Potentiation of Inositol Trisphosphate Production by Dexamethasone

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One of the mechanisms of glucocorticoid-induced hypertension has been thought to be the enhancement of vascular responsiveness to vasoconstrictors. In this regard, the effects of glucocorticoids on inositol trisphosphate production in vascular smooth muscle cells were studied. Angiotensin II and arginine vasopressin transiently increased inositol trisphosphate formation in a dose-dependent manner. Pretreatment with dexamethasone for 48 hours shifted the dose–response curves of angiotensin II– and arginine vasopressin–induced inositol trisphosphate production to the left, that is, it significantly reduced the half-maximal effective concentrations of angiotensin II (from 25 nM to 5 nM) and arginine vasopressin (from 50 nM to 25 nM). These effects of dexamethasone required a minimum of 12 hours of incubation; maximum effect was observed after 24 hours of treatment. A glucocorticoid antagonist, RU 38486, completely blocked these effects. To elucidate the interaction with prostaglandin, we used indomethacin, a potent inhibitor of prostaglandin synthesis. Treatment with indomethacin shifted the dose–response curves of angiotensin II– and arginine vasopressin–induced inositol trisphosphate production to the left. However, this shift was less than that seen after dexamethasone treatment. Indomethacin alone did not completely reproduce dexamethasone effects, and no additive effect between indomethacin and dexamethasone was observed. These results suggest, at least in part but not entirely, that the effects of dexamethasone depended on prostaglandin synthesis inhibition. We concluded that glucocorticoids altered the responsiveness of vascular smooth muscle cells to angiotensin II and arginine vasopressin through a glucocorticoid-specific receptor. These actions strongly support the mechanism by which the glucocorticoid induced hypertension through the increased sensitivity to vasoconstrictors. (Hypertension 1992;19:109–115)

Glucocorticoids are known to be involved in regulation of blood pressure and also to induce hypertension. Although the exact mechanisms of glucocorticoid-induced hypertension are poorly understood, enhancement of vascular responsiveness has been considered one of the major contributing factors. Previously, we demonstrated that the pressor response to norepinephrine is enhanced in rats with glucocorticoid-induced hypertension. Similar results were obtained from the patients with Cushing’s syndrome. Other studies also supported the notion that glucocorticoids increase vascular responsiveness. However, there are few studies elucidating the increased vascular responsiveness by glucocorticoids in vitro.

In this study, our intent was to clarify the mechanisms of glucocorticoid-induced hypertension at the cellular level, that is, we investigated the effects of glucocorticoids on vascular responsiveness by using cultured vascular smooth muscle cells (VSMCs). Because VSMC contraction can be induced by cytosolic calcium elevation mediated by inositol phosphate–specific phospholipase C activation, we determined the effect of glucocorticoid treatment on vasoconstrictor-induced inositol trisphosphate (IP₃) production in VSMCs.

To our knowledge, the present study provides the first direct evidence that glucocorticoids alter the vascular responsiveness to angiotensin II (Ang II) and arginine vasopressin (AVP) by modulating IP₃ production. Also, these results strongly suggest that glucocorticoid treatment would increase sensitivity to Ang II and AVP.

Materials

Dexamethasone, collagenase, elastase, trypsin, ethylenediaminetetraacetate (EDTA), dimethylsulf-
oxide (DMSO), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo. Indomethacin, norepinephrine, perchloric acid, and KOH were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Ang II, AVP, and [Sar²,Ala³]-Ang II were from the Peptide Institute, Osaka, Japan. Fetal calf serum (FCS) was purchased from Gibco Laboratories, Grand Island, N.Y. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Hanks' solution were obtained from Nissui Pharmaceutical Co., Ltd., Tokyo. The IP₃ assay kit (TRK 1000) was purchased from Amersham International, Ltd., Buckinghamshire, England. RU 38486, a glucocorticoid antagonist, was kindly provided by Roussel Uclaf, Paris.

