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Short communication

Impairment of endothelium-dependent relaxation in the arteries cultured with fetal bovine serum

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Abstract

Effects of chronic treatment with fetal bovine serum on the function of vascular endothelium were examined using an organ culture system. In the rabbit mesenteric arteries cultured with 10% fetal bovine serum for 7 days, the substance P- or ionomycin-induced endothelium-dependent relaxation was significantly attenuated compared to the arteries cultured in the serum-free condition. The effects of the serum were concentration- and time-dependent. By the treatment with the serum, the amounts of nitric oxide (NO) production and total mRNA for endothelial NO synthase were reduced, whereas the sodium nitroprusside-induced relaxation was rather augmented. These results suggest that chronic treatment of rabbit mesenteric artery with fetal bovine serum decreases endothelial NO synthase mRNA, reduces NO production and impairs endothelium-dependent relaxation.

Keywords: Endothelium-dependent relaxation; Nitric oxide (NO); Endothelial NO synthase; Fetal bovine serum; Organ culture

1. Introduction

Release of nitric oxide (NO) is an important endothelial function because it regulates vascular tone by acting on smooth muscle. Furthermore, NO prevents smooth muscle proliferation (Garg and Hassid, 1989), and platelet aggregation and adhesion (Gryglewski et al., 1988), suggesting that NO has also an important protective actions for vascular wall. Pathological conditions in which vascular remodeling occurs, including hypertension, atherosclerosis and diabetes, are often associated with activation of proliferation and differentiation of vascular endothelium. For these conditions, various growth factors serve to modify the growth state of the endothelial cells (Ross, 1993). As the status of cell growth is known to modulate expression of numerous tissue-specific functions, we hypothesized that stimulation of endothelial cell with growth factors may alter the property to produce NO and subsequent endothelium-dependent relaxation. Previous studies in cultured endothelial cells have demonstrated that basic fibroblast growth factor (Kostyk et al., 1995), vascular endothelial growth factor (Hood et al., 1998), and transforming growth factor β (Inoue et al., 1995) up-regulate endothelial NO synthase whereas tumor necrosis factor α (Yoshizumi et al., 1993) down-regulates it. As isolated cells in culture are known to rapidly lose their native characteristics by their lack of cellular interactions, evaluation of the results obtained with cultured cells is difficult. Additionally, in the pathological conditions, a complex of autocrine and paracrine growth factors produced by vascular wall themselves, rather than a single factor, acts to modify the endothelial cell growth through a network of cellular interactions. Organ culture system has the advantages to obtain the conditions that better preserve tissue architecture, cell-to-cell interactions, the extracellular matrix, and the morphology and function of differentiated cells and thereby to reproducibly study behavior of the whole tissue in response to environmental stimuli (Rogers et al., 1993). To gain insight into the possible roles of growth stimulation on the function of vascular endothelium, we examined the effects of chronic treatment of whole tissue with fetal bovine serum, an unspecified but mixed source of various growth factors, on the NO-mediated endothelium-dependent relaxation, NO production, and total RNA for endothelial NO synthase using the organ culture system.

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2. Materials and methods

2.1. Tissue preparation and organ culture procedure

Male Japanese White rabbits (2–3 kg) were sacrificed by a sharp blow on the neck and exsanguination. Main branches of superior mesenteric arteries were isolated. After the removal of fat and adventitia in sterile Hanks' balanced salt solution, each artery was cut into rings (approximately 2 mm wide) for measurements of muscle tension and helical strips (approximately 1.5 mm wide, 5 mm long) for NO bioassay experiments. Strips were then placed in 2 ml Dulbecco's Modified Eagle Medium (DMEM) without or with 10% fetal bovine serum supplemented with 1% penicillin–streptomycin. Arterial preparations were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 for up to 7 days.

2.2. Measurement of muscle tension

After incubation, the arterial rings were placed in normal physiological salt solution (PSS), which contained the following (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5. Ethylenediaminetetraacetic acid (EDTA), 1 μM was also added to remove contaminating heavy metal ions which catalyze oxidation of organic chemicals. The PSS was saturated with 95% O_2–5% CO_2 mixture at 37°C and pH 7.4. In some experiments, the endothelium was removed by gently rubbing the intimal surface with the forceps just before the experiments. Each muscle ring was attached to a holder under a resting tension of 10 mN in a 2-ml organ bath. Muscle tension was recorded isometrically. Concentration–response curves were obtained by the cumulative application of relaxants during a norepinephrine-induced submaximal contraction. Data were shown as a percent relaxation of the steady-state preconstriction.

