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AUTOCRINE PRODUCTION OF ENDOTHELIN-1 PARTICIPATES IN THE GLUCOCORTICOID-INDUCED Ca^{2+} INFLUX INTO VASCULAR SMOOTH MUSCLE CELLS

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Summary: We determined whether endothelin-1 (ET-1) is associated with glucocorticoid-induced Ca^{2+} influx into vascular smooth muscle cells by examining the effects of the ET_A receptor antagonist FR139317 on dexamethasone-induced $^{45}Ca^{2+}$ uptake and dihydropyridine binding by rat A7r5 cells. FR139317 inhibited the dexamethasone-induced $^{45}Ca^{2+}$ uptake and [*methyl*-^{3}H]PN 200-110 binding in a dose-dependent manner. Slot blot analysis revealed that dexamethasone increased protein kinase C-α in A7r5 cells and that this effect was also abolished by FR139317. Dexamethasone stimulated the release of immunoreactive endothelin-1 from A7r5 cells into the culture medium. These results suggest that endothelin participates in the glucocorticoid-induced Ca^{2+} influx through dihydropyridine-sensitive channels in an autocrine manner, possibly linked to the activation of protein kinase C-α.

Glucocorticoids enhance the response of vascular smooth muscle to vasopressor substances (1-5), and cause hypertension *in vivo* (6,7). It is generally recognized that vascular smooth muscle contraction is initiated by an increase in intracellular free Ca^{2+}. We previously reported that glucocorticoids increase Ca^{2+} uptake into rat A7r5 vascular smooth muscle cells (8). We are currently examining the interactions between glucocorticoids and vasoconstrictive substances.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated from the culture medium of porcine aortic endothelial cells (9). Many investigators have reported that ET is released from various tissues such as the trachea (10), kidney (11) and cells, such as neurons (12) and breast epithelial cells (13). Rat aortic smooth muscle cells are also capable of secreting ET-1 (14). Growth factors and vasoactive peptides induce prepro ET-1 mRNA expression in vascular smooth muscle cells (VSMC) isolated from spontaneously hypertensive rats which is

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Abbreviations: ET-1, endothelin-1; VSMC, vascular smooth muscle cells.

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accompanied by the release of immunoreactive ET-1 into the culture medium (15). Human VSMC also produce ET mRNA and the peptide (16). Thus, it is possible that ET-1 acts not only as a paracrine hormone, but also an autocrine hormone in VSMC.

These observations led us to examine whether ET-1 plays a role as an autocrine regulator of glucocorticoid-induced Ca^{2+} influx into VSMC. In the present study, we investigated the autocrine role of ET-1 in $^{45}Ca^{2+}$ uptake and [^{3}H]dihydropyridine binding in dexamethasone-treated A7r5 cells, using an ET_A receptor antagonist.

MATERIALS AND METHODS

Dexamethasone was purchased from Sigma (St. Louis, MO). (R)2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid (FR139317) was a gift from Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan). Isopropyl-4-(2,1,3-benzoxadiazol-4yl)-1,4-dihydro-5-methoxy-carbamyl-2,6-dimethyl-3-pyridinecarboxylate (PN 200-110) was obtained from Sandoz (Basel, Switzerland). $^{45}Ca^{2+}$ (0.6-1.6 mCi/mmol) and [*methyl*-^{3}H]PN 200-110 (85.9 Ci/mmol) were obtained from New England Nuclear (Wilmington, DE). Mouse monoclonal antibodies against protein kinase C-α, protein kinase C-β and protein kinase C-γ were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

Cell culture The A7r5 rat aortic smooth muscle cell line (17) was obtained from the American Type Culture Collection. Cells were grown in 75 cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in 5% CO_2 and humidified air at 37 °C. Cells were subcultured into 12-well plates (24 mm diameter per well) for the $^{45}Ca^{2+}$ uptake, [*methyl*-^{3}H]PN 200-110 binding and measurements of immunoreactive ET-1. For a slot blot analysis of protein kinase C isozymes, they were subcultured to Petri dishes (90 mm diameter). Phase-contrast microscopy revealed that the A7r5 cells had typical smooth muscle morphology, including hills and valleys.

Treatment with dexamethasone and FR139317 After cells reached confluence, the medium was changed to DMEM containing 10% charcoal treated FCS to eliminate the effects of endogenous steroids (8). Dexamethasone (100nM) and/or various concentrations of FR139317 were added to each 12-well plate. Cells treated with these agents for 24 hours were used for measurements of $^{45}Ca^{2+}$ uptake, [*methyl*-^{3}H]PN 200-110 binding and slot blot analysis of protein kinase C isozymes.