Cell Culture

VSMCs were prepared from the thoracic aorta of 6-week-old Wistar-Kyoto rats by an enzyme method using a modification of the technique described by Smith and Brock.¹¹ Briefly, the thoracic aorta was dissected from the surrounding tissues. After the adipose tissues around the aorta were removed under sterile conditions, the aorta was incubated in Hanks' solution containing 1 mg/ml collagenase and 3.3 units/ml elastase at 37°C for 30 minutes. After enzyme treatment, vascular tissues were cut into small pieces and further digested in Hanks' solution containing 1 mg/ml collagenase and 10 units/ml elastase at 37°C for 1.5-2 hours. The isolated cells were washed and then were suspended in DMEM containing 10% FCS, penicillin at 100 units/ml, and streptomycin at 100 µg/ml. The cells were plated in 35-mm culture dishes. The cultures were kept at 37°C in a humid atmosphere consisting of 5% CO₂ in air, and the medium was changed every 3 or 4 days. After the cells reached confluence, the medium was changed to culture media containing various doses of dexamethasone or vehicle (0.1% DMSO) as a control, for 48 hours before the experiments. Cells between the third to tenth passage were used for the experiments.

Measurement of 1,4,5-Inositol Trisphosphate Production in Vascular Smooth Muscle Cells

Production of 1,4,5-IP₃ in VSMCs was measured with a radioimmunoassay kit (TRK 1000). Confluent VSMCs were trypsinized (0.25% trypsin, 0.03% EDTA in PBS) and then were suspended in cold PBS containing 1 mg/ml BSA. After the VSMCs were rewarmed at 37°C for 10 minutes, 0.01 volume of various doses of vasoconstrictors was added and vortexed. After a designated time for each study, 0.2 volume ice-cold 20% perchloric acid was added, and the mixture was kept on ice for 20 minutes. Protein was removed by centrifugation at 2,000g for 15 minutes at 4°C; the supernatant was titrated to pH 7.5 with 10N KOH and was kept on ice for 60 minutes. After removal of insoluble KClO₄ by centrifugation, 100 µl supernatant was mixed with the assay buffer containing 0.1 M Tris buffer, 4 mM EDTA, and 4 mg/ml BSA (pH 9.0). Then 100 µl tracer (³Hinositol 1,4,5-trisphosphate) and 100 µl bovine adrenal binding protein were added. The mixture was kept on ice for 15 minutes and centrifuged again at 2,000g for 15 minutes at 4°C. The supernatant was carefully decanted and discarded. The deposit was then resuspended in 200 µl distilled water, and the radioactivity was measured by scintillation counting. On the basis of competition between unlabeled IP₃ and the fixed quantity of tritium-labeled IP₃ for the limited number of binding sites on the bovine adrenal binding preparation, the amount of IP₃ could be determined by interpolation from a standard curve. The cross-reactivities of the binding protein are d-myo-inositol 2,4,5-trisphosphate, less than 1.0%; d-myo-inositol 1,3,4-trisphosphate, 0.22%; d-myo-inositol 1,4-bisphosphate, 0.015%; d-myo-inositol 1,3,4,5-tetrasphosphate, 1.0%.

Protein Determination

The protein levels in VSMCs were determined by the method of Lowry et al,¹² using BSA as a standard.

Statistical Analysis

The results were expressed as the mean±SEM, and the statistical significance was assessed by Student's t test. Values of p<0.05 were considered to be significant.

Results

Cell Culture

The cells prepared from the thoracic aorta of Wistar-Kyoto rats had typical smooth muscle morphology including the presence of hills and valleys seen under a phase-contrast microscope. Additionally, we demonstrated the presence of smooth muscle actin by indirect immunofluorescence microscopy.¹³

Effects of Dexamethasone on Angiotensin II–Induced Inositol Trisphosphate Production

