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ENDOTHELIN-1 INHIBITS AND ENHANCES CONTRACTION OF PORCINE CORONARY ARTERIAL STRIPS WITH AN INTACT ENDOTHELIUM

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Using front-surface fluorometry and fura-2-loaded porcine coronary arterial strips with an intact endothelium, changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]i$) and tension of smooth muscle were simultaneously monitored in an attempt to determine the vasoactive properties of endothelin-1 (ET-1). ET-1 in low concentrations (0.1-1nM) caused a significant transient decrease in $[Ca^{2+}]i$ and tension of the strips precontracted with 10^{-7}M U-46619. The maximal decreases in $[Ca^{2+}]i$ and tension were obtained with 0.6nM ET-1. In higher concentrations (1nM-100nM), there was no reduction in $[Ca^{2+}]i$ or tension; the contraction induced by U-46619 was potentiated. The decreases in $[Ca^{2+}]i$ and tension induced by ET-1 were inhibited by the mechanical removal of the endothelium or by pretreatment with N^G-nitro-L-arginine and were slightly attenuated by indomethacin. Thus, ET-1 in low concentrations can induce endothelium-dependent transient relaxations accompanied by transient reductions of $[Ca^{2+}]i$ in isolated porcine coronary arteries. This effect is mainly mediated by the release of endothelium-derived relaxing factor.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide present in conditioned medium of cultured porcine endothelial cells (1). It has been reported that, prior to a prolonged increase, intravenous injection of ET-1 into the intact animal caused an initial transient decrease in systemic blood pressure (2,3). In the perfused mesenteric artery, ET-1 induced a dose-dependent reduction in perfusion pressure (4-7). This vasodilator response was thought to be induced by endothelium-derived relaxing factor (EDRF)(8) or by prostacyclin (9) released by ET from endothelial cells (4, 6). However, the precise mechanism of the vasodilating effects of ET-1 is a matter of controversy. Despite the well-documented vasodilator effect seen *in vivo* and in *in vitro* perfusion experiments, there is little documentation concerning the ET-1-induced vasorelaxation in *in vitro* tension studies (10,11). Furthermore, there has been no report in which changes in cytosolic Ca^{2+} concentrations

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ABBREVIATIONS: BK, bradykinin; $[Ca^{2+}]i$, cytosolic Ca^{2+} concentrations; EDRF, endothelium-derived relaxing factor; ET-1, endothelin-1; Fura-2/AM, acetoxymethyl ester form of fura-2; L-NNA, N^G-nitro-L-arginine.

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([Ca^{2+}]i) have been recorded. We simultaneously recorded changes in smooth muscle [Ca^{2+}]i and the tension induced by ET-1, using front-surface fluorometry and fura-2-loaded porcine coronary arterial strips with an intact endothelium. To assess the possible involvement of EDRF and/or prostacyclin, we examined the effect of N^G-nitro-L-arginine (L-NNA)(an inhibitor of nitric oxide formation)(12) and indomethacin (a cyclooxygenase inhibitor) on the ET-1-induced vasorelaxation.

MATERIALS AND METHODS

Left circumflex coronary arteries were excised from hearts of pigs immediately after slaughter. Segments of 2-3 cm from the origin were cut into circular strips (1x5 mm). Strips with or without endothelium were loaded with the Ca^{2+} indicator dye, fura-2, by incubating in oxygenated (95% O_2/5% CO_2) Dulbecco's modified Eagle's medium containing 25μM fura-2/AM (an acetoxymethyl ester form) and 2.5% fetal bovine serum for 3-4 h at 37°C. The strips were then rinsed with normal physiological salt solution (normal PSS: NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $CaCl_2$ 1.25 and D-glucose 11.5, in mM) for purposes of equilibration prior to start of measurements. Isometric tension of the strips, mounted vertically in a quartz organ bath, was measured using a strain gauge transducer (TB-612T Nihon Koden, Tokyo, Japan), simultaneously with [Ca^{2+}]i. During one hour of equilibration, the strips were stimulated with 118mM K^+ every 15 min, and the resting tension was increased stepwise to 250 mg. The steady responsiveness of each strip to 118mM K^+ was obtained before starting the experiment. When the contraction stabilized, bradykinin (10^{-6}M) was added to test for the presence of functional endothelium. Indomethacin (10^{-5}M) and L-NNA (10^{-4}M) were added at the beginning and the end of the equilibration period, respectively. As a control experiment, the endothelium of the coronary arterial strips was mechanically removed. The fluorescence of the fura-2-Ca^{2+} complex was monitored with front-surface fluorometry (13,14), using equipment specifically designed for fura-2 fluorometry (CAM-OF1, Japan Spectroscopic Co., Tokyo, Japan). The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored at 500 nm emission with the sampling rate of 400 Hz and expressed as a percentage, assuming the steady-state values in normal (at rest :5.9mM K^+) and 118mM K^+ PSS (at high K^+-depolarization) recorded at the beginning of each measurement to be 0% and 100%, respectively. The absolute values of [Ca^{2+}]i were calculated using the method described by Grynkiewicz et al (15).