2.3. NO bioassay

We referred to the methods previously described by Furchgott and Zawadzki (1980). Helical strips of freshly isolated rabbit mesenteric artery without endothelium (NO recipient) and cultured artery with endothelium (NO donor) were mounted together in sandwich form with their entire intimal surfaces opposed. They were attached to a holder in an organ bath (10 ml) under a resting tension of 10 mN. Thus, the amount of NO released from the NO donor endothelium was bioassayed as the changes of the NO recipient muscle tension measured with an isometric force transducer connected only to the NO recipient.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from endothelium-intact artery through the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method (Chomczynski and Sacchi, 1987) using the TRIzol Reagent. RT-PCR was performed using Takara RNA PCR Kit Ver. 2.1 (Takara, Japan). The first strand cDNA was synthesized using random 9 mers and AMV Reverse Transcriptase XL at 30°C for 10 min, 55°C for 30 min, 99°C for 5 min and 4°C for 5 min. PCR amplification was performed using rTaq DNA polymerase. The oligonucleotide primers used were TAC CAG CCG GGG GAC CAC (sense) and CGA GCT GAC AGA GTA GTA (antisense) for eNOS (Sessa et al., 1992) and TCC CTC AAG ATT GTC AGC AA (sense) and AGA TCC ACA ACG GAT ACA TT (antisense) for GAPDH (Fort et al., 1985). After initial denaturation and activating DNA polymerase at 94°C for 2 min, 28–48 cycles (5-cycle interval) of amplifications at 94°C for 0.5 min, 60°C for 0.5 min and 72°C for 1.5 min were done using thermal cycler (Takara Thermal Cycler PERSONAL TP 240, Takara, Japan). PCR products in each cycle were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. The possible contamination of DNA was excluded by performing a PCR with total RNA without reverse transcription step. Detectable fluorescent bands were visualized by UV-transilluminator.

2.5. Chemicals and statistics

The chemicals used were as follows: penicillin–streptomycin, TRIzol Reagent, ethidium bromide solution (Gibco BRL, USA), norepinephrine, N^G-monomethyl-L-arginine (L-NMMA), sodium nitroprusside (Wako Pure Chemical, Japan), substance P, ionomycin calcium salt, indomethacin (Sigma, USA), DMEM (Nissui Pharmaceutical, Japan), fetal bovine serum (Hyclone, USA), EDTA (Dojindo Laboratories, Japan), and rTaq DNA polymerase (Takara, Japan). The results of the experiments are expressed as means ± S.E.M. Student's *t*-test was used for statistical analysis of the results and $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of fetal bovine serum on the endothelium-dependent relaxation

In the arteries cultured in the serum-free condition for 7 days (serum-free arteries), substance P (0.1–100 nM) relaxed the norepinephrine (1 μM)-induced submaximal contraction in a concentration-dependent manner (Fig. 1A, $n = 8$). In the arteries cultured with 10% fetal bovine serum for 7 days (serum-treated arteries), the relaxant effect of substance P on the norepinephrine (1 μM)-induced submaximal contraction was almost completely inhibited ($n = 9$). In both of these arteries, substance P was

