

## Epidermal Growth Factor, a Vascular Smooth Muscle Mitogen, Induces Rat Aortic Contraction

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### Abstract

Atherosclerotic arteries have enhanced reactivity to vasoconstrictors, which suggests that features of the atherosclerotic process itself may result in this abnormal responsiveness. Since vascular smooth muscle proliferation is a prominent feature of atherosclerosis, we postulated that vasoactive agonists and smooth muscle mitogens may share certain common cellular mechanisms of action which potentially contribute to this hyperreactivity. To test this hypothesis, we studied the effects of epidermal growth factor (EGF), a well-characterized mitogen, on rat aortic vascular smooth muscle, both in intact aortic strips and in culture. EGF caused contraction ( $EC_{50} = 19$  nM) of rat aortic strips which maximally was equivalent to 40% of that induced by angiotensin II, a potent vasoconstrictor. EGF increased  $^{45}Ca$  efflux ( $EC_{50} = 3$  nM) from cultured rat aortic smooth muscle cells, which was an effect shared by angiotensin II and thought to reflect increased cytosolic-free calcium concentration. EGF (7.5 nM) also stimulated growth of these cultured cells to the same extent as 10% calf serum. These results demonstrate that EGF is both a vasoconstrictor and mitogen for rat aortic smooth muscle cells. The similarities in the effects of EGF and angiotensin II suggest that certain common intracellular mechanisms of action may exist for vasoactive agonists and growth factors which may contribute to the altered vasoreactivity of atherosclerotic vessels.

### Introduction

Experimental and clinical evidence suggests that atherosclerotic arteries exhibit enhanced reactivity to vasoactive substances, which may be a consequence of the atherosclerotic process itself (1–3). Although there have been significant increases in our understanding of the pathogenetic mechanisms in atherosclerosis (4), there are few observations that relate these mechanisms to development of altered vasoreactivity. Proliferation of vascular smooth muscle cells (VSMC)<sup>1</sup> in response

to mitogens derived from a variety of sources, including platelets, macrophages, and endothelial cells (5), is a prominent feature of the atherosclerotic process (6). As an approach to understanding the abnormal reactivity of atherosclerotic vessels, we have postulated that vasoactive agonists and VSMC mitogens share certain common cellular mechanisms of action. We chose to investigate epidermal growth factor (EGF) because it is readily available in a highly purified form (7) and is present in human platelets (8). It is also mitogenic for bovine vascular smooth muscle cells (9), and its cellular actions are associated with changes in cation movement, including calcium flux in fibroblasts (10). We reasoned that these effects on calcium flux, if present in vascular smooth muscle, might result in contraction (11). Our prediction that EGF would be vasoactive was confirmed by demonstrating that this mitogen caused contraction of rat aortic strips, with a maximal effect equivalent to 40% of that induced by the potent vasoconstrictor, angiotensin II (ang-II). This contractile effect may reflect EGF-mediated changes in calcium flux, as demonstrated in cultured rat aortic VSMC.