Effect of dexamethasone on dose–response curve of angiotensin II–induced inositol trisphosphate production. The basal intracellular IP₃ content measured by our method was 5.8±0.3 pM/10⁶ cells. When VSMCs were stimulated by Ang II, IP₃ rapidly increased and reached the maximal level 10 seconds after the addition of Ang II, then declined to the basal level within 1 minute. The maximal IP₃ level induced by 10 nM Ang II stimulation was 252.0±10.0% of the basal IP₃ (Figure 1). These results were consistent with earlier observations.¹⁴,¹⁵ The treatment with 1 µM dexamethasone for 48 hours did not affect the time course of IP₃ formation after Ang II stimulation or the basal IP₃ level (data not shown). However, pretreatment with dexamethasone significantly increased (p<0.01) the maximal IP₃ level at 10 seconds after the addition of 10 nM Ang II, which was 345.0±9.8% of the basal IP₃ level. Figure 2 shows the dose–response curve of Ang II–induced IP₃ production in 1 µM dexamethasone-treated and untreated cells, which was measured 10 seconds after the
addition of Ang II. Ang II increased IP₃ production in a dose–response manner with the half-maximal and maximal effects found at 25 nM Ang II and 1 µM Ang II, respectively. The maximal level of Ang II–induced IP₃ production was 404.5±9.3% of the basal IP₃. The treatment with dexamethasone for 48 hours shifted the dose–response curve to the left. The half-maximal effect was found at 5 nM Ang II, significantly reduced (p<0.01), but the maximal level of Ang II–induced IP₃ production was the same as that of the control, 412.0±9.0% of the basal IP₃ level.

**Angiotensin II increased inositol trisphosphate production through its specific receptor.** To determine whether Ang II–induced IP₃ production was mediated by its specific receptor, VSMCs were treated with or without the Ang II receptor antagonist [Sar¹,Ala⁸]-Ang II. One micromolar [Sar¹,Ala⁸]-Ang II alone had no effect on the basal intracellular IP₃ level in control and dexamethasone-treated cells. The IP₃ production stimulated by 10 nM Ang II was completely blocked by 1 µM [Sar¹,Ala⁸]-Ang II, irrespective of the presence of dexamethasone. These results showed that Ang II induced IP₃ production through its specific receptor (Figure 3).

**Effects of different concentrations of dexamethasone on inositol trisphosphate production stimulated by angiotensin II.** VSMCs treated with different concentrations of dexamethasone exhibited a dose-related increase in the IP₃ response to 10 nM Ang II. The increased IP₃ response became apparent as low as 10 nM dexamethasone and became maximal at 1 µM dexamethasone (Figure 4).

**Effect of dexamethasone was through the glucocorticoid-specific receptor.** To demonstrate whether the effect of dexamethasone was through the intracellular glucocorticoid receptor, VSMCs were incubated with 10 µM RU 38486, a glucocorticoid receptor–specific antagonist, with or without 1 µM dexamethasone for 48 hours. Although RU 38486 alone had no effect on basal and Ang II–induced IP₃ levels, it completely blocked the effect of 1 µM dexamethasone (Figure 5). These results suggested that this dexamethasone effect was through the glucocorticoid-specific receptor.

**Time course of the effect of dexamethasone on Ang II–induced inositol trisphosphate production.** VSMCs were treated with 1 µM dexamethasone for various periods of time before Ang II stimulation. An increased IP₃ response was detected as early as 12 hours, and the maximum effect was observed at 24...
FIGURE 4. Bar graph shows effects of various doses of dexamethasone (DX) on angiotensin II (Ang II)–stimulated inositol trisphosphate (IP₃) production. IP₃ was measured 10 seconds after the addition of 10 nM Ang II. Data represent mean±SEM of three independent experiments. **p<0.01 vs. the value in the control experiment.

