Involvement of tyrosine kinase in citrate-stimulated aldosterone production in bovine glomerulosa cells

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Received 25 October 1999; accepted in final form 3 February 2000

Kigoshi, Toshikazu, Noriko Imaizumi, Junko Yoshida, Atsushi Nakagawa, Shigeru Nakano, Matomo Nishio, and Kenzo Uchida. Involvement of tyrosine kinase in citrate-stimulated aldosterone production in bovine glomerulosa cells. Am J Physiol Endocrinol Metab 279:E140–E145, 2000.—The present study was designed to assess whether citrate stimulates aldosterone production by isolated bovine adrenal glomerulosa cells in vitro. When the cells were incubated with graded concentrations of citrate up to 4.0 mM, basal aldosterone production was significantly elevated, with a gradual reduction of extracellular ionized calcium concentration. Without citrate, however, adding increasing amounts of calcium chloride to a calcium-free medium did not reproduce the citrate’s effect on basal aldosterone production. Genistein, an inhibitor of tyrosine kinases, inhibited the citrate (4 mM)-induced aldosterone production in a dose-dependent manner, with 89.8% of inhibition at a concentration of 10 μM. When the cells were exposed to citrate (4 mM) for 5, 10, and 30 min, tyrosine in Mr 105,000 endogenous protein was dominantly phosphorylated. This study demonstrates for the first time that citrate stimulates aldosterone production in bovine adrenal glomerulosa cells in vitro and also suggests a crucial involvement of protein tyrosine kinase in the steroidogenic action of citrate in the cells.

tyrosine phosphorylation; membrane potential; genistein; steroidogenesis

ALDOSTERONE BIOSYNTHESIS in adrenal glomerulosa cells is stimulated by a variety of factors, including the main two regulators, angiotensin II (ANG II) and potassium (5, 7, 9, 10, 16, 27). Because ANG II and potassium do not stimulate aldosterone production in a calcium-free medium (6, 8–10, 16), the regulation of steroidogenesis in the cells is critically dependent on the presence of extracellular calcium. In contrast, we found in preliminary studies that, when bovine adrenal glomerulosa cells were incubated with graded concentrations of citrate, which is known to possess a calcium-chelating potency, basal aldosterone production was dose dependently stimulated in parallel with a reduction of extracellular ionized calcium concentration and a stimulation of tyrosine phosphorylation of endogenous protein tyrosine kinase has recently been suggested to have a critical role in the steroidogenic action of ANG II in bovine (4) and rat (12) adrenal glomerulosa cells. The present study demonstrates for the first time that citrate stimulates aldosterone production by isolated bovine adrenal glomerulosa cells in vitro.

MATERIALS AND METHODS

Citrate, isocitrate, fumarate, succinate, malate, α-ketoglutarate, and HEPES were obtained from Sigma Chemical (St. Louis, MO). Crude collagenase (type I) was purchased from Worthington Biochemical (Freehold, NJ). BSA (fraction V) was obtained from Peptide Institute (Tokyo, Japan). Synthetic [Ile⁶]ANG II was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Antiphosphotyrosine (PY-20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Genistein was purchased from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade.

Bovine adrenal tissues were obtained from a local abattoir. Isolated adrenal glomerulosa cells were prepared by a collagenase digestion technique as previously described (13). Dispersed cells were suspended in Medium 199 (GIBCO) containing 4 mM potassium chloride, 1.25 mM calcium chloride, 16 mM sodium bicarbonate, 0.1% (wt/vol) BSA, and 20 mM HEPES to a uniform concentration of 1 × 10⁶ cells/ml. Citrate, isocitrate, fumarate, succinate, malate, and α-ketoglutarate were dissolved in 20 mM HEPES buffer solution; each stock solution of 100 mM, with pH adjusted to 7.4 with NaOH, was prepared immediately before the experiments. Cell viability, determined by trypan blue exclusion before and at the end of the experiments, was ~90%. One-milliliter aliquots were then incubated with citrate (from 0.4 mM to 8 mM) in the absence or presence of genistein (from 10 nM to 10 μM) for 2 h at 37°C under 95% O₂-5% CO₂ gas. In some experiments, one-milliliter aliquots were incubated with ANG II (from 10 pM to 10 nM) or potassium chloride (from 0 mM to 12 mM) in the absence or presence of genistein (1, 2, and 4 mM) for 2 h at 37°C under 95% O₂-5% CO₂ gas. In other experiments, after the cells were prepared in a calcium-free solution [modified Krebs-Ringer bicarbonate solution with-

