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European Journal of Pharmacology 515 (2005) 134 – 141
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Dexamethasone prevents impairment of endothelium-dependent relaxation in arteries cultured with fetal bovine serum

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Received 3 December 2004; accepted 2 February 2005

Abstract

In the present study, we assessed the effects of dexamethasone on fetal bovine serum-induced dysfunction of mesenteric endothelial cells using an organ culture procedure. In rabbit mesenteric arteries cultured in the presence of 10% fetal bovine serum for 7 days, the endothelium-dependent, nitric oxide (NO)-mediated relaxations caused by substance P and ionomycin were decreased as compared to those in non-treated arteries. Dexamethasone (3 μM) inhibited the proliferative stimuli-induced endothelial dysfunction without affecting the contractility or NO susceptibility of smooth muscle cells. Cross-sectioned hematoxylin–eosin staining and whole-mount CD31 staining indicated that chronic proliferative stimulation induced detachment of endothelial cells from the tunica intima in some regions, and also caused thickening of the arterial wall and shortening of the internal diameter. Endothelial NO synthesis (eNOS) mRNA expression was also decreased by the treatment with fetal bovine serum. The dexamethasone treatment did not inhibit the smooth muscle hypertrophy, but it inhibited the peeling of endothelial cells and recovered the eNOS mRNA expression. These results suggest that DEX ameliorate the impairments of arterial relaxation induced by proliferative stimuli and that these beneficial effects may be mediated by maintaining the adhesion of endothelial cells to the vascular wall and/or by recovering eNOS mRNA expression.
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Keywords: Organ culture; Endothelium-dependent relaxation; Dexamethasone

1. Introduction

The vascular endothelium plays important roles in (1) local regulation of vascular tone, (2) regulation of smooth muscle cell proliferation, and (3) prevention of platelet aggregation and adhesion via the release of vasoactive products such as nitric oxide (NO) and prostacyclin. Impairment of the endothelium has long been considered to be a pathogenic mechanism of various vascular diseases, such as arteriosclerosis (Ross, 1993), and improvement of endothelial impairment is very important for therapeutic progress against these diseases.

In vascular pathophysiological sites, including those of arteriosclerosis, various growth factors generated in the lesion have been shown to play a primary role in the pathogenesis of disease. Growth factors on blood vessels act chronically on the tissue to cause damaging structural changes (Ross, 1986, 1993). In our previous study, we successfully established a serum-induced endothelial dysfunction model using an organ culture procedure (Yamawaki et al., 1999a,b). The organ culture method makes it possible to dissociate the influence of other factors from the direct effect of particular agents. This method allows us to readily examine the morphological and functional effects of various agents on tissue. Using this unique procedure, we previously established that chronic exposure to low oxygen impaired the endothelial-dependent relaxation mediated by NO in the pulmonary arteries, but not in the mesenteric arteries of rabbits (Murata et al., 2001b).

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doi:10.1016/j.ejphar.2005.04.005

Glucocorticoids such as dexamethasone are used to treat a wide variety of inflammatory diseases. The actions of glucocorticoids are mediated by an intracellular receptor, the glucocorticoid receptor, a member of the nuclear receptor family of ligand-dependent transcription factors (Hollenberg et al., 1985). Upon activation by their respective ligands, these receptors act as transcription factors; i.e., they can alter the expression of specific target genes. Several studies have reported that glucocorticoids have protective effects on the vascular endothelium, such as inhibition of lipopolysaccharide- and tumor necrosis factor α (TNFα)-induced apoptosis (Messmer et al., 1999) and inhibition of interleukin-1β- and TNFα-induced endothelial inflammatory responses (Suzuki et al., 2000; Wheller and Perretti, 1997). We recently revealed the molecular mechanisms of the dexamethasone-induced protective effects against endothelial dysfunction induced by chronic hypoxia using organ-cultured pulmonary arteries (Murata et al., 2004).

The goal of this study was to determine whether dexamethasone prevents endothelial dysfunction in an in vitro serum-induced vascular proliferative model using rabbit mesenteric arteries. The results showed that DEX has beneficial effects on chronic fetal bovine serum-induced mesenteric endothelial dysfunction through inhibition of endothelial detachment and the recovery of eNOS mRNA.

