Repression of DAX-1 and Induction of SF-1 Expression

TWO MECHANISMS CONTRIBUTING TO THE ACTIVATION OF ALDOSTERONE BIOSYNTHESIS IN ADRENAL GLOMERULOSA CELLS*

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Angiotensin II (Ang II) and adrenocorticotropic hormone stimulate aldosterone biosynthesis in the zona glomerulosa of the adrenal cortex through induction of the expression of the steroidogenic acute regulatory (STAR) protein, which promotes intramitochondrial cholesterol transfer. To understand the mechanism of this induction of the STAR protein, we have examined the effect of Ang II and forskolin, a mimicker of adrenocorticotropic hormone action, on two transcription factors known to modulate STAR gene expression in opposite ways, DAX-1 and SF-1, in bovine adrenal glomerulosa cells in primary culture. Ang II markedly inhibited DAX-1 protein expression in a time- and concentration-dependent manner (to 38.7 ± 12.9% of controls at 3 nm after 6 h, p < 0.01), an effect that required de novo protein synthesis and ERK2/1 activation. This effect was associated with a concomitant decrease in DAX-1 mRNA and an increase in mitochondrial STAR protein levels. Similarly, forskolin dramatically repressed DAX-1 protein and mRNA expression (to 19.6 ± 1.8 and 50.3 ± 4.7% of controls, respectively, p < 0.01). Neither Ang II nor forskolin affected DAX-1 protein and mRNA stability.

The aldosterone response to Ang II was markedly reduced (to 59 ± 4% of controls, p < 0.01) in transiently transfected cells overexpressing DAX-1. Whereas Ang II was without effect on SF-1 expression, forskolin significantly increased SF-1 protein and mRNA levels in a cycloheximide-sensitive manner (to 167.4 ± 16.6 and 173.1 ± 25.1% of controls after 6 h, respectively, p < 0.01). These results demonstrate that the balance between repressor and inducer function of DAX-1 and SF-1 are of critical importance in the regulation of adrenal aldosterone biosynthesis.

The biosynthesis of aldosterone, the main mineralocorticoid hormone, in the zona glomerulosa cells of the adrenal cortex is placed under the control of three principal physiological factors, the octapeptide hormone, angiotensin II (Ang II),¹ extra-}

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‡ The abbreviations used are: Ang II, angiotensin II; ACTH, adrenocorticotropic hormone; DAX-1, dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X-chromosome, gene 1; IOD, integrated optical density; P450scc, cholesterol side chain cleavage cytochrome P450; SF-1, steroidogenic factor-1; StAR protein, steroidal-

genic acute regulatory protein; RT, reverse transcriptase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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1259
In addition to response elements for SF-1, the StAR gene promoter also bears a binding site for another orphan member of the nuclear receptor superfamily, DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1) (23, 24). DAX-1 has been shown to act as a powerful repressor of StAR gene expression. Indeed, overexpression of DAX-1 in Y-1 mouse adrenal tumour cells inhibits steroid synthesis, and DAX-1 represses both basal and cAMP-induced StAR promoter activity by binding to DNA hairpin secondary structures on the StAR gene promoter or to the SF-1 protein itself (25). Furthermore, overexpression of DAX-1 in Y-1 adrenocortical cells impairs basal and cAMP-activated steroid production (26). Conversely, cAMP down-regulates DAX-1 expression in cultured rat Sertoli cells (26). Finally, SF-1 and DAX-1 are co-localized in various endocrine and steroidogenic tissues, suggesting that these two nuclear proteins may be linked in function.

Because Ang II and ACTH stimulate StAR protein expression, the present study was undertaken to investigate whether the mechanism of this response involves a modulation of DAX-1 and SF-1 expression. We report here that both Ang II, a calcium mobilizing hormone, and forskolin, used as a mimicker of ACTH action to generate a pure CAMP signal, markedly inhibit DAX-1 expression at the mRNA and protein level. We also show that forskolin significantly increases SF-1 expression. This study provides evidence that the removal of the suppressor effect of DAX-1 on StAR expression is an important mechanism through which activators of aldosterone biosynthesis increase StAR expression.