Measurements of immunoreactive ET-1 in culture medium Aliquots of culture medium from dexamethasone-treated and control cells were obtained at the times indicated in the figure legends. Radioimmunoassay for ET-1 was performed as described by Ando et al. (18), using ET-1 antiserum and ^{125}I-ET-1 (Amersham Corp., United Kingdom). The antibody did not cross-react with ET-2 or ET-3 although there was a 2% crossreactivity with human big ET-1.

Measurements of $^{45}Ca^{2+}$ uptake $^{45}Ca^{2+}$ uptake measurements were performed as described in our previous study (8). Cells were washed three times using buffer A : 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)- tris(hydroxymethyl)aminomethane(Tris)buffer, pH7.4, containing (in mM) 135 NaCl, 5 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, and 10 dextrose at 37 °C. After a brief pre-incubation, $^{45}Ca^{2+}$ (0.75 μCi) was added to each well, and then incubated at 37 °C for 2 min. Incubations were terminated by aspirating the $^{45}Ca^{2+}$ solution and washing each well three times with ice-cold buffer A containing 3 mM $LaCl_3$. Cells were solubilized in 1 ml of 0.2% (wt/vol) sodium dodecyl sulfate (SDS) (1 ml), and the aliquot (700 μl) was counted in a scintillation vial with 4 ml Ecoscint scintilator (National Diagnostics, Somerville, NJ) using an Aloka scintillation spectrometer.

Dihydropyridine receptor determination The binding of [*methyl*-^{3}H]PN 200-110 to intact cells was determined as previously described (8). [*methyl*-^{3}H]PN 200-110 was added to each well (0.04 nM final) and the cells were incubated for 60 min at 37°C. The reaction was terminated by washing cells with a solution containing 0.9% (wt/vol) NaCl, 5% (vol/vol) dimethyl sulfoxide, and 1% (wt/vol) bovine serum albumin at 4°C. Specific binding was defined as the difference between the amounts of [*methyl*-^{3}H]PN 200-110 bound in the presence and absence of 10 μM unlabeled PN 200-110.

Slot blot analysis of protein kinase C isozymes Subtype-specific monoclonal antibodies were used for the characterization of the protein kinase C-α, protein kinase C-β and protein kinase C-γ. Aliquots of cytosol or membrane fractions, prepared as previously described (19), were spotted on nitrocellulose membranes (Bio-Rad, Richmond, CA) using the MilliBlot S slot blot system (Millipore Corp., Bedford, MA) as per the manufacure's instructions. The nitrocellulose membranes were then incubated with each subtype-specific monoclonal antibody overnight at 4 °C. Immunoreactivity was detected using VECTASTAIN ABC Mouse IgG kits (VECTOR Laboratories, Burlingame, CA).

Statistical analysis Statistical analyses were done using two-way analysis of variance (ANOVA) for the time course study and the concentration-dependency studies, and the Bonferroni simultaneous multiple comparison method was used for the test of the significance of the differences between means in groups (20).

RESULTS

Effects of dexamethasone on the release of ET-1 into culture medium Figure 1 shows the time-course of the release of immunoreactive ET-1 into the culture medium of dexamethasone-treated and control cells. Dexamethasone stimulated the release of immunoreactive ET-1 into the culture medium gradually, and the concentration of ET-1 reached maximum of 51 pg/ml at 24 h, over 2-fold higher than that of control cells (23 pg/ml).

Effects of ETA receptor antagonist on dexamethasone-induced $^{45}Ca^{2+}$ uptake Figure 2 shows the concentration dependent inhibitory effects of FR139317 on $^{45}Ca^{2+}$ uptake by dexamethasone-treated cells. Without FR139317, there was a significant increase in $^{45}Ca^{2+}$ uptake in dexamethasone-treated cells. Dexamethasone produced a 1.5-fold increase over control values (1.18±0.04 to 0.77±0.06 nmoles/mg protein, mean ±SE, $p<0.05$). FR139317 inhibited the dexamethasone-induced $^{45}Ca^{2+}$ uptake in a dose-dependent manner. The approximate dose of FR139317 for half-maximal inhibition was less than 100 nM.