2. Materials and methods

2.1. Tissue preparation and organ culture procedure

Male Japanese White rabbits (2–3 kg) were euthanized by stunning and exsanguination. The organ culture procedure was performed as described previously (Murata et al., 2001a). In brief, the mesenteric arteries were isolated. After fat and adventitia had been removed in sterile Hanks' balanced salt solution, each artery was cut into rings (approximately 1.5 mm wide). The arterial strips were then placed in 2 ml of Dulbecco's Modified Eagle Medium (DMEM) with or without 10% fetal bovine serum supplemented with 1% penicillin–streptomycin in the presence or absence of 3 μM DEX. The muscle rings were maintained at 37 °C under an atmosphere of 95% air and 5% CO_2 for 7 days. The medium was changed every 2 days until the start of the experiments. Animal care and treatment were conducted in conformity with the institutional guidelines of The University of Tokyo.

2.2. Measurement of muscle tension

In the experiments to examine the effect of DEX on vascular smooth muscle, the endothelium was removed by gently rubbing the intimal surface with forceps after the organ culture procedure. The arterial rings with or without endothelium were placed in normal physiological salt solution of the following composition (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 would have catalyzed the oxidation of organic chemicals. The physiological salt solution was saturated with a 95% O_2 and 5% CO_2 mixture of pH 7.4 at 37 °C. Muscle tension was recorded isometrically with a force-displacement transducer connected to a strain amplifier (Model 3134 or 3170; Yokogawa, Japan) and an ink-writing recorder (Model 3056 or 3711; Yokogawa) under a resting tension of 10 mN. Data are shown as percentage of relaxation of the steady-state preconstriction elicited by 35-mM-high K^+-containing physiological salt solution.

2.3. Cross-section hematoxylin–eosin staining

After the incubation, arterial rings were fixed with 10% formaldehyde and embedded in paraffin. The 4-μm-thick sections were stained with hematoxylin–eosin and examined under a light microscope. Wall thickness was calculated as the diameter of the internal elastic lamina divided by the diameter of the external elastic lamina.

2.4. Whole-mount immunostaining

Whole-mount immunostaining was performed as previously described (Murata et al., 2002). The arterial rings were fixed with 2% paraformaldehyde. Fixed arteries were incubated in 0.3% Triton X-100 and 10% normal goat serum dissolved in phosphate-buffered saline (PBS) for 1 h and then probed with anti-CD31 monoclonal antibody (1 : 10 dilution) (Dako, Glostrup, Denmark) and a second reaction, using fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin G (Ig G) (1 : 200 dilution) (Vector Laboratories, Burlingame, CA, USA), was performed and then viewed. The images were captured using a Carl Zeiss confocal laser scanning microscope LSM510 imaging system (× 630). The microscope was equipped with excitation (458–488 nm) and emission (520 nm) filters. Five-micrometer-thick images from the endothelial cell surface were digitized under constant exposure time, gain, and offset.

2.5. Semi-quantitative RT-PCR analysis of eNOS mRNA expression

Total RNA was extracted from the arterial rings using the acid guanidinium thiocyanate–phenol–chloroform method with the TRIzol reagent. The concentration of total RNA was adjusted to 0.2 μg/μl with RNase-free distilled water. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (Nakazawa et al., 2001). The oligonucleotide primers for eNOS and GAPDH were designed as described previously (Nishida et al., 1992). The forward primers and reverse primers for

endothelial NO synthase and GAPDH were as follows: ATA GAA TTC ACC AGC ACC TTT GGG AAT GGC GAT (forward primer for eNOS), ATA GAA TTC GGA TTC ACT GTC TGT GTT GCT GGA CTC CTT (reverse primer for eNOS), TCC CTC AAG ATT GTC AGC AA (forward primer for GAPDH), and AGA TCC ACA ACG GAT ACA TT (reverse primer for GAPDH). After denaturation at 95 °C for 10 min, 28–44 cycles of amplification at 94 °C for 0.4 min, 55 °C for 1 min, and 72 °C for 1.5 min were performed using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedicals, Tokyo, Japan). The PCR products were electrophoresed onto 2% agarose gel containing 0.1% ethidium bromide. The possibility of their containing DNA was excluded by a PCR with total RNA without the reverse transcription step. The detectable fluorescence bands were visualized using an UV-transilluminator. The densitometric intensity of 260 base pairs for eNOS and of 308 base pairs for GAPDH at 40 cycles was quantified using an image-processing program, NIH Image 1.55. The results are expressed as the ratio of the optical density of eNOS to that of GAPDH.

