Aldosterone's Rapid, Nongenomic Effects Are Mediated by Striatin: A Modulator of Aldosterone's Effect on Estrogen Action

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The cellular responses to steroids are mediated by 2 general mechanisms: genomic and rapid/nongenomic effects. Identification of the mechanisms underlying aldosterone (ALDO)'s rapid vs their genomic actions is difficult to study, and these mechanisms are not clearly understood. Recent data suggest that striatin is a mediator of nongenomic effects of estrogen. We explored the hypothesis that striatin is an intermediary of the rapid/nongenomic effects of ALDO and that striatin serves as a novel link between the actions of the mineralocorticoid and estrogen receptors. In human and mouse endothelial cells, ALDO promoted an increase in phosphorylated extracellular signal-regulated protein kinases 1/2 (pERK) that peaked at 15 minutes. In addition, we found that striatin is a critical intermediary in this process, because reducing striatin levels with small interfering RNA (siRNA) technology prevented the rise in pERK levels. In contrast, reducing striatin did not significantly affect 2 well-characterized genomic responses to ALDO. Down-regulation of striatin with siRNA produced similar effects on estrogen's actions, reducing nongenomic, but not some genomic, actions. ALDO, but not estrogen, increased striatin levels. When endothelial cells were pretreated with ALDO, the rapid/nongenomic response to estrogen on phosphorylated endothelial nitric oxide synthase (peNOS) was enhanced and accelerated significantly. Importantly, pretreatment with estrogen did not enhance ALDO's nongenomic response on pERK. In conclusion, our results indicate that striatin is a novel mediator for both ALDO's and estrogen's rapid and nongenomic mechanisms of action on pERK and phosphorylated eNOS, respectively, thereby suggesting a unique level of interactions between the mineralocorticoid receptor and the estrogen receptor in the cardiovascular system. (***Endocrinology*** **155: 2233–2243, 2014)**

Classically, steroid hormones bind and activate intracellular receptors that function as nuclear transcription factors inducing the expression of specific mRNA transcripts within steroid-responsive cells. More recently, there has been increasing evidence that steroid hormones also can initiate rapid and initially nongenomic responses

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Abbreviations: ALDO, aldosterone; CAV_1, caveolin-1; CM-H_2DCFDA, 5–6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; EC, endothelial cell; EGF, epidermal growth factor; EM, electron microscopy; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GR, glucocorticoid receptor; IP, immunoprecipitation; KO, knockout; MR, mineralocorticoid receptor; MRA, MR antagonist; peNOS, phosphorylated eNOS; pERK, phosphorylated ERK; PP2A, protein phosphatase 2A; PTGIS, prostaglandin I2; PTGS1, prostaglandin-endoperoxide synthase 1; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; SGK1, glucocorticoid-regulated kinase 1; siRNA, small interfering RNA; STRN, striatin; STRN3, striatin-calmodulin binding protein 3; WNK4, lysine-deficient protein kinase 4.

(1). For example, in addition to aldosterone (ALDO)'s well-characterized effects on gene transcription and sodium homeostasis, it also has rapid nongenomic effects that appear to involve cross talk between the mineralocorticoid receptor (MR) and other signaling cascades, including c-Src kinase, MAPK, and the G protein-coupled receptor, G protein-coupled estrogen receptor 1 (2, 3). Specifically, ALDO rapidly increases phosphorylated ERK1/2 (pERK) (4) and production of reactive oxygen species (ROS), such as superoxide (5).

In part, the controversy concerning the validity and/or significance of the nongenomic effects of steroids is largely the result of 2 facts: the substantial body of data concerning mediators of their genomic effects and the limited data concerning potential mediators of their nongenomic effects. Recently, 2 studies have raised the possibility that striatin may be such a mediator. In one, striatin was proposed to be a critical intermediary in the nongenomic actions of estrogen in endothelial cells (ECs) (6). In the other, we reported that activation of MR leads to increases of striatin levels in the vasculature (7).