**EXPERIMENTAL PROCEDURES**

**Bovine Adrenal Zona Glomerulosa Cell Culture and Treatments**—Bovine adrenal glands were obtained from a local slaughterhouse. Zona glomerulosa cells were prepared by enzymatic dispersion with dispase and purified on Percoll density gradients as previously reported (27). Bovine adrenal glands were obtained from a local slaughterhouse. Zona glomerulosa cells were maintained in Dulbecco's modified Eagle's medium as described in detail elsewhere (27). The cells were grown on 6-well tissue culture plates (3 × 10^5 cells/well) and kept in serum-free medium for 24 h before experiments, which were performed on the third day of culture. Cells were then washed and incubated at 37 °C in serum-free medium containing various agents for varying periods of time as appropriate. At the end of the incubation period, the media were collected, and cells were processed for protein or varying periods of time as appropriate. At the end of the incubation period, the media were collected, and cells were processed for protein or}

**Reverse Transcription-Polymerase Chain Reaction—**RT-PCR was used to evaluate DAX-1 and SF-1 mRNA abundance in response to various treatments. RT-PCR measurements of mRNA were performed using Promega reagents and 500 ng of total RNA per reaction. Reverse transcription with avian myeloblastosis virus RT (5 units/reaction, 45 °C for 30 min) primed with specific primers was followed by PCR using DNA polymerase (5 units/reaction) with the following cycling parameters: denaturation for 5 min at 95 °C followed by 30 and 25 cycles for DAX-1 and SF-1, respectively, of 95 °C for 1 min, 56 °C for 1 min, and 68 °C for 30 s and a final extension at 68 °C for 10 min. The primers were bovine SF-1 (5'-GCCAAGAGGCCACCAGAATCC-3' (forward) and 5'-TGGTGATCAGACTGTGATGC-3') (reverse). Primers corresponded to positions +439-719 and 693-139 bp of SF-1 mRNA (NCBI/GenBank* accession number NM_000457). The PCR product was purified from a 1% agarose gel, cloned into pGEM*-T Easy vector (Promega), and amplified in JM 109 competent cells. The plasmid insert was sequenced by automatic sequencing using the DyEnamics Terminator sequencing kit (Amersham Biosciences) and Applied Biosystem 3100 sequencer.

**Determination of Aldosterone Production—**Aldosterone content in incubation media was measured by direct radioimmunoassay using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX). Aldosterone production was normalized and expressed per milligram of cellular protein.

**Western Blot Analysis—**For the determination of protein expression levels, bovine glomerulosa cells were washed twice in ice-cold phosphate buffer (PBS) and lysed in PBS containing 1% (w/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) deoxycholate, 1 mm phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. The cell debris were removed by centrifugation at 20,000 × g at 4 °C for 15 min, and supernatants were used as cell lysates. Proteins were quantified using a protein microassay (Bio-Rad). Equal amounts of protein (200 μg) were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Western blot analysis of DAX-1 and SF-1 proteins was carried out with a mouse monoclonal antiserum directed against DAX-1 (kindly provided by Dr. Enzo Laflif, Strasbourg, France) or rabbit polyclonal antiserum to Ad4BP/SF-1 (kindly supplied by Dr. Ken Morohashi, University of Tsukuba, Ibaraki Prefecture, Japan). Mitochondrial StAR protein levels were determined by Western blot as previously described (8). Immunoreactive proteins were visualized by the enhanced chemiluminescence method (ECL, Amersham Biosciences). Band intensity was quantified with a Molecular Dynamics Computing Densitometer.

**Angiotensin II Inhibits DAX-1 Protein Expression**—DAX-1 protein levels were high in unstimulated bovine adren al glomerulosa cells. When cells were treated with Ang II for 6 h, a significant, concentration-dependent inhibition of DAX-1 pro-
tein expression was observed between 0.1 and 10 nM Ang II, as determined by Western blot analysis. Maximal inhibition (to 38.7 ± 12.9% of controls, n = 3–9, p < 0.01) was achieved with 3 nM Ang II (Fig. 1, A and B).