### Methods

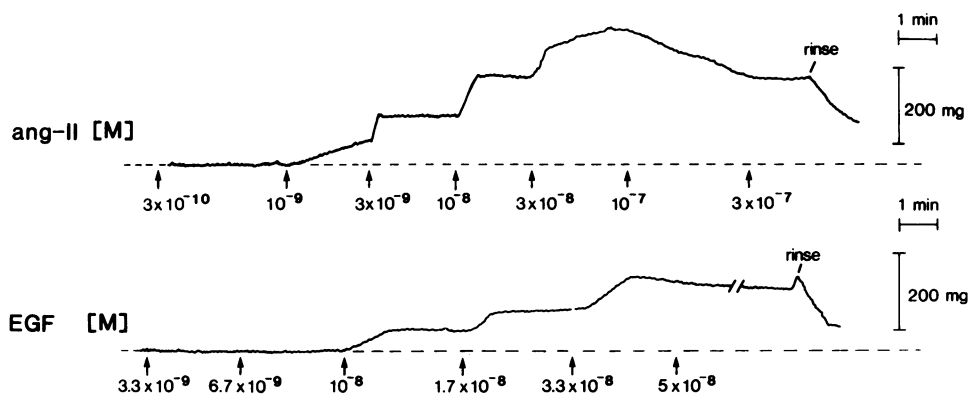
To assay contractile activity, helical strips (1 × 10 mm) were prepared from the thoracic aortae of adult male, Sprague-Dawley rats (300–350 g), and were mounted in a stainless steel holder in a muscle bath. The resting tension on each strip was adjusted to 1,500 mg. Before the start of experiments, the strips were allowed to equilibrate for 2 h in buffer under conditions as previously described (12). Endothelium was demonstrated to be functionally intact at the start of each experiment as assessed by the relaxation of precontracted strips in response to acetylcholine (13). Contractile responses to increasing concentrations of EGF purified from mouse submaxillary glands (Biomedical Technologies, Inc., Cambridge, MA, or a gift of Dr. R. Murphy, Dept. of Anatomy, Harvard Medical School, Cambridge, MA) or ang-II (synthetic human, Sigma Chemical Co., St. Louis, MO) were recorded after sequential additions of increasing amounts of agonist to the bath as previously described (12). A contractile response was allowed to reach a stable plateau (2–5 min) before each successive addition. Protein purity was estimated to be >95% by electrophoresis of the EGF preparation. On sodium dodecyl sulfate polyacrylamide gel electrophoresis, monomer and dimer bands of EGF were seen as reported by Bridgen (14), while on acetic acid-urea gels only a single band was seen (15).

VSMC were cultured from thoracic aortae of male Sprague-Dawley rats (150–200 g) using a modification of an enzymatic dissociation described previously (16). Passaged cells were replicate plated in 35-mm dishes and grown to near confluence ( $3-4 \times 10^4$  cells/cm<sup>2</sup>) in Dulbecco's modified Eagle's medium (DME), which contained 10% calf serum (M. A. Bioproducts, Walkersville, MD). To study  $^{45}Ca$  efflux, the medium was changed 24 h before the experiment to serum-free DME which was supplemented with a mixture of 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 5 ng/ml selenous acid (ITS Premix, Collaborative

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1. Abbreviations used in this paper: ang-II, angiotensin II;  $EC_{50}$ , concentration causing half-maximal effect; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; VSMC, vascular smooth muscle cell(s).



**Figure 1.** Contraction of isolated rat aorta induced by EGF or ang-II. Helical strips of rat aorta were prepared and allowed to equilibrate for 2 h in the muscle bath as described above. Contractile responses to increasing concentrations of EGF or ang-II were recorded after sequential addition of increasing amounts of agonist to the bath. Contractile response was allowed to reach a stable plateau (2–5 min) before each successive addition. Concentrations are expressed as the cumulative molar concentration. These are representative tracings from one of eight contraction experiments.

washing with a buffered salt solution which contained 1.5 mM  $\text{CaCl}_2$ , and then by adding 1 ml of buffer which contained 1 mg/ml BSA (control) and EGF (16.4 nM) or ang-II (10 nM). Determination of cell-associated  $^{45}\text{Ca}$  was carried out as previously described (17). No visible effect of EGF on cell adhesion (18) was observed during the 20-min incubations used here.

To assay for an EGF mitogenic effect, rat aortic VSMC (passage 17) were replicate plated at  $3 \times 10^4$  cells/16-mm dish in DME-10% calf serum. After 1 d, the medium was changed to DME-1% calf serum. 2 d later the medium was changed to DME which was supplemented with 1% or 10% calf serum, or 1% calf serum and EGF (7.5 nM). Cell counts were obtained approximately every 2 d until growth plateaued.

## Results

The contractile effects of purified mouse submaxillary EGF on isolated strips of rat aorta are illustrated in Fig. 1. Cumulative addition of EGF to the muscle bath produced contractile responses in all strips tested. Maximal force development in response to EGF ( $144 \pm 23$  mg,  $n = 8$ ) occurred within 5 min, and was  $\sim 40\%$  of that observed with ang-II, a positive control ( $335 \pm 41$  mg,  $n = 8$ ). Comparison of the time course of tension development showed that for a given amount of tension the rate of contraction was always slower for EGF than ang-II. For example, the time required to reach 150 mg force was 96 s for EGF ( $3 \times 10^{-8}$  M) vs. 13 s for ang-II ( $3 \times 10^{-9}$  M). The concentrations of agonist causing half-maximal effect ( $\text{EC}_{50}$ ) for force development were  $19 \pm 1.0$  and  $5.9 \pm 1.3 \times 10^{-9}$  M for EGF and ang-II, respectively, as shown in Fig. 2. These observations show that EGF is a contractile agonist for rat aortic smooth muscle.