Effect of indomethacin on dose–response curve of inositol trisphosphate production induced by angiotensin II. To elucidate the interaction with prostaglandin, we used indomethacin, a potent inhibitor of prostaglandin synthesis. Like dexamethasone treatment, treatment with 1 μM indomethacin for 48 hours shifted the dose–response curve of Ang II–induced IP₃ production to the left. However, this shift was not as marked as that observed after the treatment with 1 μM dexamethasone. The half-maximal effect was seen at 10 nM Ang II. However, that was significantly lower (p<0.05) than that of the control. When 1 μM indomethacin and 1 μM dexamethasone were administered simultaneously, the dose–response curve of Ang II–induced IP₃ production was identical with that obtained after treatment with 1 μM dexamethasone alone (Figure 7). Indomethacin

FIGURE 5. Bar graph shows effect of RU 38486 on angiotensin II (Ang II)–stimulated inositol trisphosphate (IP₃) production. Vascular smooth muscle cells were incubated with 10 μM RU 38486 with or without 1 μM dexamethasone (DX) for 48 hours. Reactions were stopped 10 seconds after 10 nM Ang II stimulation. Data are mean±SEM of three independent experiments. **p<0.01 vs. the value in the control experiment.

FIGURE 6. Line graph shows time course for the effect of dexamethasone (DX) on angiotensin II (Ang II)–stimulated inositol trisphosphate (IP₃) production. Vascular smooth muscle cells were incubated for the indicated time periods with (●) or without (○) 1 μM DX. Reactions were stopped 10 seconds after the addition of 10 nM Ang II. Data represent mean±SEM of three independent experiments. **p<0.01 vs. the value in the control experiment.

FIGURE 7. Dose–response curve for the effect of indomethacin on the inositol trisphosphate (IP₃) production stimulated by angiotensin II (Ang II). Vascular smooth muscle cells were incubated with 1 μM indomethacin (●), 1 μM dexamethasone (DX) (○), 1 μM indomethacin plus 1 μM DX (▲), and vehicle (●) for 48 hours. IP₃ was then measured after 10 seconds of Ang II. Data are mean±SEM of three independent experiments. *p<0.05, **p<0.01 vs. the value in the control experiment.
alone did not completely reproduce the dexamethasone effect on Ang II-induced IP₃ production even at higher concentrations (data not shown). Moreover, no additive effect between indomethacin and dexamethasone was observed. These results suggest that, at least in part, the effect of dexamethasone on Ang II–stimulated IP₃ production is dependent on inhibition of prostaglandin synthesis.

**Effects of Dexamethasone on Arginine Vasopressin–Induced Inositol Trisphosphate Production**

Effect of dexamethasone on dose–response curve of arginine vasopressin–induced inositol trisphosphate production. AVP stimulated IP₃ production in a manner very similar to that of Ang II. IP₃ increased rapidly, reached the maximal level in 10 seconds, and declined to the baseline level within 1 minute. However, the maximal IP₃ level induced by stimulation with 100 nM AVP was 251.9±5.2% of the basal IP₃ level, which was significantly lower than that induced by the same concentration of Ang II and was consistent with the results of previous studies.¹⁵ The treatment with 1 μM dexamethasone for 48 hours did not affect the time course of IP₃ production after AVP stimulation (data not shown). However, in dexamethasone-treated cells, 100 nM AVP induced a significantly higher (p<0.05) IP₃ level at 10 seconds than in untreated cells (306.8±6.4% versus 251.9±5.2%). Figure 8 shows the dose–response curve of AVP-induced IP₃ production at 10 seconds. AVP induced the increased IP₃ in a dose–response manner with the half-maximal and maximal effects found at 50 nM and 1 μM AVP, respectively. The maximal level of AVP-induced IP₃ production was 305.8±8.2% of the basal IP₃. The treatment with dexamethasone for 48 hours shifted this dose–response curve to the left.

The half-maximal effect was found at 25 nM AVP, significantly reduced (p<0.05), but the maximal level of AVP-induced IP₃ production was the same as that of the control, 310.7±7.5% of the basal IP₃ level. The effect of dexamethasone on AVP-induced IP₃ production was significantly more modest than that on Ang II.