Activation of nongenomic effects by estrogen has been identified in several cell types (8–10), and the mechanisms underlying these effects are linked to the association of estrogen receptor (ER)α with caveolin-1 (CAV_1), presumably in membrane-associated caveolae, and/or with striatin (6). Striatin, a 780-amino acid protein, was identified and cloned more than a decade ago (11). In adult mammals, striatin is mainly expressed in neurons of both the central and peripheral nervous systems with very high levels of expression in the striatum, hence its name. Two other striatin family members are described in higher eukaryotes: striatin-calmodulin binding protein 3 (STRN3) and zinedin (12). At the molecular level, striatin has 4 protein-protein interaction domains: a caveolin-binding domain, a coiled-coil structure, a Ca^{2+}-calmodulin binding domain, and a large WD-repeat domain at the C-terminal region of the protein.

Based on the information that striatin has a CAV_1 binding domain and that both ERα (6) and the MR also bind to CAV_1 (13), we hypothesized that striatin is a universal mediator of the nongenomic effects of the steroids receptors, MR and ERα. In this study, we test the hypothesis that not only is striatin involved in the nongenomic effects of steroids but also is a mediator of cross talk between steroids at a previously unrecognized level. We propose that striatin's involvement in the nongenomic effects of steroids is broad based and that it may serve as an integrator of rapid nongenomic effects of various steroids.

Materials and Methods

Animals

Twelve-week-old C57BL/6J male mice were purchased from The Jackson Laboratory. Animals were housed in the animal facility in a 12-hour light, 12-hour dark cycle at an ambient temperature of 22 ± 1°C and were maintained on ad libitum Purina rodent chow (0.8% NaCl, 5053; Purina) and tap water. After 3 days of acclimatization, 5 mice were killed for aorta collection. All experimental procedures followed the guidelines of and were approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Cell culture

EA.hy926 cells were maintained in DMEM essentially as described by others (6, 14) and detailed in Supplemental Materials. Mouse aortic ECs were isolated from male mice under sterile conditions as previously described (7, 15, 16) and maintained on DMEM (see Supplemental Materials for details). Our cell cultures showed positive staining for EC markers, because they were positive for Von Willebrand factor and platelet-endothelial cell adhesion molecule-1 (CD31) using immunofluorescence techniques (Supplemental Figure 1).

Measurement of ROS

The oxidative fluorescent indicator dye 5–6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (C6827; Invitrogen) was used as a probe to measure the ROS production essentially as previously described (17–19). Confluent EA.hy926 ECs in 6-well plates were washed with fresh DMEM and incubated with DMEM premixed with 10μM CM-H_2DCFDA for 1 hour at 37°C. Cells were washed twice with PBS to remove the loading buffer and cells allowed to recover for 10 minutes at 37°C for cellular esterases to deacetylate H_2DCFDA. Cells were then stimulated with or without 1nM ALDO for 30 minutes releasing a variety of ROS species, which oxidize H_2DCF into DCF. Intracellular DCF fluorescence was recorded in a FlexStation III Microplate Reader (Molecular Device) after excitation at 492 nm and emission 527 nm. To normalize fluorescence measurements, background fluorescence of cell-free mixtures was taken, and total proteins were measured using the Pierce BCA protein assay kit (Thermo Scientific).

RT and quantitative real-time PCR (qPCR)

Total RNA was extracted using the RNeasy Mini kit (QIAGEN Sciences) following the manufacturer's instructions as described by us (16). cDNA was synthesized from 3 μg of total RNA with the First Strand cDNA Synthesis kit (Amersham). PCR amplification reactions were performed with TaqMan gene expression assays in duplicate with the use of the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The $\Delta\Delta$ cycle threshold method was used to determine mRNA levels. Target gene expression was normalized to 18S rRNA levels. TaqMan Probes used were from Applied Biosystems: zinedin (STRN4, Hs00183850_m1), STRN3 (Hs00205827_m1), striatin (STRN, Hs00162404_m1), lysine-deficient protein kinase 4 (WNK4, Hs00260769_m1), serum glucocorticoid-regulated kinase 1 (SGK1) (Hs00178612_m1), prostaglandin I2 (PTGIS) (Hs00919949_m1), and prostaglandin-endoperoxide synthase 1 (PTGS1) (Hs00168776_m1).