Calcium has been clearly implicated in mediating vascular contraction in response to various agonists including ang-II (11, 19). To study the effects of EGF on VSMC calcium homeostasis, we measured  $^{45}\text{Ca}$  efflux from preloaded cultured rat aortic VSMC which were stimulated by EGF as described in Fig. 3 A, again comparing EGF with ang-II. Both EGF and ang-II stimulated calcium efflux, which was measured as a decrease in preloaded cellular  $^{45}\text{Ca}$  content. Ang-II-stimulated  $^{45}\text{Ca}$  efflux was significantly different from control ( $P = 0.00003$ ) at the earliest time point tested (30 s). However, EGF-mediated efflux was not significantly different from control until 3–5 min after incubation. This delay in EGF-stimulated  $^{45}\text{Ca}$  efflux may be related to the slow development of tension that we observed in response to EGF in the rat aortic strips (Fig. 1).

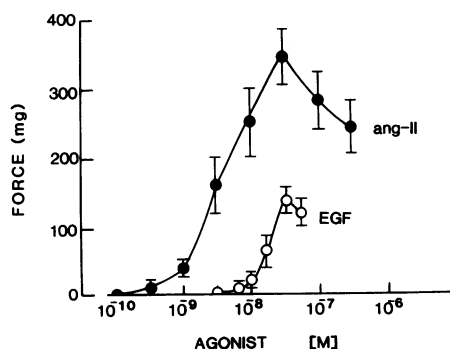
Maximal stimulation of  $^{45}\text{Ca}$  efflux by EGF ( $0.58 \pm 0.15$

nmol/mg protein) occurred at  $2 \times 10^{-8}$  M EGF, and was  $\sim 20\%$  of that observed with  $1 \times 10^{-8}$  M ang-II ( $3.84 \pm 0.20$  nmol/mg protein) (Fig. 3 B). These EGF-stimulated changes in cultured rat aortic VSMC compare favorably with those reported by other workers in A431 cells (18) and fibroblasts (20, 21).  $\text{EC}_{50}$  values for  $^{45}\text{Ca}$  efflux that were stimulated by EGF and ang-II were  $3.0 \pm 0.8$  and  $2.1 \pm 0.2 \times 10^{-9}$  M, respectively (Fig. 3 B).

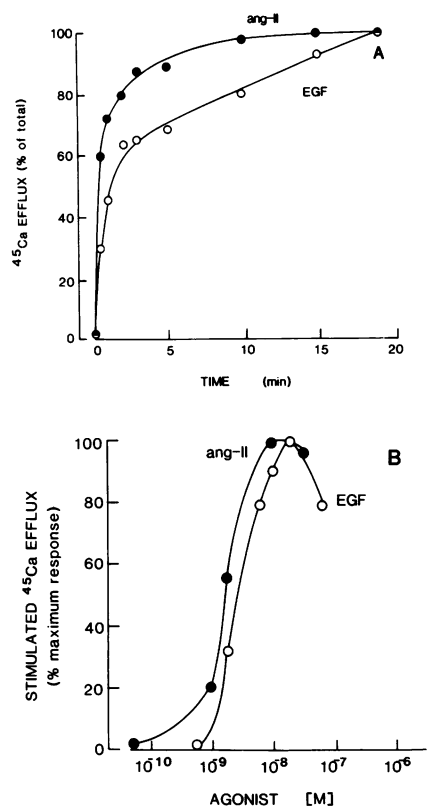
Purified EGF (7.5 nM), in the presence of a relatively low concentration of calf serum (1%), stimulated the proliferation of cultured rat aortic VSMC to the same extent as a maximally effective growth stimulus, 10% calf serum (Fig. 4). These observations are in agreement with published data which shows EGF to be mitogenic for certain cell types including aortic smooth muscle cells (9).