Other effects of dexamethasone on arginine vasopressin–induced inositol trisphosphate production. Other effects of dexamethasone on AVP-induced IP₃ production were very similar to the effects on Ang II–induced production. The increased IP₃ response was apparent as low as 10 nM dexamethasone and was maximal at 1 μM dexamethasone. Although 10 μM RU 38486 alone had no effect on the basal and AVP-induced IP₃ levels, it completely blocked the effect of 1 μM dexamethasone. An increased IP₃ response was detected as early as after 12 hours of treatment with dexamethasone, and the maximum effect was observed at 24 hours. This effect of dexamethasone was still apparent after 72 hours of treatment. The treatment with 1 μM indomethacin for 48 hours shifted the dose–response curve of AVP-induced IP₃ production to the left. However, this shift was not as marked as that seen after treatment with 1 μM dexamethasone. Moreover, no additive effect between indomethacin and dexamethasone was observed (Table 1). Indomethacin alone did not completely reproduce the effect of dexamethasone on AVP-induced IP₃ production even at higher concentrations.

**Effects of dexamethasone on norepinephrine-induced inositol trisphosphate production.** Addition of norepinephrine increased the amount of IP₃ within 10 seconds, reaching a peak value at 30 seconds, and then falling to the level before the addition of norepinephrine within 2 minutes. The IP₃ level 30 seconds after the addition of 10 μM norepinephrine was 132.0±6.5% of the basal IP₃ level. The treatment with 1 μM dexamethasone for 48 hours did not have any effect on norepinephrine-induced IP₃ production and metabolism. We did not observe any effect of dexamethasone on norepinephrine-induced IP₃ production; it was seen only with addition of Ang II and AVP.

**Effects of dexamethasone on cell number and cell protein.** Dexamethasone had no significant effects on cell number and cell protein in VSMCs incubated for 48 hours, as compared with the control.

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**TABLE 1. Effect of Indomethacin on Arginine Vasopressin–Induced Inositol Trisphosphate Production**

<table>
<thead>
<tr>
<th>Cell group</th>
<th>% of basal IP₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>251.9±5.2</td>
</tr>
<tr>
<td>DX (1 μM)</td>
<td>306.8±6.4*</td>
</tr>
<tr>
<td>Indomethacin (1 μM)</td>
<td>276.1±4.5</td>
</tr>
<tr>
<td>DX+indomethacin</td>
<td>310.5±5.0*</td>
</tr>
</tbody>
</table>

Reactions were measured 10 seconds after the addition of 100 nM arginine vasopressin. Data represent the mean±SEM of three independent experiments. IP₃, inositol trisphosphate; DX, dexamethasone.

*p<0.05 vs. the value in the control experiment.
Discussion

In the present study, we first demonstrated the direct effects of glucocorticoids on VSMCs, which could potentially explain how glucocorticoids induce hypertension. Glucocorticoids altered the responsiveness of VSMCs to Ang II and AVP through the glucocorticoid-specific receptor. Treatment with dexamethasone shifted the dose–response curve of Ang II- and AVP-induced IP₃ production to the left, strongly suggesting increased sensitivity to Ang II and AVP after dexamethasone treatment. These effects were partially dependent on inhibition of prostaglandin synthesis. This finding is very meaningful in terms of demonstrating one of the mechanisms of glucocorticoid-induced hypertension at the cellular level.

The precise mechanisms of the effects of glucocorticoids on these results are still unclear. The potent inhibition of prostaglandin synthesis by glucocorticoids is one of them, as mentioned earlier. However, the results of this study show that glucocorticoids may exert their effects in other ways. Both Ang II and AVP bind to their own specific receptors on VSMCs and produce two second messenger molecules, IP₃ and diacylglycerol.16,17 Dexamethasone shifted the dose–response curves of IP₃ production stimulated by Ang II and AVP to the left; the most plausible explanation is that dexamethasone increases the affinity but not the number of Ang II and AVP receptors. However, since there is little information about Ang II or AVP receptor affinity increased by glucocorticoids, it is less likely that the changes in receptor affinity induce the shift. An alternative explanation may reside in changes at the level of guanine nucleotide binding (G) protein since glucocorticoids are reported to modulate the function of G protein.18–21 This notion is supported by the experimental evidence that the action of dexamethasone appeared after 12 hours of incubation. This relatively long duration could account for the involvement of G protein with respect to protein synthesis. However, at present, modulation of G protein in receptor affinity or its participation in phospholipase C activation cannot be made because of the paucity of definitive information concerning the specificity of G protein involvement in agonist-induced transmembrane signaling.

