number. Competitive binding curves were analyzed using the LINPLOT software package.

Determination of Intracellular Ca^{2+}

Free intracellular Ca^{2+} was determined using dual excitation wavelength spectrofluorometric measurement of myocytes loaded with fura 2 as recently described by this laboratory. Intracellular Ca^{2+} was examined in the presence of HEPES-buffered salt solution in cells that were either maintained in 10% fetal calf serum until immediately before use or in cells that were serum-deprived for a 24-hour period.
Figure 1. Graph showing 7-day growth rate of mesenteric artery myocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Myocytes were plated in 35-mm dishes at a density of 40,000 cells/plate, and at the indicated times, the cells were detached using a versene/trypsin mixture and counted using either a hemocytometer chamber or an automated cell counter. *Significant difference in cell number attained between the two strains at \( p < 0.05 \).

Absolute values of Ca\(^{2+}\) were estimated using constants for maximal (\( R_{\text{max}}^\prime \)) and minimal (\( R_{\text{min}}^\prime \)) fluorescence ratios (340:380 nm) that were determined for each preparation as previously described.\(^{15} \)

Data Analysis

Results are presented as the mean±SEM of the indicated number of observations. Observations were made on myocytes derived from at least six different vials of frozen cells, and each observation was treated statistically as a separate \( n \) value. Differences between strains or effects of growth factors were assessed using either one- or two-way analysis of variance (ANOVA), and a repeated-measures design was used when appropriate (SYSTAT software, Evanston, Ill.). In all instances, a value of \( p < 0.05 \) was assumed to indicate a statistical difference.

Results

Figure 1 illustrates the rate of growth of SHR and WKY myocytes maintained in DMEM plus 10% fetal calf serum over a 7-day period after exposure to serum-free medium. Two specific points are noteworthy. The first is that the rate of growth during the log phase was not different between cells of the two strains, as evidenced by the slopes of the growth curves between days 3 and 7. The second is that the total number of cells achieved in the SHR culture was significantly greater than the total in the WKY culture. The most likely explanation for the latter observation is either a difference in the initial attachment rate or a delay of the WKY myocytes in entering the cell cycle.

In contrast to these results, which suggest that SHR and WKY myocytes have the capacity to grow at similar rates, when the growth response of SHR and WKY cells to varying levels of EGF was examined in the presence of 2% fetal calf serum, cells of SHR responded with an increase in growth over a wide concentration range, whereas WKY myocytes were essentially unresponsive (Figure 2).

To determine whether the differential growth response to EGF is the result of a difference in EGF receptor number or affinity, competitive \([\text{\textsuperscript{125I}}]\)EGF binding was examined (Figure 3). No difference in either the number of \([\text{\textsuperscript{125I}}]\)EGF binding sites (\( B_{\text{max}}^\prime \)) for SHR, 6.27±1.82 pg/10\(^6\) cells; for WKY, 4.38±1.03 pg/10\(^6\) cells; \( n = 5 \) and 6) or affinity for EGF (\( K_d^\prime \); for SHR, 0.72±0.31 nM\(^{-1}\); for WKY, 0.40±0.05 nM\(^{-1}\)) was detected between cells of the two strains, sug-

Figure 2. Graph showing effect of epidermal growth factor (EGF) on vascular myocyte growth. BSA, bovine serum albumin; FCS, fetal calf serum; SHR, spontaneously hypertensive rats; WKY, normotensive Wistar-Kyoto rats. Cells were plated at 40,000 cells/35-mm dish in Dulbecco’s modified Eagle’s medium plus 10% FCS and then growth-arrested for 48 hours in Dulbecco’s modified Eagle’s medium with 0.1% BSA. Addition of 0.5% FCS with the indicated concentrations of EGF (ng/ml) were made on day 0, and then the cells were counted at days 1, 3, and 5. Significant responses to EGF were detected beginning at day 3 in SHR. Values are mean±SEM.
suggesting that a postreceptor mechanism might be responsible for the differential growth response to EGF. Therefore, protocols were developed to assess the growth response of SHR and WKY myocytes to EGF in the presence of varying levels of second messenger pathway activity, which was induced by adding varying levels of fetal calf serum or insulin. Initial experiments examined the 4-day growth rate of cells in the presence of varying concentrations of fetal calf serum. At lower concentrations of serum, no differences in growth of cells of the SHR and WKY rats were observed (Figure 4). At levels of serum >7.5%, however, the growth rate of SHR myocytes was greater than that of WKY myocytes. When the effect of varying levels of fetal calf serum on the growth response to 10 ng/ml EGF was examined, SHR myocytes gave a pronounced response at low levels of serum (i.e., 0.5%), whereas WKY myocytes showed a more gradual increase in growth response, approaching that of the SHR at a concentration of 15% (Figure 5).