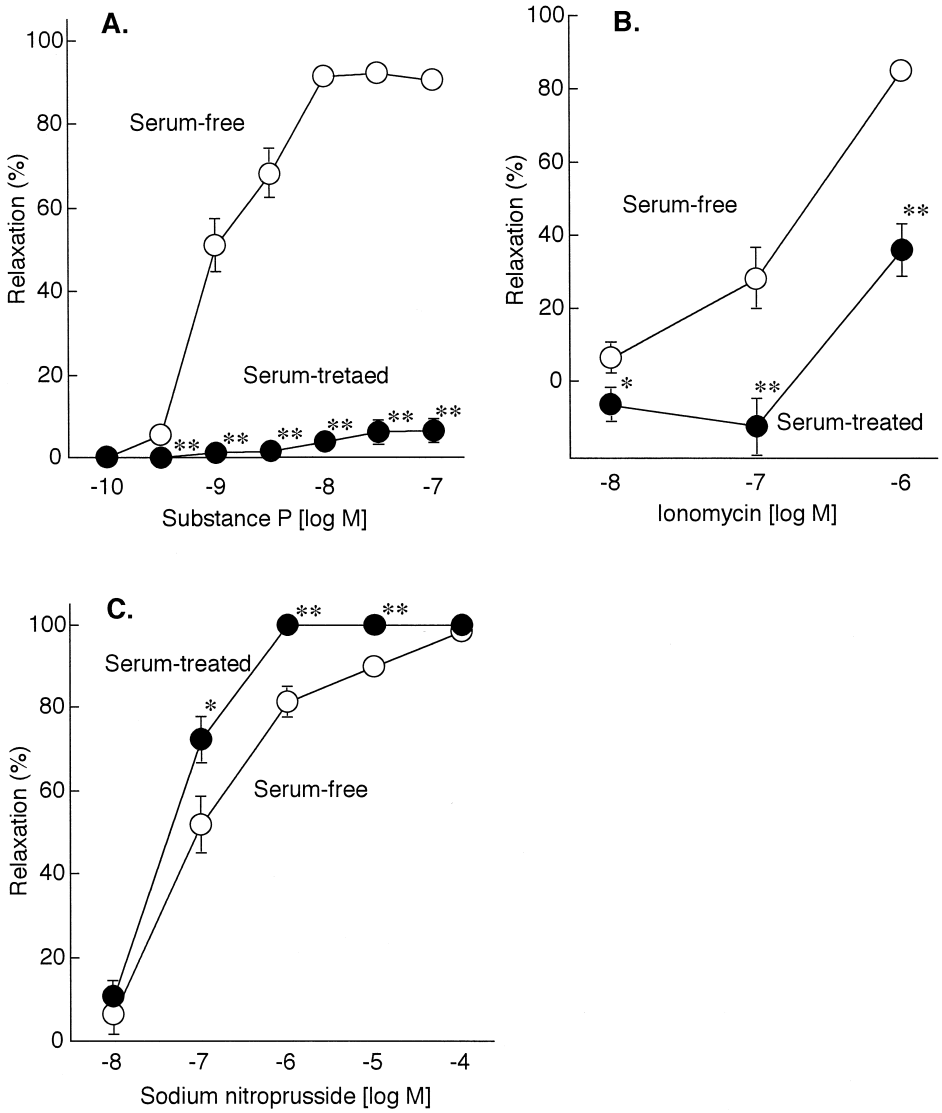

Fig. 1. Concentration–response relationship for the relaxant effect of substance P (0.1–100 nM, $n = 8$–9) (A), ionomycin (0.01–1 μM, $n = 14$) (B), and sodium nitroprusside (0.01–100 μM, $n = 8$) (C) on the norepinephrine (1 μM)-induced submaximal contraction in the rabbit mesenteric arteries cultured without (Serum-free, open circle) or with 10% fetal bovine serum (Serum-treated, closed circle) for 7 days. The relaxants were cumulatively added after the norepinephrine-induced contraction had reached a steady state. Effect of sodium nitroprusside was examined using the endothelium-denuded arteries whereas the effects of other relaxants were examined using the endothelium-intact arteries. One hundred percent represents the steady-state preconstriction. Results are expressed as means ± S.E.M. *, **: Significantly different from serum-free with $P < 0.05$ and $P < 0.01$, respectively.

ineffective when endothelium was removed whereas the treatment with indomethacin (10 μM, 30 min) had no effect on the relaxation ($n = 4$). The substance P (100 nM)-induced relaxation in the serum-free arteries was significantly inhibited by the treatment with L-NMMA (200 μM, 30 min) (by 70.5 ± 4.1%, $n = 15$). Although the treatment with serum for 3 days had no effect on the relaxant effect of substance P, treatment for 4 days significantly attenuated the relaxation (peak relaxation was 95.8 ± 1.9% in the arteries cultured without serum and 33.5 ± 7.4% with serum, $n = 6$ each, $P < 0.01$). The inhibitory effect of serum was further augmented by the treatment for 7 days (peak relaxation was 92.2 ± 0.7% in the serum-free arteries and 6.6 ± 2.9% in the serum-treated arteries, $P < 0.01$, Fig. 1A). We also examined the concentration–response relationship in the inhibitory effect of fetal bovine serum. The inhibitory effect of the 10% fetal bovine serum for a 7-day treatment was significantly larger than that of 2% fetal bovine serum (peak relaxation was 18.0 ± 4.1% in the 2% serum-treated arteries and 5.7 ± 1.8% in the 10% serum-treated arteries, $n = 4$ each, $P < 0.01$).