Another possible factor that could explain our data is the interactions with prostaglandin synthesis. In our previous studies,5,22 we showed that prostaglandins are modulators of vascular reactivity and that glucocorticoids inhibit prostaglandin synthesis resulting in enhancement of vascular reactivity. Furthermore, studies using an antiglucocorticoid, RU 38486, showed that diminished stimulation of vascular procacyclin synthesis might contribute to vascular hyperreactivity in glucocorticoid-induced hypertension.23

In our present study, the treatment with indomethacin shifted the dose–response curves of Ang II– and AVP-induced IP₃ production to the left. However, these alterations in responsiveness were not as pronounced as that seen after treatment with dexamethasone. When indomethacin and dexamethasone were administered simultaneously, the dose–response curves of Ang II– and AVP-induced IP₃ production were identical to that obtained after dexamethasone treatment alone. From these results, it is possible that these dexamethasone actions were partially dependent on inhibition of prostaglandin synthesis. Moreover, the basal concentrations of prostaglandins in cultured VSMCs are reported to be relatively low,24 which might explain why dexamethasone had no effect on the basal intracellular IP₃ concentration in the present study. In addition, calcium-mobilizing vasoconstrictors are shown to potently induce prostaglandin release,24 suggesting that dexamethasone might suppress such vasoconstrictor-induced prostaglandin release. However, since both dexamethasone and indomethacin inhibit all kinds of prostaglandin synthesis, we cannot exclude the possibility of involvement of prostaglandin. Accurately, our current study showed that the effects of dexamethasone were not fully explained by the inhibition of cyclooxygenase activity alone. Nevertheless, a recent study on renal mesangial cells revealed that prostaglandin E₂ attenuated IP₃ production in response to Ang II and AVP, thus indicating that indomethacin could restore the Ang II– and AVP-induced IP₃ content.25 The role of prostaglandin synthesis during dexamethasone treatment in IP₃ production induced by vasoconstrictors remains indistinct, and further study will be needed.

Interestingly, dexamethasone failed to have any effect on norepinephrine-induced IP₃ production. Norepinephrine, like Ang II and AVP, is a potent vasoconstrictor. The involvement of prostaglandin might be related to this finding, since Hassid and Williams24 demonstrated that Ang II and AVP stimulated prostaglandin synthesis in VSMCs, whereas norepinephrine did not. Previously, we reported that Ang II and AVP but not norepinephrine increased collagen synthesis in the same culture of VSMCs as used in the present study.20 Taken together, these results can suggest that the connection of α₁-receptor of VSMCs with G protein and/or the effects of prostaglandin synthesis might be different from that of Ang II and AVP.

Another important finding in the present study is that this glucocorticoid effect was through an intracellular glucocorticoid-specific receptor. The steroid derivative RU 38486 has antiglucocorticoid activity and high binding affinity for a cytosolic steroid receptor.27 The treatment with RU 38486 had no effect on basal or Ang II– or AVP-induced IP₃ production itself. However, the effects of dexamethasone on Ang II– and AVP-induced IP₃ production were completely blocked by RU 38486. It suggested that these dexamethasone effects were through glucocorticoid-specific receptors.

In conclusion, we first demonstrated that glucocorticoids altered the responsiveness to Ang II– and AVP-induced IP₃ production through its specific...
receptor, which strongly suggested increased sensitivity to Ang II and AVP after the glucocorticoid treatment. Moreover, these effects were partially dependent on inhibition of prostaglandin synthesis. This evidence was to make clear, by using VSMCs, the mechanisms of vascular hypersensitivity in glucocorticoid-induced hypertension.

References


Key Words • dexamethasone • norepinephrine • inositol phosphates • angiotensin II • arginine vasopressin • vascular smooth muscle
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