Chemicals Synthetic ET-1 was obtained from Peptide Institute Co. Ltd. (Osaka, Japan), U-46619 (11-dideoxy-9-methanoepoxy-prostaglandin F2α), N^G-nitro-L-arginine, indomethacin and fura-2/AM were purchased from Aldrich Chemical Co.(U.S.A), Calbiochem Co (U.S.A), WAKO (Osaka, Japan) and DOJINDO (Kumamoto, Japan), respectively. Fura-2/AM was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and diluted in the medium just before loading the dye. The final concentration of DMSO was 5%.

Data analysis Values were expressed as mean ± standard error. Student's t-test was used to determine statistical significance and P values less than 0.05 were considered to be significant.

RESULTS

Both endothelial and smooth muscle cells were loaded with fura-2. While it was reported that fura-2-fluorescence changes of endothelial cells in vascular strips might interfere with estimation of changes in [Ca^{2+}]i in smooth muscles (16), we found the fluorescence of endothelial cells, if any, is negligible, and that changes in fluorescence in the strips indicate changes in [Ca^{2+}]i in the smooth muscle cells (data not shown). 1) There was no increase in the fluorescence ratio following the application of bradykinin to endothelium-intact coronary arterial strips at rest. Bradykinin elevates [Ca^{2+}]i in cultured endothelial cells (17), 2) There was no differences in changes in fluorescence or in force during contractions induced by K^+-depolarization, in the

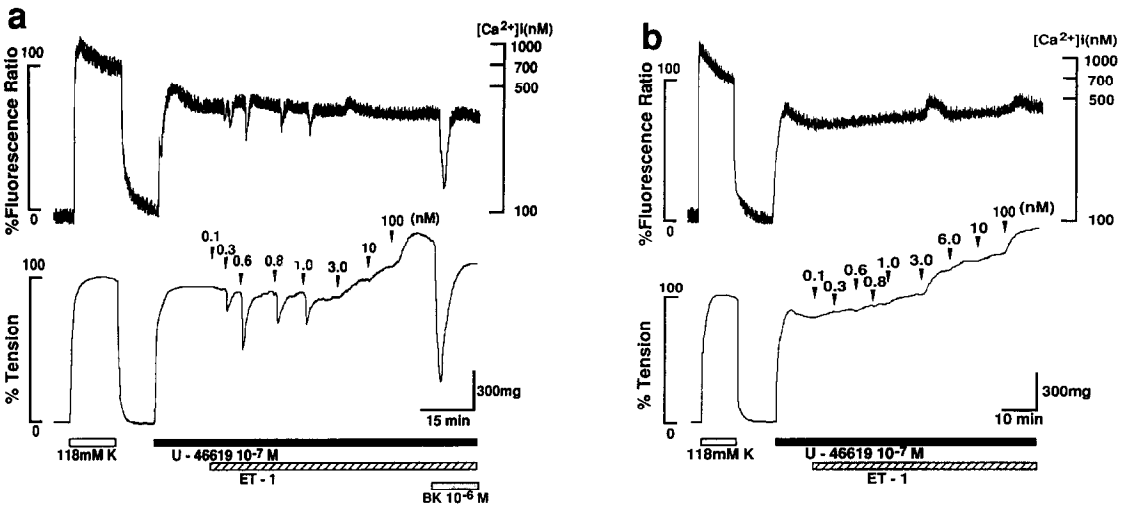

Figure 1. Representative recordings of the effects of various concentrations of ET-1 (▨) on the elevations of $[Ca^{2+}]i$ (upper trace) and tension (lower trace) of the porcine coronary arterial strips with (a) and without (b) endothelium precontracted with 10^{-7}M U-46619 (■). ET-1 (final concentrations are expressed in nM) was applied at the point indicated by arrow head. (a)Bradykinin (BK,10^{-6}M,▩) was applied at the end of this protocol to confirm that the function of endothelium was well preserved.

presence or absence of the endothelium. We found no difference in decreases in the force induced by bradykinin in strips with an intact endothelium, with and without fura-2 loading. We already reported that loading vascular strips with fura-2 had no effects on contractility (14).