Concomitantly, Ang II powerfully stimulated intramitochondrial StAR protein accumulation (Fig. 1C) and aldosterone production (Fig. 1D) over the same range of concentrations. At 10 nM Ang II, aldosterone production into the medium was increased by 71-fold (127.2 ± 19.4 pmol/mg of protein versus 1.8 ± 0.26 pmol/mg in control cells, n = 3, p < 0.01).

Kinetics of the Effect of Ang II on DAX-1 Protein Expression—To determine the kinetics of the inhibition of DAX-1 protein expression induced by Ang II, we stimulated glomerulosa cells with 10 nM Ang II for 0–6 h. As shown in Fig. 2, A and B, the inhibitory effect of Ang II was time-dependent, with DAX-1 protein levels reaching 61.5 ± 4.6% (n = 5, p < 0.01) and 40 ± 3.4% of controls (n = 5, p < 0.01) after 4 and 6 h, respectively. No further decrease in DAX-1 protein expression was observed thereafter (data not shown). The progressive inhibition of DAX-1 protein expression was accompanied with a time-dependent increase in aldosterone production from Ang II-treated adrenal glomerulosa cells (Fig. 2C).

Ang II-induced Inhibition of DAX-1 Protein Expression Requires Protein Synthesis—The inhibition of DAX-1 protein expression by Ang II depended upon de novo protein synthesis. Indeed, although glomerulosa cells stimulated with 10 nM Ang II for 6 h showed a significant decrease in DAX-1 protein levels (to 40.8 ± 2.6% of controls, n = 3, p < 0.01), simultaneous treatment with cycloheximide, a protein translation inhibitor, abolished the Ang II-induced inhibition of DAX-1 protein expression (Fig. 3, A and B). Cycloheximide also completely abolished the aldosterone response to Ang II, as previously reported by others (28) (Fig. 3C).

Involvement of ERK2/1 Activation in Ang II-induced Repression of DAX-1—Ang II is known to activate ERK2/1 in bovine adrenal glomerulosa cells (29). To determine whether inhibition of DAX-1 protein expression by Ang II is mediated by the mitogen-activated protein kinase pathway, we stimulated cells with Ang II alone or in combination with U0126 (Biomol, Plymouth Meeting, PA), an inhibitor of MEK-1, the kinase that phosphorylates and activates ERK2/1. As shown in Fig. 4, A and B, the inhibitory effect of Ang II on DAX-1 expression was completely abolished in the presence of U0126. In contrast, the p38 mitogen-activated protein kinase inhibitor, SB203580, did not affect Ang II action (data not shown).

To determine whether Ang II exerts its inhibitory effect directly on DAX-1 mRNA expression, we stimulated glomerulosa cells for 6 h with 10 nM Ang II alone or in combination with 10 μM U0126, and DAX-1 mRNA levels were then determined by semi-quantitative RT-PCR. The results shown in Fig. 4, C and D, indicate that Ang II treatment led to a significant decrease in bovine DAX-1 mRNA levels (to 68.8 ± 4.3% of controls, n = 4, p < 0.01). This down-regulation of DAX-1 mRNA by Ang II was completely prevented by the MEK-1 inhibitor U0126.

The involvement of ERK2/1 in the functional response to Ang II was also observed at the level of steroid production. Indeed, as shown in Fig. 4E, two structurally unrelated MEK-1 inhibitors, U0126 and PD98059 (Alexis Biochemicals, Läufelfingen, Switzerland), significantly reduced aldosterone production elicited by Ang II.

Forskolin Inhibits DAX-1 Protein and mRNA Expression—We next examined whether another inducer of StAR expression and aldosterone production, forskolin, used as a mimicker of ACTH, which mobilizes the cAMP messenger system, also affects DAX-1 expression. As shown in Fig. 5, A and B, treatment of adrenal glomerulosa cells with 25 μM forskolin for 6 h dramatically inhibited DAX-1 protein expression (to 19.6 ± 1.82% of controls, n = 3, p < 0.001). As for Ang II, inhibition of DAX-1 protein expression by forskolin was concentration- and time-dependent (data not shown). Furthermore, the inhibition of DAX-1 protein expression by forskolin...
was completely prevented by cycloheximide. In contrast, the MEK-1 inhibitor, U0126, had no effect on forskolin action. The inhibition of DAX-1 protein expression provoked by forskolin was associated with a 56-fold increase in aldosterone production (Fig. 5).