In the serum-free arteries, ionomycin (0.01–1 μM) relaxed the norepinephrine (1 μM)-induced preconstriction in a concentration-dependent manner. The maximum relaxation caused by 1 μM ionomycin was 85.1 ± 1.8% (Fig. 1B, $n = 14$). The concentration–response curve was shifted

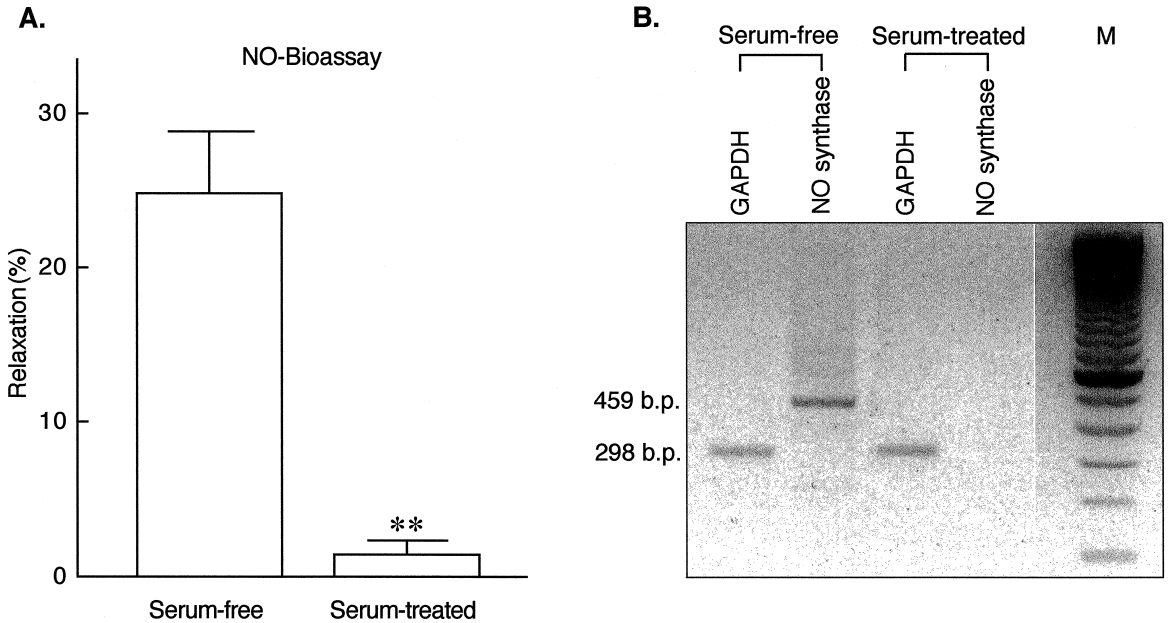

Fig. 2. (A) Mean percentage relaxation in the endothelium-denuded, freshly isolated rabbit mesenteric arteries (NO recipient) in response to nitric oxide (NO) released from the endothelium-intact arteries (NO donor), cultured without (Serum-free) or with fetal bovine serum (Serum-treated) for 7 days. Substance P (100 nM) was added after the contraction induced by norepinephrine (1 μM) in the NO recipient had reached a steady state. One hundred percent represents the steady-state preconstriction. Results are expressed as means ± S.E.M. of 10–12 experiments. ∗∗: Significantly different from serum-free with $P < 0.01$. (B) Agarose-gel electrophoresis of RT-PCR products for endothelial NO synthase and GAPDH after 48 cycles of amplification. Total RNA was isolated from the endothelium-intact rabbit mesenteric arteries cultured without (Serum-free) or with fetal bovine serum (Serum-treated) for 7 days. The amplified products were 459 basepairs (b.p.) for endothelial NO synthase and 298 b.p. for GAPDH. Lane M demonstrates 100 b.p. DNA ladder.

to the right in the serum-treated arteries, and the maximum relaxation caused by 1 μM ionomycin was significantly attenuated to 36.0 ± 6.9% ($n = 14$, $P < 0.01$).

3.2. Effects of fetal bovine serum on the sodium nitroprusside-induced relaxation in smooth muscle

In the serum-free arteries denuded with endothelium, sodium nitroprusside (0.01–100 μM) relaxed the norepinephrine (1 μM)-induced submaximal contraction in a concentration-dependent manner (Fig. 1C, $n = 8$). In the endothelium-denuded, serum-treated arteries, the sodium nitroprusside-induced relaxation was significantly augmented ($n = 8$). When these arteries had been maximally constricted by 100 μM norepinephrine, the sodium nitroprusside-induced relaxation in the serum-treated arteries was also significantly augmented (peak relaxation was 92.4 ± 1.2% in the serum-treated arteries, $n = 17$, and 56.3 ± 2.5% in the serum-free arteries, $n = 18$, $P < 0.01$).