The growth response of SHR and WKY myocytes to EGF was also examined in the presence of varying concentrations of insulin. The repeated-measures

**Figure 3.** Graph showing that specific ¹²⁵I-labeled epidermal growth factor (EGF) binding to myocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) was determined at 4°C in separate preparations of mesenteric artery myocytes (n=5 and 6). All values are mean±SEM. No significant differences in binding were detected using repeated measures analysis of variance. Please see text for $B_{\text{max}}$ and $K_d$ values.

**Figure 4.** Graph showing growth of myocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) in fetal calf serum. The indicated level of fetal calf serum was added to growth-arrested myocytes, and cell number was determined after a 4-day period. *Significant difference between SHR and WKY cells at p<0.05. Values are mean±SEM; n=6.
ANOVA indicated that insulin alone over the concentration range of 0.03–3 μg/ml had no detectable effect on growth of cells from either strain (Figure 6), although the number of SHR myocytes was significantly greater at 0.3–3 μg/ml insulin (Figure 6). When the effect of varying levels of insulin on growth induced by 10 ng/ml EGF was examined, a differential response of the two strains was observed. SHR myocytes responded to EGF at lower concentrations of insulin, reaching a maximal level at 0.1 μg/ml (Figure 7), whereas WKY myocytes achieved a maximal growth response to EGF in the presence of 1 μg/ml insulin, which was greater than that of SHR myocytes. It should also be noted that SHR myocytes responded to EGF with a significant growth response in the absence of other added factors, suggesting that under the conditions of our experiment, this peptide is a mitogen for the SHR vascular myocyte.

When viewed collectively, these results indicate that SHR myocytes have a higher sensitivity to or lesser requirement for fetal calf serum or insulin to induce a state of "competence" to respond to EGF. On the chance that this differential requirement is the result of a general activation of second messenger pathways resulting from the previously reported elevation of intracellular Ca²⁺ in these cells, we assessed the level of free intracellular Ca²⁺ in the two preparations and determined the effect of alterations in the levels of ionized extracellular Ca²⁺ on growth.

Table 1 shows the basal levels of free intracellular Ca²⁺ in cells of the two strains. It was observed that free intracellular Ca²⁺ is elevated in SHR myocytes regardless of whether they were examined within minutes of removing fetal calf serum or were serum-deprived for 24 hours. These results suggest that the elevated level of Ca²⁺ is an intrinsic property of the SHR myocyte and not an acute response to growth factors that are present in fetal calf serum. When the effect on growth of varying levels of ionized extracellular Ca²⁺ was examined, it was observed that growth was suppressed in the presence of the low extracellular Ca²⁺ (0.25 mM) in cells of both strains and that growth of SHR myocytes was greater at all levels of Ca²⁺ that were examined (Figure 8).

**Discussion**

It has long been recognized that the arterial wall becomes thickened in the hypertensive subject, and it has been proposed that this architectural change plays a role in maintaining elevated peripheral resistance. In addition, atherosclerotic lesions are characterized by the rapid proliferation of vascular smooth muscle cells in the intima of affected blood...
vessels. A defect in vascular smooth muscle growth regulation has been proposed as a link between these vascular disorders, since hypertension is a major risk factor for atherosclerosis. Thus, there may be a cellular mechanism that is common to the vascular pathology of these two diseases.

It has been established that smooth muscle of the large arteries undergoes hypertrophic growth characterized by DNA synthesis in the absence of karyokinesis and cytokinesis, resulting in a heterogeneous diploid and polyploid myocyte population. Although the events that occur in the small resistance arteries are not as well understood, it is generally assumed that hyperplastic growth takes place. Several groups have used the cultured vascular smooth muscle system to identify differential patterns of growth regulation in the SHR compared with the WKY rat. A common observation from our laboratory as well as others has been that cultured SHR myocytes grow faster than WKY myocytes in the presence of 10% fetal calf serum or growth factors such as PDGF and EGF.

The present studies were designed to examine further the mechanism underlying the enhanced growth response of SHR myocytes to EGF. EGF is a single-chain, anionic, 6,100 molecular weight peptide that binds to a specific receptor that is structurally similar to the V-erb-B oncogene product. EGF was chosen for study for several reasons: 1) This peptide is a potent growth promoter for SHR myocytes, 2) EGF may be of physiological relevance, since it has been detected in human urine and at low levels in human plasma, is of platelet origin, and has distinct vascular actions. 3) EGF-like domains that are capable of inducing cell growth have been described in the extracellular matrix protein laminin. The latter observation, coupled with the recent demonstration that EGF can promote growth in NIH 3T3-derived NR6 cells without being internalized, provides a route whereby an extracellular matrix component might play a role in growth regulation of vascular smooth muscle through an interaction at the EGF receptor.

The most important finding of this study is the apparent differential responsiveness of SHR and WKY myocytes. SHR myocytes, in contrast to WKY myocytes, grow at a more rapid rate than WKY myocytes over a range of insulin concentrations. This finding is in keeping with the observation that SHR in vitro respond to a variety of factors at a more rapid rate than WKY. A possible explanation for this finding is that SHR might respond to insulin at a lower concentration than WKY. However, this does not appear to be the case, since insulin at a concentration of 0.03 μg/ml produces a similar growth response in SHR and WKY myocytes. Furthermore, the concentration of insulin that produces a half-maximal response is essentially the same in SHR and WKY myocytes. This finding suggests that the differential responsiveness of SHR myocytes to insulin may be due to a difference in the number of insulin receptors, which is consistent with our demonstration that SHR myocytes have more insulin receptors than WKY myocytes.