The inhibition of DAX-1 expression by forskolin was also observed at the transcriptional level, as demonstrated in Fig. 5, C and D. Indeed, in glomerulosa cells stimulated with forskolin, DAX-1 mRNA levels were decreased to 50.3 ± 4.7% of control untreated cells (n = 3, p < 0.001). This effect was not reversed by the MEK-1 inhibitor, U0126. Moreover, actinomycin D did not affect the reduction of DAX-1 mRNA expression elicited by forskolin (data not shown).

**Ang II and Forskolin Do Not Affect DAX-1 Protein and mRNA Degradation**—To determine whether Ang II and forskolin decreased DAX-1 protein levels by accelerating its catabolism, we incubated bovine glomerulosa cells for 6 h in the absence or presence of either agonist and then followed DAX-1 protein levels under conditions of protein synthesis blockade. As shown in Fig. 6A, DAX-1 protein levels were quite stable...
over time under basal conditions and in the absence of cycloheximide. In contrast, when de novo protein synthesis was prevented, the levels of DAX-1 protein decreased markedly with time, with a half-life of $\frac{1}{6}$ h. Neither Ang II nor forskolin accelerated this process (Fig. 6A).

The inhibition of DAX-1 mRNA expression could result from changes in transcription rate and/or in mRNA turnover. We therefore examined whether Ang II affected DAX-1 mRNA stability. Glomerulosa cells were treated for 6 h in the absence or presence of Ang II (10 nM), and then actinomycin D was added. After 6 h, DAX-1 mRNA levels decayed to 45.2% of the zero time value in cells treated with actinomycin D alone and to a similar value (53.4% ± 4.9%, n = 3, p < 0.01) in cells that had been pretreated with Ang II (Fig. 6B). Similar results were obtained with forskolin (data not shown).

Effect of Overexpression of DAX-1 on the Aldosterone Response to Ang II—The role of DAX-1 on the aldosterone response to Ang II was confirmed in bovine glomerulosa cells that had been transiently transfected with DAX-1 cDNA. In cells transfected with the empty pBKCMV vector, Ang II induced a robust aldosterone production (Fig. 7, Mock). Although the absolute aldosterone values were somewhat lower than in non-transfected cells, possibly because of the transfection procedure itself, the fold increase over basal was not significantly different (see, for example, Figs. 1D, 3C, or 4C). This aldosterone response was associated with the expected repression of DAX-1. In contrast, in cells transiently transfected with pBKCMV-hDAX-1, which expressed 20–30-fold higher levels of the DAX-1 protein, the aldosterone response to Ang II was significantly reduced to 59.4% ± 4% of the response measured in mock-transfected cells (n = 3, p < 0.01).

Effects of Ang II and Forskolin on SF-1 Protein and mRNA Expression—Because the transcription factor SF-1 is known to regulate StAR protein expression, we examined whether the induction of StAR expression induced by both Ang II and forskolin involves changes in SF-1 protein expression in adrenal glomerulosa cells. As shown in Fig. 8, A and B, a significant increase in SF-1 protein expression was observed in cells stimulated for 6 h with forskolin (167.4 ± 16.6% of controls, n = 7, p < 0.001), whereas Ang II had no effect. The forskolin-induced increase in
SF-1 protein expression was blocked by cycloheximide.

We next examined whether forskolin and Ang II affected SF-1 mRNA expression. Bovine glomerulosa cells were stimulated for 6 h with 25 μM forskolin (FK) alone or in combination with 10 μM U0126 or 50 μM cycloheximide (CHX). DAX-1 protein analysis and DAX-1 mRNA RT-PCR were performed as described in the legends of Figs. 1 and 4. Ctrl, control. A, Western blot of DAX-1 from a representative experiment. B, densitometric analysis of DAX-1 protein expression shown as a percentage of control. C, representative ethidium bromide staining of an agarose gel after 30 cycles of RT-PCR. D, densitometric analysis of DAX-1 mRNA shown as percentage of untreated control after normalization for GAPDH mRNA. E, aldosterone production in the culture medium. Values represent the mean ± S.E. of three independent experiments. **, significantly different from control, p < 0.01. Values represent the mean ± S.E. of three independent experiments.