2.6. Chemicals

The chemicals used were as follows: dexamethasone, substance P, ionomycin calcium salt (Sigma, St. Louis, MO, USA), Hank's balanced salt solution, penicillin–streptomycin (Gibco BRL, Tokyo, Japan), and DMEM (Nissui Pharmaceutical, Tokyo, Japan).

2.7. Statistical analysis

The results of the experiments are expressed as the means ± S.E.M. Statistical evaluation of the data was performed using unpaired Student's *t*-test and a value of $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Smooth muscle contraction

First, we assessed the acute effects of dexamethasone on smooth muscle contraction and relaxation using freshly isolated rabbit mesenteric arteries with endothelium. It was found that pretreatment of the fresh arteries with 100 nM–50 μM dexamethasone for 20 min had no effects on either 15–65-mM-high K^+-induced contractions or 100 nM substance P-induced relaxation of contraction induced by 35-mM-high K^+.

We next examined the chronic effects of dexamethasone on smooth muscle contractions (Fig. 1). In mesenteric arteries with endothelium cultured under FBS-free conditions in the presence or absence of 3 μM dexamethasone for 7 days, the 15–65-mM-high K^+ solution induced contractions in a concentration-dependent manner with an amplitude similar to that obtained in fresh mesenteric arteries (maximum force: fresh arteries, 56.8 ± 3.8; DMEM-cultured arteries, 52.1 ± 3.1; DMEM-cultured arteries with dexamethasone, 51.0 ± 4.2 mN/mg wet weight; $n = 5$ each). Chronic (7 days) 10% FBS-treatment slightly decreased the high K^+-induced contraction regardless of the presence or absence of 3 μM dexamethasone (maximum force: fetal bovine serum-cultured arteries, 38.5 ± 2.0 mN/mg wet weight; fetal bovine serum-cultured arteries with dexamethasone, 40.2 ± 4.8 mN/mg wet wt.; $n = 5$ each).

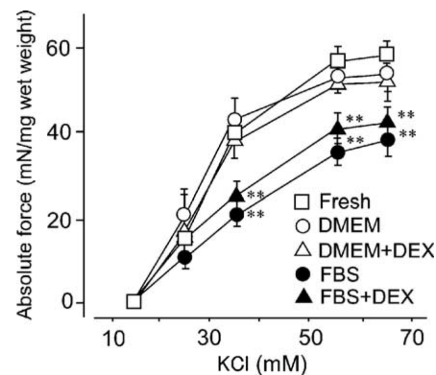

Fig. 1. Effects of 3 μM dexamethasone (DEX) on high K^+-induced contractions in mesenteric arteries cultured in the presence or absence of 10% fetal bovine artery (FBS) for 7 days. **Significantly different from the DMEM-cultured arteries without DEX (indicated as DMEM in the figure) at $P < 0.01$. Results are expressed as the means ± S.E.M. ($n = 6$ each).

3.2. Endothelium-dependent relaxation

We have previously reported that in the organ-cultured rabbit mesenteric artery, substance P and ionomycin induced endothelium-dependent relaxation in a concentration-dependent manner (Murata et al., 2001b; Yamawaki et al., 1999b). In the present study, we performed experiments by using concentrations of substance P and ionomycin to induce maximum endothelium-dependent vasodilation.

In the arteries without endothelium, 100 nM substance P was ineffective on high K^+ (35 mM)-induced contraction ($n = 4$). Compared with the freshly isolated arteries, substance P (100 nM) caused similar vasorelaxation of the muscle contraction elicited by 35-mM-high K^+ in the arteries cultured with DMEM for 7 days (fresh arteries, 50.5 ± 2.5%; DMEM-cultured arteries, 44.5 ± 8.5%; $n = 5$ each; Fig. 2A).

NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) (Busse et al., 2002) are well known as endothelium-derived relaxation factors and the production ratio of these relaxation-factors depends on the kind of stimulator, vascular and animal species. In the present study, we used 35 mM KCl-induced contraction to eliminate EDHF-induced vasodilation. We further examined whether substance P-induced vasodilation is induced by NO or prostacyclin in the DMEM-cultured rabbit mesenteric artery. The substance P-induced relaxation was almost abolished

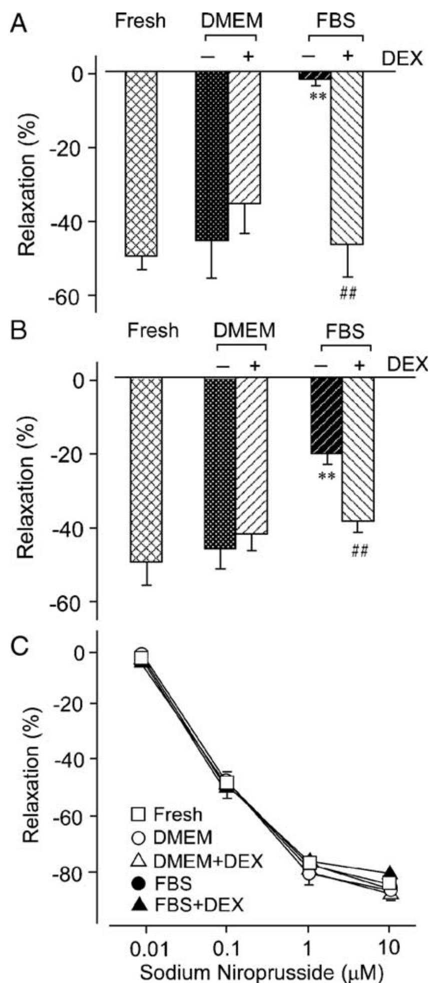

Fig. 2. (A) Effects of 3 μM dexamethasone (DEX) on substance P (0.1–30 nM)-induced relaxation in mesenteric arteries cultured in the presence or absence of 10% fetal bovine serum (FBS) for 7 days. (B) Effects of 3-μM dexamethasone on ionomycin (1–100 nM)-induced relaxation in mesenteric arteries cultured in the presence or absence of 10% FBS. Substance P or ionomycin was added after the 35-mM-high K^+-induced contraction had reached a steady-state level. Results are expressed as the means ± S.E.M. (n = 10–15). **Significantly different from the DMEM-cultured arteries without DEX (indicated as DMEM in the figure) with P < 0.01. ##Significantly different from the FBS-treated, DEX-free arteries (indicated as FBS in the figure) at P < 0.01. (C) Effects of 3 μM DEX on sodium nitroprusside (SNP)-induced relaxation in mesenteric arteries without endothelium cultured in the presence or absence of 10% FBS for 7 days. SNP was added after the 35-mM-high K^+-induced contraction had reached a steady-state level. Results are expressed as the means ± S.E.M. (n = 6 each).

by L-NMMA in the arteries cultured in DMEM (5.3 ± 2.8%, n = 6), suggesting that the endothelium-dependent relaxations of DMEM-cultured arteries are mainly attributable to NO production.

Culture of the tissue with DMEM in the presence of dexamethasone (3 μM) for 7 days did not affect the amplitude of 100 nM substance P-induced relaxation (37.2 ± 7.7%; n = 5). However, the substance P-induced endothelium-dependent relaxation was almost completely suppressed after 7 days of treatment with fetal bovine serum (2.1 ± 1.8%; n = 5). Culture of the tissue with dexamethasone (3 μM) for 7 days significantly restored the fetal bovine serum-induced inhibition of the substance P-induced relaxation (48.2 ± 7.6%; n = 5, Fig. 2A).