Figure 1a, and b show representative recordings of the effect of cumulative applications of ET-1 (0.1-100nM) on $[Ca^{2+}]i$ and tension of the strip precontracted with 10^{-7}M U-46619, a thromboxane A_2 analog. When U-46619 was applied to strips with and without endothelium, $[Ca^{2+}]i$ and tension rapidly increased and reached steady state levels within 15min. Both levels were maintained for at least 60min of observations. The application of low concentrations of ET-1 (0.1-1nM) to strips with an intact endothelium significantly inhibited the elevation of $[Ca^{2+}]i$ and tension (Figs 1a and 2). The steady-state levels of $[Ca^{2+}]i$ and tension development induced by U-46619 were 66.3±2.01% (354.4nM;n=6) and 92.3±1.75% (n=6), respectively. The maximum reductions of $[Ca^{2+}]i$ and tension ($[Ca^{2+}]i$=53.9±1.38%,n=6, 284.4nM; and tension=70.3±4.26%,n=6) were obtained with 0.6nM ET-1. The duration of ET-1-induced decreases in $[Ca^{2+}]i$ and tension was 2-3min. With a concentration of 3nM or higher, there was no inhibition of $[Ca^{2+}]i$ or tension and ET-1 enhanced the elevations of $[Ca^{2+}]i$ and tension development induced by U-46619 (Fig 2). When 10^{-6}M bradykinin was applied at the end of the specific protocols, the $[Ca^{2+}]i$ and tension were significantly decreased to nearly resting levels, thereby indicating that the endothelium was intact and functional.

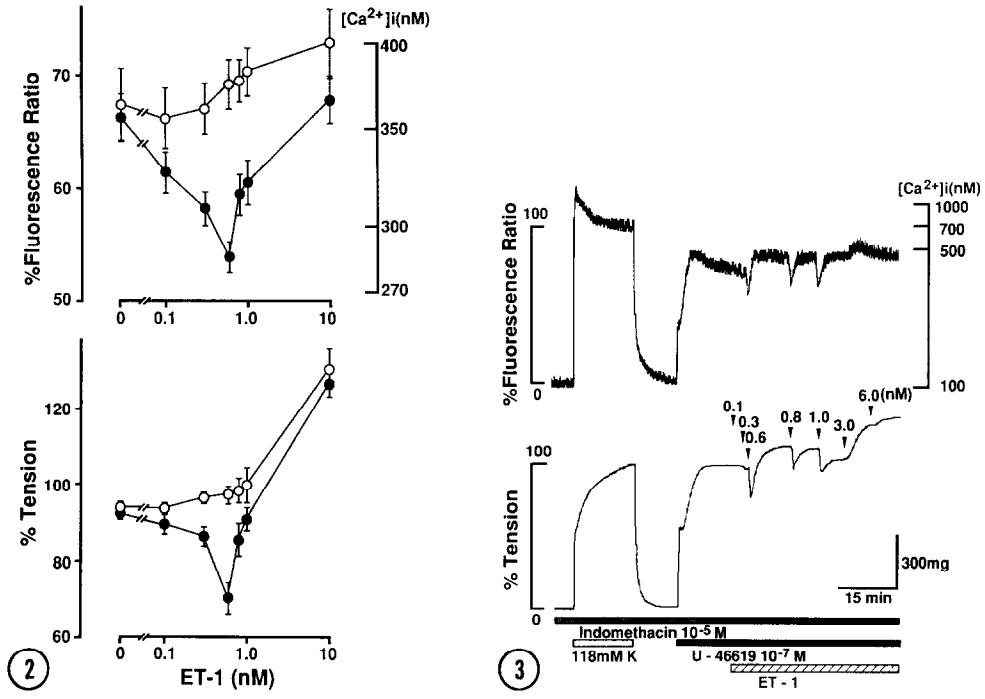

Figure 2. The concentration-response relationships for the effects of ET-1 on the changes in $[Ca^{2+}]i$ and tension development induced by U-46619, in the absence (open circles, 5 measurements) and presence (closed circles, 6 measurements) of endothelium. abscissa :final concentration of ET-1 in nM. Vertical bars represent s.e. mean.