## Discussion

The present study demonstrates that EGF mediates contraction of isolated rat aorta, induces  $^{45}\text{Ca}$  efflux from cultured rat aortic VSMC, and also stimulates the proliferation of these cells in vitro. The  $\text{EC}_{50}$  values for  $^{45}\text{Ca}$  efflux and aortic strip contraction are similar and in the nanomolar range, being slightly higher for aortic contraction. There are conflicting data in the literature concerning the effects of EGF on cellular

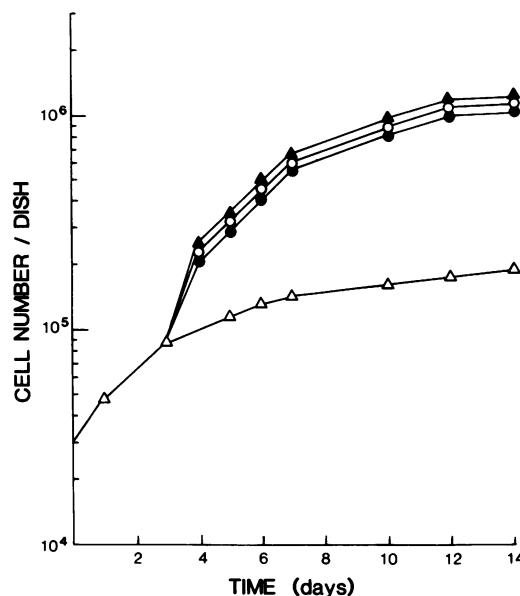


**Figure 2.** Dose response of EGF and ang-II-induced rat aortic contraction. Helical strips of rat aortae were made to contract in response to the cumulative addition of EGF (○) or ang-II (●) as described in Fig. 1. Experimental values are mean  $\pm$  SEM of eight determinations.  $\text{EC}_{50}$  values were estimated by logit-log transformation after normalization of observations to percentage of maximal response.



**Figure 3.** (A) Time-dependent  $^{45}\text{Ca}$  release from cultured rat aortic VSMC stimulated by EGF or ang-II. VSMC at near confluence were incubated for 24 h in serum-free DME that contained  $^{45}\text{Ca}$  ( $2 \mu\text{Ci}/\text{ml}$ ) supplemented with ITS Premix and BSA ( $2 \text{ mg}/\text{ml}$ ).  $^{45}\text{Ca}$  efflux was initiated by washing with a buffered salt solution that contained  $1.5 \text{ mM CaCl}_2$  and then adding  $1 \text{ ml}$  of buffer that contained  $1 \text{ mg}/\text{ml}$  BSA (control) and EGF ( $16.4 \text{ nM}$ ) or ang-II ( $10 \text{ nM}$ ). Data are plotted as percentage of maximal efflux (maximal efflux equals [ $^{45}\text{Ca}$  content at  $t = 0 \text{ min}$ ] minus [ $^{45}\text{Ca}$  content at  $t = 20 \text{ min}$ ]) for each agonist at the indicated time points.  $^{45}\text{Ca}$  content decreased by  $5.10$ ,  $5.67$ , and  $7.44 \text{ nmol}/\text{mg}$  protein for control, EGF, and ang-II, respectively, during the  $20\text{-min}$  interval for the experiment shown. These are representative tracings from one of three efflux experiments. (B) Dose response of EGF and ang-II-mediated  $^{45}\text{Ca}$  efflux in cultured rat aortic VSMC.  $^{45}\text{Ca}$  efflux from rat VSMC cultures was carried out as described above. Values are the mean  $\pm$  SEM for 3–5 dishes, normalized to percentage of maximal response. Stimulated  $^{45}\text{Ca}$  efflux for each time point was calculated as  $^{45}\text{Ca}$  content (control) minus  $^{45}\text{Ca}$  content (agonist) for 3–5 dishes and the absolute  $^{45}\text{Ca}$  contents were statistically compared by a two-tailed  $t$  test. Maximal stimulated  $^{45}\text{Ca}$  efflux was complete at  $5 \text{ min}$ , and the  $^{45}\text{Ca}$  content at this time was used for calculation of percentage of maximum response for each concentration.