Immunoprecipitation (IP)

IP assays were performed as previously described us (13). In brief, cells were homogenized in IP buffer (RIPA buffer; Santa Cruz Biotechnology, Inc), and the cell lysate was sonicated for 20 seconds (30 V) and incubated for 30 minutes at 4°C. The lysate was then centrifuged at 10 000*g* for 10 minutes, and protein content was determined by Micro BCA protein kit (Pierce). The protein (500 μg) was incubated with 1–2 μg of monoclonal or polyclonal antibodies for 1 hour at 4°C together with 50–100 μL of protein G or A/G MicroBeads (Miltenyi Biotec). The microbeads, antibody, and cell lysate mix was separated using MACSmini column and respective magnetic stand. Finally, the beads were washed with RIPA buffer and the bound immunocomplexes eluted using boiling loading dye and assessed by Western blot analysis. The antibodies used were from BD Transduction Laboratories, mouse antistriatin (catalog no. 610838) and mouse anti-CAV_1 (catalog no. 611338); and Santa Cruz Biotechnology, Inc, rabbit anti-MR (catalog no. sc11412) and rabbit anti-CAV_1 (catalog no. sc 894). The specificity of the rabbit anti-MR was assessed and compared with those provided by Dr Gomez-Sanchez (20; see also Refs. 21, 22).

Electron microscopy (EM)

ECs have caveolae. However, they lose these structures in vitro (14). To this end, EA.hy926 and mouse aortic ECs were analyzed for the detection of caveolae by EM as detailed in Supplemental Materials. Images were collected using an Advanced Microscopy Techniques digital imaging system (Supplemental Figure 2).

Small interfering RNA (siRNA) technology

An initial screen was performed using DharmaFECT 1 Transfection Reagent and 4 different oligonucleotides for striatin on EA.hy926 cells to determine which siRNA oligonucleotide most efficiently caused maximal knockdown of our target gene, striatin (Supplemental Figure 3). Details of this approach and Western blot analyses are presented in Supplemental Materials.

Data analysis

Each experiment was performed at least 3 times in triplicate determination. Data were analyzed by one-way ANOVA followed by the Tukey or Newman-Keuls post hoc test for multiple comparisons. Differences in means with probability values of less than or equal to 0.05 were considered statistically significant. Values are expressed as mean ± SEM.

Results

Striatin and MR closely interact in ECs

We have previously shown that striatin and MR proteins are expressed in cultured human ECs (EA.hy926) and in early passages of mouse aortic ECs (7). Both cell lines were further analyzed microscopically and shown to express EC markers (Supplemental Figure 1) and caveolae (Supplemental Figure 2). In addition, co-IP assays confirmed an association between striatin and MR as recently reported by us (7).

ALDO stimulates pERK through an MR-dependent mechanism

EA.hy926 cells were stimulated with varying concentrations of ALDO for 15 minutes. pERK levels were then measured by Western blot analysis and normalized to total ERK expression as reported by us (13). The lowest ALDO concentration (10^{-8} mol/L) produced a 40% increase in the pERK/ERK ratio (Figure 1A). Incubation of EA.hy926 cells with 10^{-8} mol/L ALDO for different time periods resulted in a peak of the pERK/ERK ratio at 15 minutes that was abrogated when cells were preincubated with the MR antagonist (MRA) canrenoic acid (10^{-6} mol/L) (Figure 1B). These results show that ALDO (10^{-8} mol/L) leads to a rapid increase in pERK levels through a mechanism that requires MR activation in EA.hy926 cells. It is important to note that at 10^{-8} mol/L ALDO, we consistently observed significantly increased responses that varied from 1.4- to 2.5-fold. This range in responses was probably due to the use of different batches of cells used at the different times the experiments were performed. We assume that the absolute differences were