Figure 3. Representative recordings of the effects of 10^{-5}M indomethacin (▩) on ET-1-induced (▨) decreases in $[Ca^{2+}]i$ (upper trace) and tension (lower trace) of porcine coronary arterial strips with endothelium, precontracted with 10^{-7}M U-46619 (■). After pretreatment with 10^{-5}M indomethacin, the protocol similar to Figure 1 was carried out.

In the strips without the endothelium, ET-1 of low or high concentrations did not inhibit the elevations of $[Ca^{2+}]i$ and tension but did enhance the elevations of $[Ca^{2+}]i$ and tension induced by U-46619, in a dose-dependent manner (Fig 1b and 2). As shown in Figure 2, the steady-state levels of $[Ca^{2+}]i$ and tension development after 10nM ET-1 application to strips without endothelium ($[Ca^{2+}]i$=72.9±2.88%,n=5, 400.2nM; tension=130.6±5.18%,n=5) did not significantly differ from those observed in strips with the endothelium ($[Ca^{2+}]i$=67.7±1.98%,n=6, 363.6nM; tension=125.7±3.31%,n=6). Thus, an inhibition of the contraction induced by low concentrations of ET-1 depends on the presence of an intact endothelium and the enhancing effects of high concentrations of ET-1 on U-46619-induced contractions are independent of endothelial cells.

To examine the contribution of prostacyclin and EDRF in the ET-1-induced relaxation, strips with an intact endothelium were pretreated with 10^{-5}M indomethacin or 10^{-4}M L-NNA. Decreases in $[Ca^{2+}]i$ and tension induced by ET-1 were slightly attenuated by indomethacin (Fig

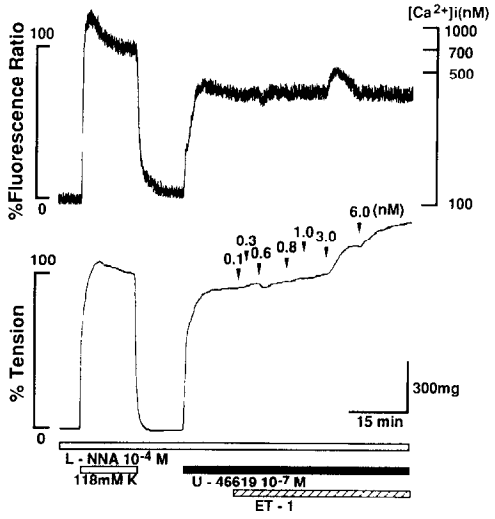

Figure 4. Representative recordings of the effects of 10^{-4}M L-NNA (▨) on ET-1-induced (▨) decreases in $[Ca^{2+}]i$ (upper trace) and tension (lower trace) of porcine coronary arterial strips with endothelium precontracted with 10^{-7}M U-46619 (■). After pretreatment with 10^{-4}M L-NNA, the protocol similar to that for Figure 1 was carried out.

3), but were significantly inhibited by L-NNA (Fig 4). Figure 5 shows the inhibitory effects of indomethacin and L-NNA on the maximal decreases of $[Ca^{2+}]i$ and tension induced by 0.6nM ET-1. The extent of decrease in $[Ca^{2+}]i$ was slightly attenuated by indomethacin and was markedly inhibited by L-NNA. ($P<0.05$ and $P<0.001$, respectively). The decrease in tension was slightly,