In the freshly isolated arteries, ionomycin (100 nM) caused the same amplitude of vasorelaxation of contraction elicited by 35-mM-high K^+ in the arteries cultured in DMEM for 7 days (fresh arteries, 49.2 ± 8.8%; DMEM-cultured arteries, 43.2 ± 6.8%; n = 5 each; Fig. 2B). The vasodilator effect of ionomycin (100 nM) on 35-mM-high K^+-induced contraction was also significantly attenuated in the fetal bovine serum-cultured arteries (18.0 ± 2.8%, n = 6). The dexamethasone treatment for 7 days almost completely recovered the inhibition of ionomycin-induced vasodilation by the fetal bovine serum treatment (38.5 ± 3.8%; n = 5).

3.3. Sodium nitroprusside-induced relaxation

In all of the mesenteric arteries without endothelium, sodium nitroprusside (1 nM–10 μM) caused vasorelaxation of the contractions elicited by 35-mM-high K^+ solution in a concentration-dependent manner (Fig. 2C). The maximum relaxation due to sodium nitroprusside was 90.6 ± 3.8% in DMEM-cultured arteries, 87.43 ± 3.8% in fetal bovine serum-cultured arteries, 89.2 ± 4.3% in DMEM-cultured arteries with dexamethasone, and 90.4 ± 1.8% in fetal bovine serum-cultured arteries with dexamethasone (n = 5 each).

3.4. Morphological changes of endothelial cells in cross-sectioned hematoxylin–eosin staining

Fig. 3A shows representative micrographs of cross-sectioned and hematoxylin–eosin stained tissue samples from arteries exposed to one of four experimental procedures. Endothelial cells of fresh mesenteric arteries adhered tightly to the inner surface. In the arteries cultured without dexamethasone, endothelial cells were also located along the tunica intima and no morphological change was observed. In the fetal bovine serum-treated arteries (also without dexamethasone), in contrast, many endothelial cells adhered to the vascular wall (Fig. 3, FBS 1), but in some regions of the arterial lumen, floating and detachment of endothelial cells and clots of endothelial cells were observed (Fig. 3, FBS 2). Dexamethasone treatment completely prevented the fetal bovine serum-induced endothelial detachment from the surface of the tunica interna (fetal bovine serum + dexamethasone).

In the medial layer of the fresh, DMEM-cultured arteries and the DMEM-cultured arteries with dexamethasone, most smooth muscle cells were arranged in an orderly fashion and the shape of the nucleus was generally flat. In the serum stimuli-treated arteries with or without dexamethasone, in contrast, arterial thickening of arterial walls with a shortening of internal diameter and loss of smooth muscle cell orientation and nuclear rounding were observed (Fig. 3B).