FIG. 5. Forskolin inhibits DAX-1 protein and mRNA expression in adrenal glomerulosa cells. Adrenal glomerulosa cells were stimulated for 6 h with 25 μM forskolin (FK) alone or in combination with 10 μM U0126 or 50 μM cycloheximide (CHX). DAX-1 protein analysis and DAX-1 mRNA RT-PCR were performed as described in the legends of Figs. 1 and 4. Ctrl, control. A, Western blot of DAX-1 from a representative experiment. B, densitometric analysis of DAX-1 protein expression shown as a percentage of control. C, representative ethidium bromide staining of an agarose gel after 30 cycles of RT-PCR. D, densitometric analysis of DAX-1 mRNA shown as percentage of untreated control after normalization for GAPDH mRNA. E, aldosterone production in the culture medium. Values represent the mean ± S.E. of three independent experiments. **, significantly different from control, p < 0.01. Values represent the mean ± S.E. of three independent experiments.

DISCUSSION

The present study was undertaken in an attempt to investigate the mechanism of the known induction of StAR protein expression in adrenal glomerulosa cells in response to two physiological activators of aldosterone biosynthesis, the octapeptide hormone angiotensin II and adrenocorticotropic hormone, ACTH (10, 30). We have focused our attention on two orphan members of the nuclear receptor family of transcription factors, DAX-1 and SF-1. Indeed, on one hand, DAX-1 has been shown to repress StAR gene expression by binding to a hairpin structure located in the StAR gene promoter and to block steroidogenesis (25). On the other hand, multiple binding elements for SF-1 have been reported in the 5′-flanking region of the StAR gene and are required for maximal promoter activity (4).

Two main conclusions can be drawn from the present work. 1) Ang II, which recruits the calcium messenger signaling system (31), and forskolin, used as a mimicker of ACTH action via cAMP generation, both markedly repressed DAX-1 expression, and 2) forskolin significantly increased the expression of SF-1. It is worth stressing that these observations were obtained in bovine glomerulosa cells in primary culture, expressing normal levels of only endogenous DAX-1 and SF-1, rather than in artificial overexpression systems.

The inhibition of DAX-1 exerted by Ang II and forskolin was manifest at both the protein and mRNA levels. In fact, although most studies have focused on the developmental and tissue distribution of DAX-1 expression in various species (32, 33), on its mechanism of action (25, 34, 35), and on the incidence of mutations in the DAX-1 gene on adrenal and reproductive functions (23, 24, 36, 37), information on the regulation of DAX-1 expression in steroidogenic tissues is practically non-existent. In our hands, the aldosterone response was correlated with the extent of the repression of DAX-1, the highest amounts of the mineralocorticoid being produced when

SF-1 protein expression was blocked by cycloheximide.

We next examined whether forskolin and Ang II affected SF-1 mRNA expression. Bovine glomerulosa cells were stimulated for 6 h with 10 nM Ang II or 25 μM forskolin, and SF-1 mRNA levels were then determined by semi-quantitative RT-PCR. The results shown in Fig. 8, C and D, indicate that forskolin treatment led to a significant increase in bovine SF-1 mRNA levels (to 173.1 ± 25.1% of controls, n = 5, p < 0.05). In contrast, Ang II had no significant effect on SF-1 mRNA expression. The forskolin-induced increase in SF-1 mRNA expression was almost completely abolished by actinomycin D (Fig. 8, C and D), suggesting a direct action of forskolin on SF-1 mRNA at the transcriptional level.
DAX-1 protein levels were the lowest. In principle, two main mechanisms can account for the inhibition of DAX-1 expression observed in response to Ang II and forskolin; they are 1) an inhibition of DAX-1 gene transcription and/or translation, leading to reduced mRNA and protein levels, and 2) a decrease in DAX-1 mRNA and/or protein stability. Our data indicate that the latter mechanism is much less likely to occur. Indeed, neither Ang II nor forskolin accelerated the decay rate of DAX-1 mRNA or protein under conditions of blockade of transcription with actinomycin or blockade of protein translation with cycloheximide; a half-life of ~6 h was measured for the DAX-1 protein whether the agonists were present or not. Therefore, a more likely mechanism should be the induction by Ang II and forskolin of a repressor protein, which blocks DAX-1 gene transcription. Consistent with this hypothesis, the repression exerted by Ang II and forskolin was abolished under conditions of protein synthesis inhibition.