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out calcium; 0.1% (wt/vol) BSA, 0.1% (wt/vol) glucose, and 4 mM potassium), the effect of adding an increasing amount of calcium chloride to the calcium-free solution on basal aldosterone production in the absence or presence (4 mM) of citrate was studied. The cells were then precipitated by centrifugation, and the media were stored at −20°C to measure the aldosterone and electrolyte concentrations. Aldosterone levels in the incubation media were determined by radioimmunoassay as previously described (13) by use of kits from Daiichi Radioisotope Institute (Tokyo, Japan). The sensitivity was 25 pg/ml. The intra- and interassay variations were 2.9 and 4.7%, respectively.

The levels of pH in the incubation media before and 2 h after the addition of citrate (7.4 ± 0.1; n = 3) were determined with a digital pH meter (HM-30V, TOA Electronics, Tokyo, Japan). Ionized calcium levels in the incubation media (corrected for pH 7.4) were measured with a NOVA 8 calcium analyzer (NOVA Biomedical). Sodium, potassium, chloride, and magnesium levels in the incubation media were determined with an autoanalyzer (Hitachi 7450E, Hitachi City, Japan).

The whole cell patch-clamp technique was used in the present experiments. The membrane potentials were recorded with an Axopatch ID amplifier and a Digidata 1200 (Axon Instruments, Forster City, CA) under the control of P-CLAMP 6. The glomerulosa cells suspended in Medium 199 were placed on the bottom of a 0.5-ml volume chamber maintained at 37°C. The chamber was continuously perfused at a rate of ~1 ml/min via a gravity-flow system with modified Tyrode solution containing 145 mM NaCl, 4 mM KCl, 1.28 mM CaCl2, 1.13 mM MgCl2, 10 mM HEPES, and 0.1% (wt/vol) glucose (pH was adjusted to 7.4 with NaOH). The reagents tested were added to the modified Tyrode solution. The patch pipettes were fabricated from glass capillaries (1.5 mm OD) using a two-stage puller (PP-83, Narishige, Japan) to give a pipette resistance of 3.8–4.8 MΩ when filled with pipette solution containing (in mM) 130 KCl, 5 K2ATP, 5 phosphocreatine (disodium salt), 2.5 MgCl2, 5 HEPES, and 5 EGTA (pH was adjusted to 7.2 with KOH).

To investigate the existence of endogenous substrate protein(s) for tyrosine kinase(s) in bovine adrenal glomerulosa cells, the cells (106 cells) were stimulated with citrate (4 mM) for three time periods (5, 10, and 30 min) at 37°C under 95% O2-5% CO2 gas, washed twice with ice-cold PBS containing 1 mM Na2VO4 and then lysed in 1.0 ml of lysis buffer containing 1% (wt/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 1 μg/ml aprotinin. Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates (1 mg of protein) with the antiphosphotyrosine antibody (4 μg). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immuno-blotted with the antiphosphotyrosine antibody (1 μg).

Results are expressed as means ± SE. Data with only one grouping variable were analyzed statistically by one-way ANOVA followed by the Bonferroni post hoc test. Comparisons of aldosterone responses between two groups were assessed by repeated-measures ANOVA. Dose-response curves for aldosterone production were analyzed with the curve-fitting program of Delta Graph for Power Macintosh.