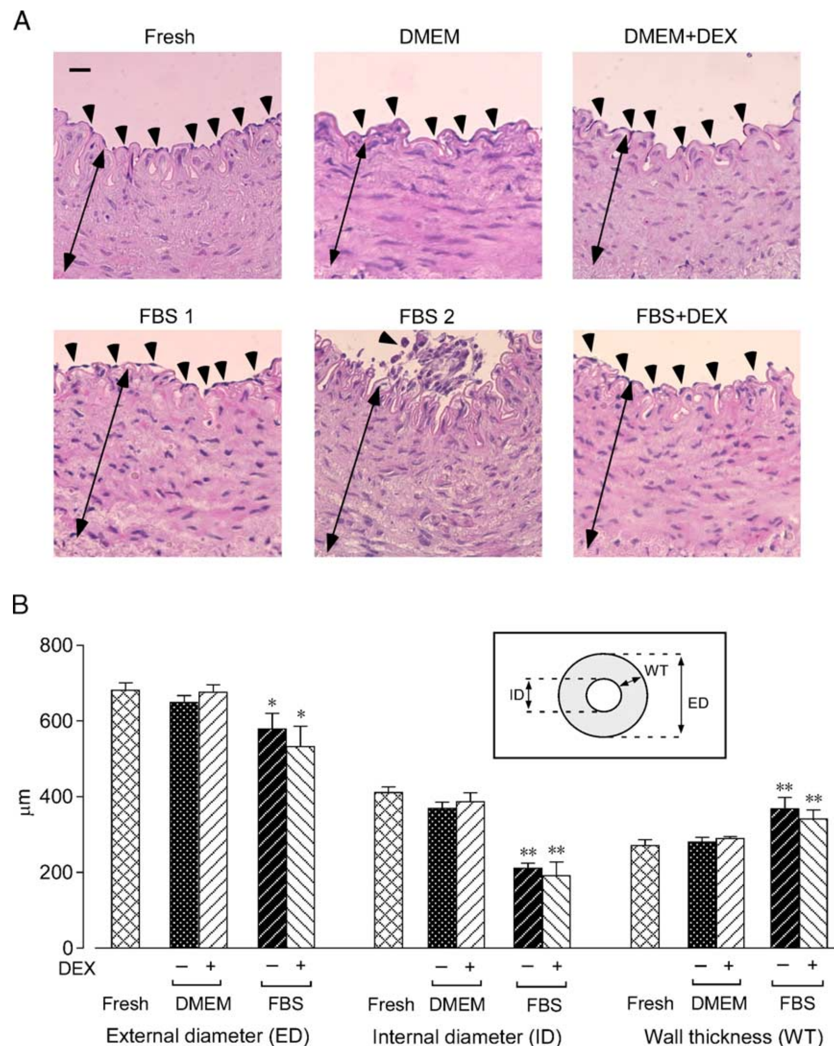

Fig. 3. (A) Effects of 3-μM dexamethasone (DEX) treatment on endothelial morphology (cross-sectioned hematoxylin–eosin staining image) after 7-day culture in the presence or absence of 10% fetal bovine serum (FBS). Arrowheads indicate endothelial cells and arrows indicate the medial layer in the vascular wall. Bar = 50 μm. A typical trace from the experiments is shown. (B) Effects of 3 μM DEX treatment on wall thickness after 7-day culture in the presence or absence of 10% FBS in hematoxylin–eosin staining. Results are expressed as the means ± S.E.M. ($n = 6$ each). *, **Significantly different from the DMEM-cultured arteries without DEX (indicated as DMEM in the figure) at $P < 0.05$ or 0.01, respectively.

3.5. Morphological changes of endothelial cell in whole-mount immunostaining

We conducted whole-mount immunostaining using CD31, an endothelial cell marker antibody to stain the endothelial surface. Fig. 4 shows typical images from each of the four experiments. In the fresh and DMEM-cultured mesenteric arteries, endothelial cells were attached tightly to the tunica interna ($n = 5$) and were tightly blocked like cobblestones. Observation of the cross-sectioned and hematoxylin–eosin-stained sections of the fetal bovine serum-treated mesenteric arteries revealed that endothelial cells were excoriated from the tunica interna in some regions (Fig. 4A, FBS 1–3). In other regions, however, endothelial cells were still attached to the tunica interna, although the cobblestone appearance was distorted (Fig. 4, FBS 4 and 5). Treatment with 3 μM dexamethasone completely prevented the serum stimuli-induced endothelial detachment. However, in the fetal bovine serum-cultured arteries with dexametahsone, endothelial rippling in the transverse axis direction, i.e., against blood flow, was kept intact (Fig. 4, FBS + DEX). We further quantified the CD31-positive area of the tunica interna as shown in Fig. 4B. Results indicate that the CD31-positive area was significantly decreased in the fetal bovine serum-treated artery. Dexamethasone completely restored the decreased area.

3.6. eNOS mRNA expression

We further investigated eNOS mRNA expression by using semi-quantitative RT-PCR analysis. In the fetal bovine serum-treated artery, eNOS mRNA expression was significantly decreased. Dexamethasone treatment restored the down-regulated eNOS mRNA expression (Fig. 5).