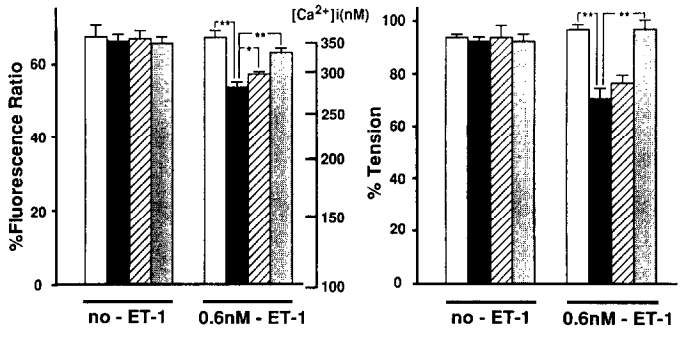

Figure 5. Elevations of $[Ca^{2+}]i$ (left panel) and tension (right panel) induced by 10^{-7}M U-46619 in the absence (left) and the presence (right) of 0.6nM ET-1.(□; strips without endothelium, ■; strips with endothelium, ▨; strips with endothelium pretreated by 10^{-5}M indomethacin, ▩; strips with endothelium pretreated with 10^{-4}M L-NNA). Each column represents mean ± s.e (shown in vertical lines; n=4-6). * $P<0.05$, ** $P<0.001$

but not significantly, attenuated by indomethacin, and was completely inhibited by L-NNA ($P<0.001$). Thus, while EDRF appears to play a major role in the ET-1-induced vasorelaxation, the minor contribution of prostacyclin in this relaxation cannot be neglected.

DISCUSSION

We obtained evidence that ET-1 causes a transient relaxation (or inhibition of the contraction) of strips of the pig coronary artery with an intact endothelium precontracted by U-46619, this relaxation is accompanied by a concomitant reduction in $[Ca^{2+}]i$. This is the first report to directly show that ET-1, the potent vasoconstrictor peptide, also induces a decrease in $[Ca^{2+}]i$ and tension of vascular smooth muscle. Since this effect disappeared with removal of the endothelium, the $[Ca^{2+}]i$ decrease induced by ET-1 is probably mediated by actions through endothelial cells. The requirement of endothelial cells for the ET-1-induced depressor or relaxing effect is in good agreement with reported data (2-7,10,11). It is now well accepted that endothelial cells play a pivotal role in regulation of the contractile state of vascular smooth muscle, by secreting not only vasoconstrictors such as ET-1 and thromboxane, but also vasodilators such as EDRF and prostacyclin. In the present study, the ET-1-induced endothelium-dependent relaxation was inhibited by L-NNA more effectively than by indomethacin (Fig 3,4 and 5), hence the ET-1-induced relaxation might be mediated mainly by EDRF in porcine coronary arteries. This result is supported by data that the ET-1 induced dose-related reduction in blood pressure (18) *in vivo* and the perfusion pressure (4,6,7) in rat isolated perfused mesenteric arteries, and these depressor effects were mediated by the release of EDRF. Topouzis et al. (19) reported that ET-1 increased the tissue content of guanosine 3',5'-cyclic monophosphate, an intracellular second messenger evoked by EDRF (20) in rat aorta with endothelium. However, it has been reported that ET-1 stimulates the release of prostacyclin *in vivo* in the anesthetized dog (21) and *in vitro* from guinea pig or rat isolated lung (4), from bovine aortic endothelial cells (22) and porcine coronary artery (11). Thus, there may be different mechanisms related to the vasorelaxation in different species as well as in experimental protocols.

It is generally accepted that the release of EDRF is regulated by a transient increase in $[Ca^{2+}]i$ of endothelial cells (23). Vigne *et al.* (24) demonstrated the presence of high-affinity binding sites for ET-1 in cultured endothelial cells and that ETs stimulate phosphatidylinositol hydrolysis and DNA synthesis concomitant with the elevation of $[Ca^{2+}]_i$. In addition, we recorded the Ca^{2+}-transient induced by ET-1 in endothelial cells *in situ* (25). Thus, it is conceivable that ET-1 induces vasorelaxation via receptors with a high affinity for ET-1.

In summary, the present findings provide direct evidence that in isolated porcine coronary arteries, low concentrations of ET-1 can act on the endothelium receptor to inhibit an increase in $[Ca^{2+}]i$ and tension, mainly through the release of EDRF, whereas high concentrations of ET-1 enhance the U-46619-induced contraction through a direct action on smooth muscle. Thus, ET-1 may play an important role in controlling vascular tonus not only by acting directly on vascular

smooth muscle cells to induce contraction in a paracrine manner, but also by acting on endothelial cells to induce relaxation in an autocrine manner.

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