**RESULTS**

The changes in basal aldosterone production and extracellular ionized calcium concentration in the presence of graded concentrations of citrate in the incubation media are shown in Fig. 1. Basal aldosterone production was significantly stimulated when the cells were incubated with graded concentrations of citrate up to 4.0 mM (345.4 ± 28.8 pg/10^5 cells at 1.6 mM of citrate, P = 0.0077; 412.8 ± 47.8 pg/10^5 cells at 2.0 mM of citrate, P = 0.0002; 497.1 ± 95.3 pg/10^5 cells at 4.0 mM of citrate, P < 0.0001 vs. corresponding basal value without citrate; 182.5 ± 9.7 pg/10^5 cells, n = 10). At concentrations of citrate >4 mM, basal aldosterone levels tended to decrease (308.3 ± 74.2 pg/10^5 cells at 6.0 mM of citrate, P = 0.0461; 259.3 ± 68.9 pg/10^5 cells at 8.0 mM of citrate, P = 0.2524). The ionized calcium concentration in the incubation medium significantly decreased (from 1.27 ± 0.09 mM at 0.4 mM of citrate to 0.22 ± 0.01 mM at 4 mM of citrate, P < 0.0005 vs. corresponding basal value without citrate, 1.66 ± 0.11 mM, n = 3) when the concentration of citrate was elevated up to 4.0 mM. At concentrations of citrate >4 mM, the ionized calcium levels were <0.1 mM. The levels of potassium, chloride, magnesium, and pH in the incubation media were not significantly altered after the administration with citrate (data not shown) with the exception of a gradual increase in sodium concentration in the incubation medium (149.5 ± 0.3 meq/l at 4.0 mM citrate; P < 0.0001 vs. corresponding basal value without citrate; 141.0 ± 0.6 meq/l, n = 3). When the cells were incubated in the presence of fumalate, succinate, malate, and α-ketoglutarate (from 2 mM to 8 mM), the levels of basal aldosterone production were not significantly altered.

Figure 2 shows the effect of addition of increasing amounts of calcium chloride (from 0.4 mM to 2 mM) to a calcium-free medium (potassium; 4 mM) on basal aldosterone production in the absence or presence (4
mM) of citrate in the incubation medium. When calcium chloride (from 0.4 mM to 2 mM) was added in the incubation medium, the levels of basal aldosterone production were markedly elevated in the presence of 4 mM citrate (a maximum level of 483.0 ± 66.9 pg/10⁵ cells at 0.8 mM calcium chloride; *P* < 0.0008 vs. corresponding basal value without calcium chloride; 35.3 ± 17.4 pg/10⁵ cells, *n* = 3). In the absence of citrate, the maximum levels of basal aldosterone production were 146.7 ± 58.2 pg/10⁵ cells at 0.4 mM calcium chloride (*P* = 0.1146 vs. corresponding basal value without calcium chloride; 26.3 ± 18.1 pg/10⁵ cells, *n* = 3). The levels of basal aldosterone production without calcium chloride in the incubation medium were not different between the absence and the presence (4 mM) of citrate in the incubation medium.

To assess the mechanism of stimulatory action of citrate on aldosterone production, the effect of citrate administration on resting membrane potential of bovine adrenal glomerulosa cells was examined in the present study. Figure 3 shows representative evidence of the effect of citrate administration on resting membrane potential. As shown in Fig. 3, the administration with 4 mM citrate did not alter a resting membrane potential, whereas the administration with 8 mM potassium chloride induced a membrane depolarization from −87 mV at 4 mM citrate (4 mM potassium) to −73 mV at 8 mM potassium.

Figure 4 shows the effect of citrate administration on a potassium-aldosterone dose-response curve in bovine adrenal glomerulosa cells. When citrate (1, 2, and 4 mM) was administered into the incubation medium, a potassium-aldosterone dose-response curve was shifted to the left (the concentration inducing 50% of the maximal response was 5.1 ± 0.2 mM in the presence of 1 mM citrate, *P* = 0.9623; 4.1 ± 0.4 mM in the presence of 2 mM citrate, *P* = 0.0106; 4.0 ± 0.4 mM in the presence of 4 mM citrate, *P* = 0.0040 vs. corresponding control value without citrate, 5.1 ± 0.1 mM, *n* = 3).

In the similar experiment shown in Fig. 5A, the levels of aldosterone production in response to doses of ANG II <1 nM were elevated, whereas those in response to doses of ANG II ≥1 nM were unaltered. When these results were expressed as a ratio of control, the response of the ratio of ANG II-stimulated aldosterone production to the corresponding basal level was significantly (*P* < 0.0001, *n* = 3) lower in the presence of citrate (2 and 4 mM) than in its absence (Fig. 5B).

To investigate whether intracellular protein tyrosine kinase(s) has a critical role in citrate-induced aldosterone production, the effect of genistein, a potent inhibitor of tyrosine kinases, on citrate (4 mM)-induced aldosterone production in bovine adrenal glomerulosa cells was examined. As shown in Fig. 6, genistein inhibited the citrate (4 mM)-induced aldosterone production in a dose-dependent manner with 14.6% (*P* = 0.2113, *n* = 3), 61.3% (*P* = 0.0038, *n* = 3), and 89.8%
(P < 0.0001, n = 3) of inhibition at concentrations of 0.1 μM, 1 μM, and 10 μM, respectively. Genistein at the concentrations used in the present study did not alter basal aldosterone production in bovine adrenal glomerulosa cells.