3.3. NO bioassay

In the bioassay system, addition of 100 nM substance P during the norepinephrine (1 μM)-induced contraction caused a transient relaxation in the recipient arteries, as reported by Zawadzki et al. (1981). Using the serum-free arteries as an NO donor, the substance P-induced relaxation in the recipient arteries was 24.8 ± 4.0% (Fig. 2A, $n = 10$). Using the serum-treated arteries as a donor, the substance P-induced relaxation was significantly reduced to 1.5 ± 0.9% ($n = 12$, $P < 0.01$).

3.4. RT-PCR

After 48 cycles of amplification, products of the expected size (459 basepairs (b.p.) for endothelial NO synthase and 298 b.p. for GAPDH) were detected in the RNA preparations obtained from the serum-free arteries (Fig. 2B). In the RNA preparations obtained from the serum-treated arteries, no visible band corresponding to endothelial NO synthase was detected whereas GAPDH products were visible at the same cycles. Similar results were obtained from other two independent sets of experiments.

4. Discussion

The present results showed that the endothelium-dependent relaxation elicited by substance P, which is attributable mainly to NO production, was significantly reduced by the chronic treatment with fetal bovine serum (Fig. 1A). It was also shown that the inhibitory effect of fetal bovine serum was both time- and concentration-dependent. The decrease in the relaxation is not attributable to the morphological damage in endothelium because we confirmed histologically that endothelium was intact in the

serum-treated arteries (data not shown). There are at least three possible explanations for the impairment of endothelium-dependent relaxation; (1) receptor which is responsible for release of NO is down-regulated, (2) NO production by NO synthase is impaired, and (3) mechanism of cGMP-dependent relaxation in smooth muscle is impaired.

Receptor agonists produce NO by increasing Ca^{2+} level in endothelium to activate endothelial NO synthase (Moncada et al., 1991). Ca^{2+} ionophore has been shown to directly increase endothelial cytosolic Ca^{2+} concentration (Vanhoutte et al., 1986). The present results showed that the Ca^{2+} ionophore-induced relaxation was also significantly reduced by the treatment with fetal bovine serum (Fig. 1B). These results suggest that the mechanisms after an increase in endothelial Ca^{2+} level would be impaired. However, possible involvement of decreases in the number of the NK_1 receptor, which mediates the effect of substance P, could not be excluded.

NO produced by endothelial cells activates guanylate cyclase to increase cGMP content in smooth muscle (Rapoport and Murad, 1983). cGMP activates cGMP-dependent protein kinase which leads to muscle relaxation by decreasing cytosolic Ca^{2+} concentration and/or Ca^{2+} sensitivity of contractile apparatus (Karaki et al., 1988). In the serum-treated arteries, the relaxant effect of an NO releaser, sodium nitroprusside, was significantly augmented compared to that in the serum-free arteries (Fig. 1 C). These results suggest that the mechanism of cGMP-dependent relaxation in smooth muscle is not impaired by the treatment with fetal bovine serum.

To further examine the mechanism of dysfunction of endothelium-dependent relaxation, we measured the amounts of NO production by NO bioassay experiments and changes in endothelial NO synthase mRNA level by RT-PCR analysis. The results from the NO bioassay suggested the decrease in the amounts of endothelial NO production in the serum-treated arteries (Fig. 2A). In addition, RT-PCR analysis indicated the decrease in the total amounts of recoverable eNOS mRNA in the serum-treated arteries (Fig. 2B). Therefore, we conclude that a chronic treatment of arteries with fetal bovine serum decreases endothelial NO synthase mRNA level, reduces NO production, and impairs endothelium-dependent relaxation. The effects might be caused not only by direct actions of serum-derived factors on endothelium but also by indirect actions of the mediators produced by the arterial wall themselves. Because we have previously reported that smooth muscle cells tended to proliferate after the organ culture with fetal bovine serum (Sato et al., 1997) and proliferating smooth muscle cells have been shown to secret endothelial stimulating mitogens (Ross, 1993).

The present study has been performed to determine the effects of fetal bovine serum, which represents the mixed potential effects of growth factors, on the endothelial function using an organ culture system in which tissue architectures and consequent cell interactions were maintained. Since vascular pathological conditions are often associated with the endothelial stimulation with a complex of growth factors through a network of cellular interactions, our findings may be related to the functional changes of vascular endothelium in disease.

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