Research, Inc., Lexington, MA), and  $2 \text{ mg}/\text{ml}$  bovine serum albumin (BSA) which contained  $^{45}\text{Ca}$  ( $2 \mu\text{Ci}/\text{ml}$ ).  $^{45}\text{Ca}$  efflux was initiated by calcium homeostasis. On the one hand, EGF at  $1.7 \text{ nM}$  has been shown to stimulate hexose transport in Swiss 3T3 fibroblasts in a calcium-dependent fashion (21), and EGF at  $\sim 20 \text{ nM}$  causes  $^{45}\text{Ca}$  efflux from A431 cells (18) and human foreskin fibroblasts (20). On the other hand, a lower concentration of EGF ( $0.8 \text{ nM}$ ) did not stimulate  $^{45}\text{Ca}$  efflux from Swiss 3T3 fibroblasts (22). In the well-studied cases of ang-II-mediated VSMC contraction (19) or adrenal glomerulosa cell secretion (23), stimulation of calcium efflux has been clearly demonstrated and is correlated with an increase in cytosolic



**Figure 4.** Proliferation of cultured rat aortic VSMC stimulated by EGF. Rat aortic VSMC (passage 17) were replicate-plated at  $3 \times 10^4$  cells per  $16 \text{ mm}$  dish in DME-10% calf serum. After one day, the medium was changed to DME-1% calf serum. Two days later, the medium was changed to DME supplemented with: (a) 1% calf serum ( $\Delta$ ), (b) 10% calf serum ( $\blacktriangle$ ), (c) and (d) 1% calf serum and EGF ( $7.5 \text{ nM}$ ) obtained from Biomedical Technologies, Inc. ( $\circ$ ) or Dr. Richard Murphy ( $\bullet$ ). Each data point is the mean cell count from three dishes.

free calcium concentration (24, 25). Experiments using the fluorescent calcium indicator quin 2 have demonstrated that EGF stimulates rapid increases in cytosolic free calcium concentration in cultured human foreskin fibroblasts (10).

Contraction of VSMC in response to ang-II has been shown to be coupled to increases in cytoplasmic calcium concentration (11, 19, 24). A presumed mechanism for ang-II-mediated VSMC contraction has been the activation of calmodulin-dependent myosin light chain kinase, with a resulting increase in myosin light chain phosphorylation (11, 26). However, although contraction has a prolonged tonic phase, the rise in calcium is transient (24, 25), and phosphorylation of the myosin light chain returns to basal levels within  $5 \text{ min}$  (26). To explain the tonic phase of contraction, some authors have suggested that protein kinase C may regulate a calcium-dependent VSMC contraction (27). There is evidence that EGF may activate protein kinase C: platelet-derived growth factor which has effects on intracellular  $\text{Ca}^{2+}$  similar to EGF (10) has been shown to increase diacylglycerol concentration in Swiss 3T3 cells (28); and, it now appears that diacylglycerol activates protein kinase C (29). We found significant differences in the time course and magnitude of EGF and ang-II-mediated  $^{45}\text{Ca}$  efflux and aortic strip contraction. These differences may reflect differential activation by EGF and ang-II of calmodulin and C-kinase related calcium-dependent mechanisms.

The concentrations of EGF used for most experiments reported here were  $\sim 150$ – $400$ -fold higher than those reported in human platelet-rich plasma (8). However, only small amounts of EGF exist free in plasma; most is associated with platelets, and one might anticipate that higher local concentrations would be available at sites of active platelet-vessel wall interactions (5).

In summary, we have demonstrated that EGF is a potent mitogen for cultured rat aortic smooth muscle cells and induces contraction of rat aorta. The similarities observed in the cellular effects of EGF and ang-II, a classic vasoconstrictor hormone, suggest that vascular mitogens and vasoactive agents may share certain intracellular mechanisms. Further study of these cellular mechanisms in vascular smooth muscle may shed light on the pathophysiology underlying the altered reactivity of atherosclerotic vessels.

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