Finally, to investigate whether citrate stimulates tyrosine phosphorylation of intracellular specific protein(s) in bovine adrenal zona glomerulosa cells, intracellular protein tyrosine phosphorylation was measured after an exposure of the cells to citrate, as described in MATERIALS AND METHODS. When the cells were exposed to citrate (4 mM) for 5, 10, and 30 min, tyrosine in Mr 105,000 endogenous protein (105 K protein, or molecular mass of 105 kDa) was dominantly phosphorylated, with a maximal response at the 10-min point (Fig. 7, lane 2). In contrast, no increase in 105 K protein tyrosine phosphorylation was detected when the cells were exposed to vehicle alone for the same time periods. Surprisingly, tyrosine in the same molecular weight protein was phosphorylated when the cells were exposed to ANG II (1 nM) for the same time periods (Fig. 7, lane 3, at the 10-min point).

Fig. 5. Effect of citrate (2 and 4 mM) administration on an angiotensin II (ANG II)-aldosterone dose-response curve (A) and a ratio of ANG II-stimulated aldosterone production level to the corresponding basal level (B) in bovine adrenal glomerulosa cells. Data are expressed as means ± SE of 3 separate experiments. Dose-response curves for aldosterone production were analyzed with the curve-fitting program of Delta Graph for Power Macintosh.

Fig. 6. Effect of genistein (10 nM–10 μM) on aldosterone production in the absence (○) or presence (●) of citrate (4 mM) in the incubation medium by isolated bovine adrenal glomerulosa cells. Data are expressed as means ± SE of 3 separate experiments.

Fig. 7. Western blot analysis of citrate- and ANG II-induced tyrosine phosphorylation of Mr 105,000 endogenous protein (molecular mass of 105 kDa) in bovine adrenal glomerulosa cells. Cells (10⁶ cells) were exposed to citrate (4 mM) or ANG II (1 nM) for 10 min at 37°C under 95% O₂-5% CO₂ gas, as described in MATERIALS AND METHODS. Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates (1 mg of protein) with the anti-phosphotyrosine antibody (4 μg). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the anti-phosphotyrosine antibody (1 μg).
DISCUSSION

The present study demonstrates for the first time that citrate stimulates aldosterone production in bovine adrenal glomerulosa cells in vitro. The present work also suggests that protein tyrosine kinase(s) is crucially involved in the citrate-stimulated aldosterone production in the cells.

In the present study, the elevation of the basal aldosterone production rate induced by the graded administration with citrate was associated with a gradual reduction of extracellular ionized calcium concentration. However, the administration with citrate (4 mM) did not stimulate basal aldosterone production in a calcium-free medium (Fig. 2). These results suggest that the presence of calcium in the incubation medium, even if its concentration is very low, is essential in the stimulatory action of citrate on aldosterone production in bovine adrenal glomerulosa cells. Although the sodium concentration was significantly elevated in the presence of citrate, it is unlikely that this event contributes to the citrate-induced elevation of aldosterone production, because the effect of a high sodium concentration has been known to be rather suppressive on the aldosterone secretion rate (22). In addition, adding increasing amounts of calcium chloride (from 0.4 mM to 2 mM) to a calcium-free medium without citrate did not reproduce the citrate’s effect on basal aldosterone production (Fig. 2). This finding strongly suggests that the citrate-induced elevation of basal aldosterone production is predominantly due to the presence of citrate itself, rather than lowering calcium concentration in the incubation medium. Although the physiological concentration of cytosolic citrate in bovine adrenal glomerulosa cells is unclear, the concentrations of citrate used in the incubation media in the present study seem to be adequate compared with those in other reports (3, 21).