Effects of ETA receptor antagonist on dexamethasone-induced [methyl-^{3}H]PN 200-110 binding Without antagonist, pretreatment with dexamethasone increased binding of [*methyl*-^{3}H]PN 200-110 to A7r5 cells (Figure 3). Dexamethasone produced a 2.7-fold increase over control values (14.2±1.2 to 5.2±0.6 fmoles/mg protein, mean ±SE, $p<0.01$). The ETA receptor antagonist FR139317 also inhibited the binding of [*methyl*-^{3}H]PN 200-110 to dexamethasone-treated cells. This inhibitory effect was similar to that of FR139317 on $^{45}Ca^{2+}$ uptake by dexamethasone-treated cells. One micromolar of FR139317 almost abolished the effects of dexamethasone.

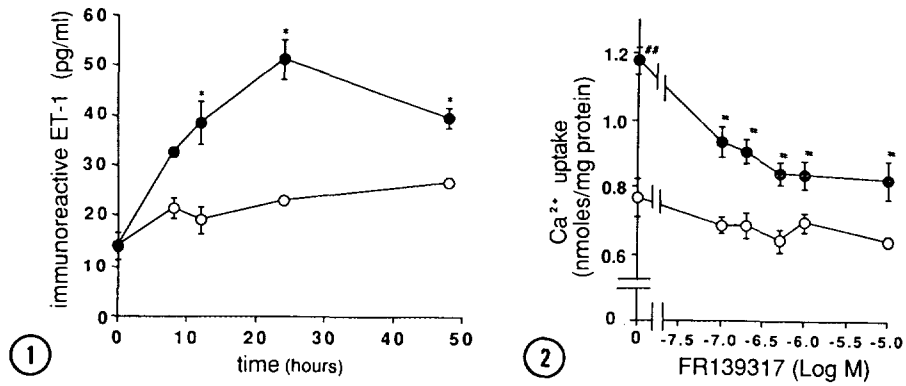

Figure 1. Effects of dexamethasone on the release of immunoreactive ET-1 into the culture medium of A7r5 cells. A7r5 cells were incubated with (•) or without (o) 100 nM dexamethasone. At the indicated times, aliquots of culture medium were taken for measurements of immunoreactive ET-1. Each bar represents the mean ±SE (n=4). *p<0.05; versus control cells.

Figure 2. Dose-dependent inhibitory effects of FR139317 on $^{45}Ca^{2+}$ uptake for 2 min by A7r5 cells. A7r5 cells were incubated with (•) or without (o) 100 nM of dexamethasone and various concentrations of FR139317 for 24 h. Each point represents the mean ±SE (n=4). ##p<0.05, versus control cells without FR139317. #p<0.05; versus dexamethasone-treated cells without FR139317.

Effects of ETA receptor antagonist on dexamethasone-induced protein kinase C immunoreactivity Dexamethasone increased immunoreactivity of protein kinase C-α in A7r5 cells (Figure 4). The effect of dexamethasone was inhibited by the ETA receptor antagonist FR139317. Immunoreactivity of protein kinase C-β in the dexamethasone-treated cells was not significantly different from control cells (data not shown). Protein kinase C-γ was not detected in A7r5 cells.

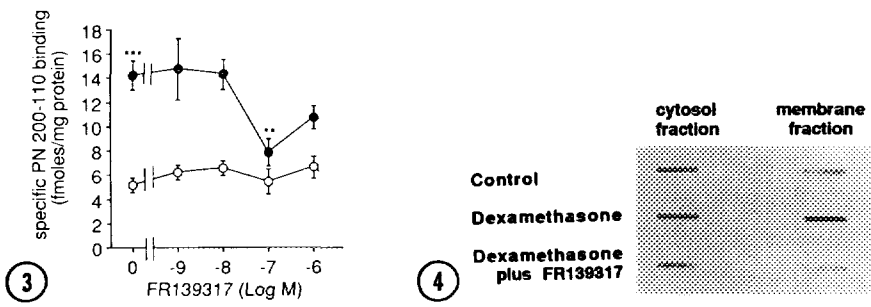

Figure 3. Inhibitory effects of FR139317 on binding of [methyl-^{3}H]PN 200-110 to A7r5 cells. A7r5 cells were incubated with (•) or without (o) 100 nM dexamethasone and various concentrations of FR139317 for 24 h. Each bar represents the mean ±SE (n=4). ***p<0.01, versus control cells without FR139317. **p<0.01, versus dexamethasone-treated cells without FR139317.