A number of studies suggest that the mitogen-activated protein kinase cascade is involved in the regulation of steroidogenesis. For example, ERK2/1 activation mediates the stimulation of steroid production in porcine (35), murine (38), and human (39) ovarian granulosa cells. In bovine adrenal glomerulosa cells, Ang II stimulates ERK2/1 (p42/44) activity via protein kinase C and Ras/Raf-1 kinase (29). Whether this activation contributes only to the trophic effect of Ang II on adrenal cells or also to the steroidogenic response was not known. In the present study, the ERK2/1-mediated inhibition of DAX-1 expression induced by Ang II would suggest a major role for ERK2/1 in Ang II-induced aldosterone production. In contrast, the role of ERK2/1 in the activation of adrenal steroidogenesis by ACTH or forskolin is controversial. Thus, forskolin was found to increase StAR expression and steroidogenesis in Y1 mouse adrenocortical tumor cells and to activate ERK2/1 (21). In contrast, in bovine fasciculata and rat glomerulosa cells, ACTH treatment did not lead to p42/44 mitogen-activated protein kinase (ERK2/1) activation (40, 41). In agreement with the latter reports, in our hands the massive inhibition of DAX-1 expression elicited by forskolin was insensitive to two unrelated inhibitors of p42/44 activation, a finding that rules out an involvement of ERK2/1 in the steroidogenic response to cAMP.

The role of DAX-1 in the steroidogenic response to Ang II was corroborated in cells overexpressing DAX-1. Indeed, Ang II-induced aldosterone production was markedly reduced in cells expressing 20–30-fold higher levels of DAX-1. This finding is in agreement with a previous report showing that steroid production is impaired in Y-1 adrenocortical cells expressing DAX-1 (34) and, to the contrary, with the demonstration that mice lacking DAX-1 show an increased adrenal responsiveness to

![Fig. 6. Lack of effect of Ang II and forskolin of DAX-1 protein and mRNA stability.](image)

![Fig. 7. Effect of DAX-1 overexpression on the aldosterone response to Ang II.](image)
that forskolin markedly increases SF-1 protein expression, a result that is also compatible with an increase in the amount of phosphorylated SF-1.

In contrast to forskolin, Ang II had no effect on SF-1 expression. In human H295R adrenocortical carcinoma cells, deletion analysis of the StAR gene promoter has shown that Ang II and dibutyryl cAMP activation of reporter gene expression requires the presence of one critical SF-1 binding site located proximally to the transcriptional start site, within the 150 first bases (10, 13). No effect of Ang II or forskolin on endogenous SF-1 protein expression or phosphorylation was reported. The proximal SF-1 binding element overlaps with a hairpin structure (nucleotides −61 to −27), which is a binding site for DAX-1 and mediates DAX-1-induced StAR gene repression (25). In fact, DAX-1 is likely to act as a suppressor of SF-1-induced transcription. Indeed, recent studies demonstrate a direct interaction between DAX-1 and SF-1, potentially recruiting co-repressors such as NcoR (nuclear receptor co-repressor) to the transcription factor complex (48, 49). Our results demonstrating that Ang II and forskolin block DAX-1 expression are, therefore, compatible with a model in which binding of DAX-1 to the hairpin structure and/or heterodimer formation between DAX-1 and SF-1 is prevented, the latter thus becoming able to enhance StAR gene expression.

In summary, we have shown that two activators of aldosterone biosynthesis in adrenal zona glomerulosa cells mobilizing distinct signaling pathways markedly lower the expression levels of DAX-1, a known repressor of the STAR protein, which plays a crucial role in the activation of steroidogenesis. Our studies provide the first evidence that the balance between DAX-1 and SF-1 expression levels, i.e., between repressor and inducer functions, may play a key role in the fine tuning of the mineralocorticoid response. In view of the specific distribution of these two transcription factors, this mechanism may be of general relevance in all steroid-producing tissues.

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