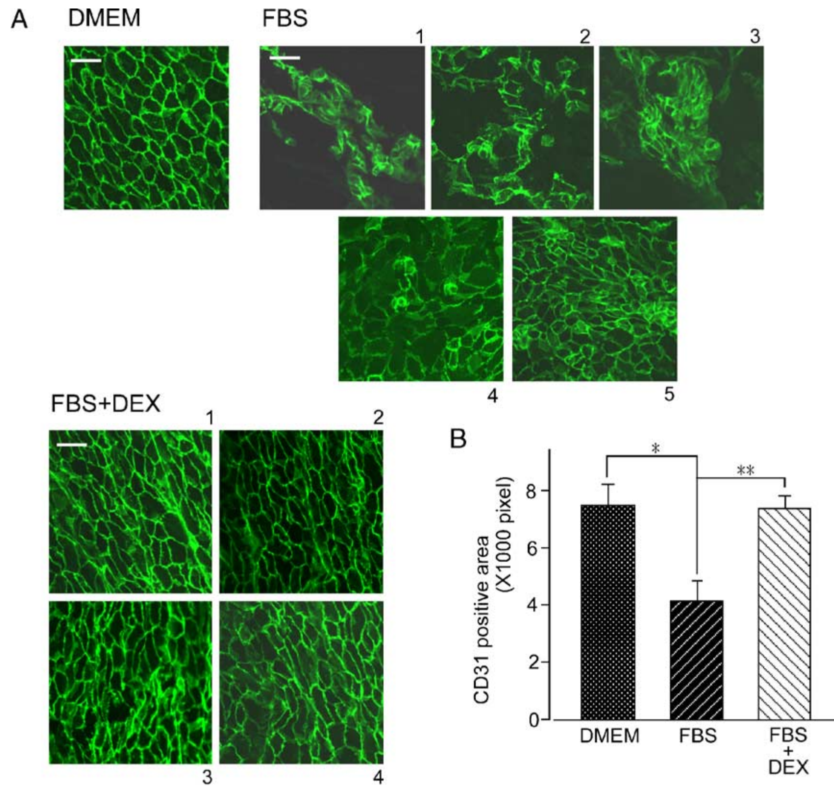

Fig. 4. (A) Effects of 3-μM dexamethasone (DEX) treatment on endothelial morphology (surface image) after 7-day culture in the presence or absence of 10% fetal bovine serum (FBS). Endothelial cells were stained with a specific endothelial cell marker, CD31. The images were captured with a Carl Zeiss confocal laser scanning microscope LSM510 imaging system. To compare the cell area in situ, 5.1-μm-thick sections showing the widest area in each cell were captured under constant exposure time, gain, and offset. Bar = 20 μm. (B) The CD31-positive fluorescent area was also quantified by calculating the CD31-positive pixel area/total area. Each column indicates the means ± S.E.M. ($n = 4–5$). **, *Significantly different at $P < 0.01$ or $P < 0.05$.

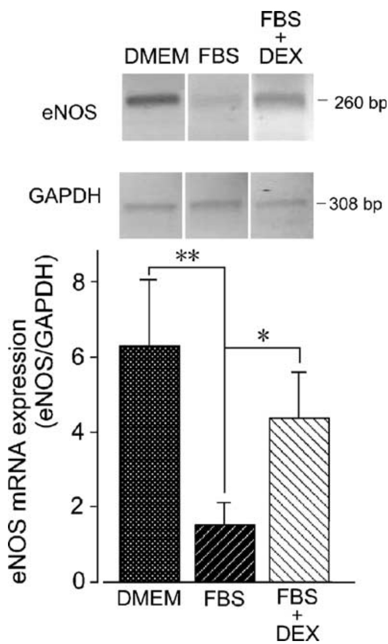

Fig. 5. Effect of 3-μM dexamethasone (DEX) treatment on eNOS mRNA expression level after 7-day culture in the presence or absence of 10% fetal bovine serum (FBS). Each column indicates the means ± S.E.M. ($n = 4$). **, *Significantly different at $P < 0.01$ or $P < 0.05$.

4. Discussion

In the present experiments, using an organ culture procedure, we revealed that treatment with 3 μM dexamethasone prevented fetal bovine serum-induced endothelial dysfunction and morphological changes. A lower dose (0.3 μM) of dexamethasone had no such protective effects against the fetal bovine serum-induced endothelial dysfunction ($n = 4$, data not shown). On the other hand, a higher concentration (30 μM) of dexamethasone yielded no additional improvement over that observed by 3 μM dexamethasone ($n = 4$, data not shown). Consequently, we chose 3-μM dexamethasone administration throughout this study and investigated the mechanism of its beneficial effect on the serum stimuli-induced endothelial dysfunction.