Figure 4. Slot blot analysis of immunoreactive protein kinase C-α in cytosol and membrane fractions of A7r5 cells. A7r5 cells were incubated with or without 100 nM dexamethasone and 1 μM of FR139317 for 24 h. Protein kinase C-α was detected using a subtype specific mouse monoclonal antibody.

DISCUSSION

We previously demonstrated that glucocorticoids increase Ca^{2+} uptake into A7r5 vascular smooth muscle cells which is completely blocked by nifedipine (8). We also reported that glucocorticoids increase the number of higher affinity binding sites for [*methyl*-^{3}H]PN 200-110 (8). Therefore, it is thought that glucocorticoid-induced Ca^{2+} uptake may be mediated by an opening of dihydropyridine-sensitive Ca^{2+} channels.

The potent vasoconstrictor peptide ET-1 has been shown to stimulate a modest release of Ca^{2+} from intracellular storage sites, as well as to activate the dihydropyridine-sensitive, voltage dependent Ca^{2+} channels in VSMC (21), following enhanced Ca^{2+} influx. These mobilization of Ca^{2+} are implicated as a mechanism by which ET-1 causes sustained vascular smooth muscle contraction. In the present study, we assessed the association between ET-1 and glucocorticoid-induced Ca^{2+} influx into VSMC, using a potent, highly specific ETA receptor antagonist FR139317 (22). Aramori et al. demonstrated that FR139317 has a high affinity for ETA receptors expressed in transfected Chinese hamster ovary cells (23). In the present study, the ETA receptor antagonist FR139317 inhibited dexamethasone-induced $^{45}Ca^{2+}$ uptake and [*methyl*-^{3}H]PN 200-110 binding in a dose-dependent manner. The effects of dexamethasone were abolished at concentrations of 10 μM FR139317. These findings suggest that glucocorticoid-induced Ca^{2+} influx through dihydropyridine-sensitive channels is, at least partially, dependent on an ETA receptor mediated pathway.

We have recently demonstrated a link between the dexamethasone-induced Ca^{2+} influx and the activation of protein kinase C in A7r5 cells (19). This is consistent with our present hypothesis that ET-1 participates in the glucocorticoid-induced Ca^{2+} influx into vascular smooth muscle cells. First, ET-1 initiates hydrolysis of the plasma membrane inositol phospholipids (24), resulting in the generation of inositol triphosphate (25) and diacylglycerol. In turn, these serve as second messengers for intracellular Ca^{2+} mobilization and protein kinase C activation, respectively (26). Griendling et al. reported that ET causes a robust stimulation of the diacylglycerol/protein kinase C pathway in cultured rat VSMC (27). Protein kinase C is a Ca^{2+} /phospholipid-dependent enzyme that mediates a variety of physiological and pathological cell functions (28). The activation of protein kinase C can modulate the dihydropyridine-sensitive Ca^{2+} channels by phosphorylation in VSMC, following enhanced Ca^{2+} influx. Fish et al. (29) demonstrated that the conductance of dihydropyridine-sensitive Ca^{2+} channels is modulated by activation of protein kinase C in VSMC, using the whole-cell voltage-clamp technique. Other investigators reported that protein kinase C plays an important role in mediating tonic ET-stimulated VSMC responses, including contraction (30,31).

In the present study, we have shown that dexamethasone-induced protein kinase C-α immunoreactivity in A7r5 cells is abolished by an ETA receptor antagonist. In addition, dexamethasone stimulated the release of ET-1 from A7r5 vascular smooth muscle cells into the culture medium. Takahashi et al. reported that plasma concentrations of immunoreactive ET-1 in the dexamethasone-treated rats were significantly higher than in controls (32). Subsequently, Kanse et al. have shown that dexamethasone stimulates the release of ET-1 from VSMC but not endothelial cells (33). Therefore, glucocorticoids may increase protein kinase C activity in VSMC via an ET-1 autocrine loop, resulting in the opening of dihydropyridine-sensitive Ca^{2+} channels.

In summary, dexamethasone stimulates autocrine production of immunoreactive ET-1 in A7r5 cells, directly or indirectly, and causes an increase in [*methyl*-^{3}H]PN 200-110 binding, following enhanced $^{45}Ca^{2+}$ uptake. These results suggest that autocrine production of ET-1 participates in the glucocorticoid-induced Ca^{2+} influx into vascular smooth muscle cells, possibly linked to the activation of protein kinase C.

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