To assess the mechanism(s) of stimulatory action of citrate on aldosterone production, we first evaluated an alteration in resting membrane potential for bovine adrenal glomerulosa cells before, during, and after the administration with 4 mM citrate by a whole cell patch-clamp method. The resting membrane potential for the cell was −89 mV, and the electrical response of the cell to 8 mM potassium was a rapid depolarization. These results are consistent with previous reports (17, 19, 26). During the administration with 4 mM citrate, however, the resting membrane potential was not altered. The administration with citrate at this concentration can induce a marked elevation of aldosterone production in the present study. These findings indicate that an activation of voltage-dependent calcium channels, including L type and T type channels (2, 20), is not involved in the mechanism of stimulatory action of citrate on aldosterone production in the cells. Because we did not measure intracellular free Ca$^{2+}$ concentration during the administration with citrate into the incubation medium, it is unclear whether an intracellular Ca$^{2+}$-dependent system including protein kinase C and calmodulin is involved in the mechanism of action of citrate in the present study.

To assess further the mechanism(s) of stimulatory action of citrate on aldosterone production, we next evaluated the mode of action of citrate on agonist (potassium and ANG II)-stimulated aldosterone production (Figs. 4 and 5). In these experiments, a potassium-aldosterone dose-response curve was shifted to the left by the addition of citrate in the incubation medium, suggesting an increased sensitization of the potassium-stimulated aldosterone production by citrate. Moreover, without potassium as well as calcium in the incubation medium, citrate did not stimulate basal aldosterone production. In addition, the responsiveness of aldosterone production to ANG II was blunted in the presence of citrate (Fig. 5B). Because similar findings are also observed by the use of ANG II instead of citrate, according to previous reports (9–11, 28), the mode of action of citrate on basal and stimulated aldosterone production resembles that of ANG II. Although ANG II is well known to stimulate aldosterone biosynthesis via activating its specific cell-surface receptor, known as the AT$_1$ receptor (1, 23), the specific AT$_1$ receptor antagonist losartan did not inhibit the citrate-induced aldosterone production (unpublished observation, data not shown). Taken together with these findings, we postulate a hypothesis that citrate stimulates aldosterone production via activating a part of the ANG II-induced intracellular signaling pathways at a postreceptor site in bovine adrenal glomerulosa cells in vitro. On the other hand, protein tyrosine kinase has recently been suggested to have a critical role in the steroidogenic action of ANG II in bovine (4) and rat (12) adrenal glomerulosa cells. For these reasons, we investigated the role of protein tyrosine kinase in the citrate-stimulated aldosterone production in bovine adrenal glomerulosa cells. In the present study, genistein, a potent inhibitor of protein tyrosine kinase, inhibited citrate-stimulated aldosterone production in a dose-dependent manner without a significant alteration of basal aldosterone production, suggesting a nontoxic effect of genistein. The doses of inhibitor used in the present study are adequate compared with those in previous reports (4, 12, 18, 24). These results thus suggest that protein tyrosine kinase has a crucial role in citrate-induced aldosterone production in bovine adrenal glomerulosa cells. To confirm our observations, we further investigated whether citrate activates tyrosine phosphorylation of a specific substrate protein in the cells. In the present study, tyrosine in Mr 105,000 protein was dominantly phosphorylated by the citrate administration (Fig. 7). This result demonstrates that citrate stimulates a specific protein tyrosine kinase system in bovine adrenal glomerulosa cells in vitro. Because tyrosine in the same molecular weight protein was also phosphorylated by ANG II stimulation, these results support the hypothesis we have described. We previously reported that the calcium chelator EGTA stimulates aldosterone production in vitro (15). The modes of action on the agonist (potassium, 8 mM, and ANG II, 10 nM)-stimulated
aldosterone production, however, differ between citrate and EGTA (14), suggesting different intracellular signals between the two substances. Although the biological significance of citrate’s effect on aldosterone production is unclear in the present study, it might be possible to speculate that citrate within glomerulosa cells can serve as an intracellular modulator of ANG II-induced aldosterone synthesis, inasmuch as citric acid cycle intermediates are suggested to stimulate the formation of aldosterone from corticosterone by the mitochondrial fraction of adrenal homogenates in vitro (25).

In conclusion, the present study suggests that citrate stimulates aldosterone production via activation of a specific protein tyrosine kinase system that includes tyrosine phosphorylation of Mr 105,000 substrate in bovine adrenal glomerulosa cells in vitro. Although the phosphorylated protein of Mr 105,000 was not analyzed precisely in the present study, its characteristics and its functional role, that of an intracellular protein tyrosine kinase responsible for citrate-induced aldosterone production as well as for the signals for citrate-induced activation of the kinase in the cells, remain to be elucidated.

This study was supported in part by a grant for Collaborative Research from Kanazawa Medical University (C96-10).

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