As demonstrated in Fig. 2, fetal bovine serum treatment impaired not only the endothelium-dependent relaxations induced by receptor stimulation but also those induced by ionomycin, a Ca^{2+} ionophore. This result suggests that the NO-producing pathway downstream Ca^{2+} increment may be impaired in the endothelial cells. Treatment with 3 μM dexamethasone restored both the impairment of endothelium-dependent relaxation induced by receptor stimulation and that induced by ionomycin (Fig. 2A and B).

Our previous studies showed that chronic fetal bovine serum treatment significantly impaired the contraction of rabbit mesenteric artery (Yamawaki et al., 2000) and this impairment was confirmed in the present study. In accordance with the impairment of the arterial smooth muscle contractility, we observed proliferative changes, such as wall thickness, by fetal bovine serum treatment. In this study, we revealed that the 3-μM dexamethasone treatment did not recover the impairment of contractility in the serum stimuli-cultured mesenteric arteries (Fig. 1). In addition, 3 μM of dexamethasone had no appreciable effect on the proliferative changes in the vascular wall (Fig. 3). These results suggest that dexamethasone treatment restored the impaired endothelial function without changing the proliferative effect on the vascular smooth muscle wall in this experimental condition.

We further investigated the effects of dexamethasone on the endothelial impairment elicited by fetal bovine serum treatment. Chronic serum treatment for 7 days induced peeling of endothelial cells in some regions. In this study, although 3 μM of dexamethasone did not prevent the fetal bovine serum-induced impairment of contractility or vascular wall hypertrophy, it completely preserved the adherence of endothelial cells to the vascular wall. In addition, the present study further demonstrated that the responsiveness of vascular smooth muscle to sodium nitroprusside was not changed by dexamethasone treatment, suggesting that the vasodilation pathway downstream of cGMP accumulation in smooth muscle cells may not be affected by dexamethasone treatment (Fig. 2C). Taken together, these results indicate that the physical detachment of the endothelium from the vascular wall may be considered part of the serum stimuli-induced endothelial dysfunction and dexamethasone treatment can completely inhibit these changes. The endothelial adhesion is mediated by a surface-bound receptor of the integrin superfamily (Juliano and Varner, 1993). In addition, extracellular matrix regulates the expression of adhesion molecules (Juliano and Haskill, 1993). Because the actions of glucocorticoids are generally considered to be mediated by alteration of the expression of specific target molecules, it can be assumed that the expression of some genes related to adhesion molecules and extracellular matrix may contribute to the occurrence of FBS-induced endothelial detachment and DEX may regulate the expression of these genes, resulting in an improvement of this impairment.

In the present study, the damage to the endothelial morphology was not ubiquitous and areas with intact endothelium were often observed (see Figs. 3A and 4, FBS 1, and 4, FBS 4 and 5). In contrast, the endothelium-dependent relaxation was almost abolished, as shown in Fig. 2A. This discrepancy indicates another possible means of inducing impairment of endothelial-dependent relaxation. We have previously found that the long-term treatment of mesenteric arteries with platelet-derived growth factor (PDGF) or FBS induced impairment of endothelium-dependent relaxation by decreasing the mRNA expression of endothelial NO synthase (Yamawaki et al., 1999a,b). In the present study, we confirmed eNOS mRNA expression is down-regulated in the serum-stimulated artery and dexamethasone restored the eNOS mRNA expression (Fig. 5). On the other hand, as shown in Fig. 2A and B, the serum stimuli-induced inhibition of vasorelaxation by substance P was more sensitive than that induced by ionomycin. It was reported that eNOS activation by receptor stimulation is regulated not only by Ca^{2+}/calmodulin but also by other mechanisms, such as eNOS phosphorylation by serine/threonine kinase Akt and heat shock protein 90 (Fleming et al., 2001; Fulton et al., 1999; Govers and Rabelink, 2001), indicating that fetal bovine serum treatment may affect these signal transduction pathways to activate eNOS activity. Further examination will be needed to clarify this point.

In conclusion, DEX has beneficial effects on the impairments of arterial relaxation due to proliferative stimuli, which may in part be mediated through its protection of endothelial adhesion to the vascular wall and its recovery of eNOS mRNA expression.

Acknowledgments

This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education (Japan) and by research fellowships from the Japan Society for the Promotion of Science for Young Scientists.

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