FIGURE 7. Graph showing effect of varying levels of insulin on the growth response of myocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) to 10 ng/ml epidermal growth factor (EGF). The indicated amount of insulin was added with 10 ng/ml EGF to growth-arrested myocytes, and cell number was determined after a 4-day period. *Significant difference between responses of SHR and WKY cells at p<0.05. Values are mean±SEM; n=6.

FIGURE 8. Bar graph showing effect of extracellular Ca²⁺ on growth of myocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) in 5% fetal calf serum. Defined Ca²⁺ medium was added to growth-arrested cells, and cell number was determined after a 72-hour period. Values are mean±SEM; n=6. *Significant difference between 0.25 and 0.75 mM at p<0.05. At all levels of extracellular Ca²⁺, values of SHR were greater than those of WKY at p<0.05.
TABLE 1. Basal Level of Free Intracellular Ca²⁺

<table>
<thead>
<tr>
<th>Condition</th>
<th>SHR Mean±SEM</th>
<th>SHR n</th>
<th>WKY Mean±SEM</th>
<th>WKY n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum free</td>
<td>109±3.5*</td>
<td>8</td>
<td>88±8.1</td>
<td>7</td>
</tr>
<tr>
<td>2% FBS</td>
<td>103±5.0*</td>
<td>6</td>
<td>75±5.0</td>
<td>5</td>
</tr>
</tbody>
</table>

SHR, spontaneously hypertensive rats; WKY, normotensive Wistar-Kyoto rats; n, number of observations; FBS, fetal bovine serum.

*Significant difference between strains at p<0.05.

WKY myocytes for factors that induce a state of competence to progress through the cell cycle, with the end result that, under appropriate conditions, growth responses of cells of the two strains can be either identical or very different. It has been proposed that quiescent or prereplicative cells do not proceed through DNA synthesis and cytokinesis until induced to do so by exposure to a competence factor such as PDGF and a progression factor, such as EGF or insulinlike growth factor.²⁻¹² PDGF is known to initiate a series of cellular events, including internalization and phosphorylation of the peptide–receptor complex, and the production of several second messengers including inositol trisphosphate,²⁹⁻³⁰ diacylglycerol,³¹ and cAMP.³² Among the secondary changes that result are alterations in intracellular Ca²⁺, Na⁺, and H⁺ content as well as specific phosphorylation events.²⁸ In addition, PDGF and other growth factors induce the expression of mRNA coding for products of the proto-oncogenes c-fos and c-myc.³₃,³₄ The net result of these cellular responses is thought to be the transcripion of a series of genes that make the cell competent to progress through the cell cycle.²⁸

The present study examined the sensitivity of SHR and WKY myocytes to fetal calf serum and insulin as promoters of the growth response to EGF. Fetal calf serum was used because it is a rich source of growth factors and is a potent growth promoter. Insulin was used because of a previous report³⁵ that demonstrated that this hormone makes mesangial cells competent to grow in response to endothelin. It is likely that in the present studies insulin exerted its action through the insulinlike growth factor receptor, since the effects were observed over a concentration range that was higher than typically observed for insulinlike growth factor.³⁶

Our results are consistent with the hypothesis that SHR myocytes are, in effect, better able to respond to EGF than are WKY myocytes, perhaps as a result of a constitutively active second messenger system. This hypothesis can also explain the enhanced growth responses that other laboratories⁹⁻¹⁴ have reported for SHR aortic myocytes. Because of our previous observation that basal levels of free intracellular Ca²⁺ are elevated in SHR myocytes, we proposed that the elevated level of free intracellular Ca²⁺ might be responsible for the differential responsiveness of SHR and WKY cells.

As an initial test of this postulate, we examined the effect of three levels of extracellular Ca²⁺ on the 3-day growth rates of SHR and WKY myocytes in 5% fetal calf serum. The results indicate that exposure of vascular myocytes to low Ca²⁺ medium causes a significant reduction in cell growth rate (Figure 8). Furthermore, there is a differential response of the two cell lines to extracellular Ca²⁺, with SHR cells exhibiting an enhanced response to each level of Ca²⁺. Although these findings indicate that extracellular Ca²⁺ is a modulator of vascular myocyte growth, the precise role that alterations in intracellular Ca²⁺ content play in the response to progression factors such as EGF remains to be determined.

In summary, the present study demonstrates that there are specific differences in vascular smooth muscle responses to growth factors. It is postulated that these differences result from alterations in second messenger pathway activity. Additional experiments are required to determine whether the cellular differences contribute to arterial wall thickening in the hypertensive animal or play a role in the enhanced risk of the hypertensive individual for the development of atherosclerosis.³⁷

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