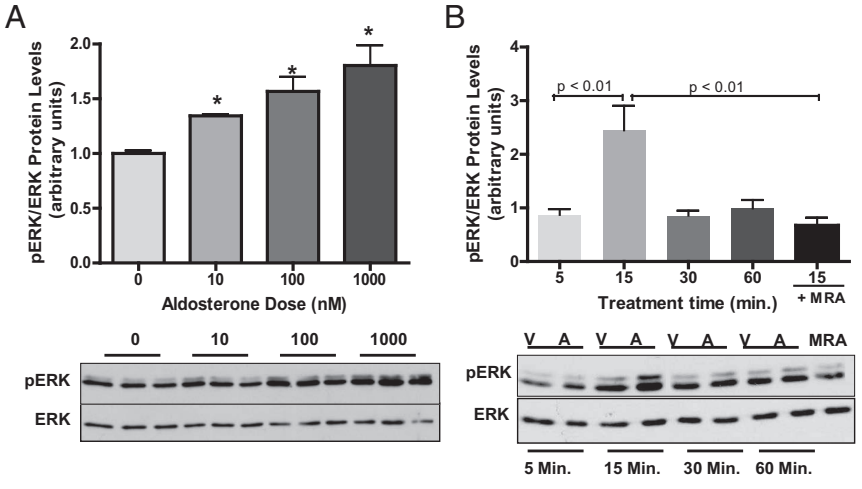

Figure 1. ALDO increases pERK levels. A, EA.hy926 cells were stimulated with different concentrations of ALDO for 15 minutes. Protein levels were measured by Western blot analysis; *, $P < .05$ compared with time point 0. The minimum ALDO concentration (10^{-8} mol/L) produced a 40% increase in the pERK/ERK ratio. B, ALDO (10^{-8} mol/L) leads to a rapid increase in pERK/ERK ratio with maximum stimulation at 15 minutes in EA.hy926 cells. Preincubation with 10^{-6} mol/L canrenoic acid (MRA), an MRA, prevents the increase in the pERK/ERK ratio, suggesting that ALDO is acting through the MR. Values shown in the graph were calculated comparing the ALDO (A)-treated cells with the respective vehicle (V) and plotted as mean ± SEM.

secondary to minor differences in the cells used. Importantly, even though the absolute percentage of changes differed, the changes were all in the same direction and significantly so. We propose that this further strengthens the validity of our findings because of the replication achieved by studying different batches of EA.hy926 cells.

Striatin is essential in mediating MR-dependent nongenomic/rapid responses to ALDO

Using siRNA technology to reduce the levels of striatin, EA.hy926 cells were transfected and subsequently stimulated with ALDO as described in Supplemental Figure 3. The transfection process itself did not modify the pERK/ERK levels, as assessed in cells transfected with scrambled siRNA and untransfected cells, which had nearly identical pERK/ERK levels, basally and in response to ALDO. The results show that in the presence of lower levels of striatin protein, the peak in pERK/ERK ratio is abrogated (Figure 2, A and B). This observation is reinforced by examining the effect of the siRNA on the levels of pERK. As anticipated by the data shown in Figure 2B, this effect is due mainly to increases in pERK in cells treated with ALDO relative to pERK in those treated with vehicle. In contrast, in the presence of striatin siRNA, the ratio of pERK in ALDO to placebo-treated cells did not increase. In fact, the ratios were reduced below control levels, suggesting that knocking down striatin may actually suppress de novo activity of this phosphorylation possibly via alterations on protein phosphatase 2A (PP2A) function, because this phosphatase is reported to be physically associated with striatin (36). However, this effect does not seem to be secondary to the experimental procedure, because cells treated with scrambled siRNA did not produce this response (Figure 2, A–C). Studies were performed in the presence of scrambled siRNA where ALDO increased pERK/ERK levels as observed in untransfected cells (Figure 2, A and C). These data suggest that striatin is needed

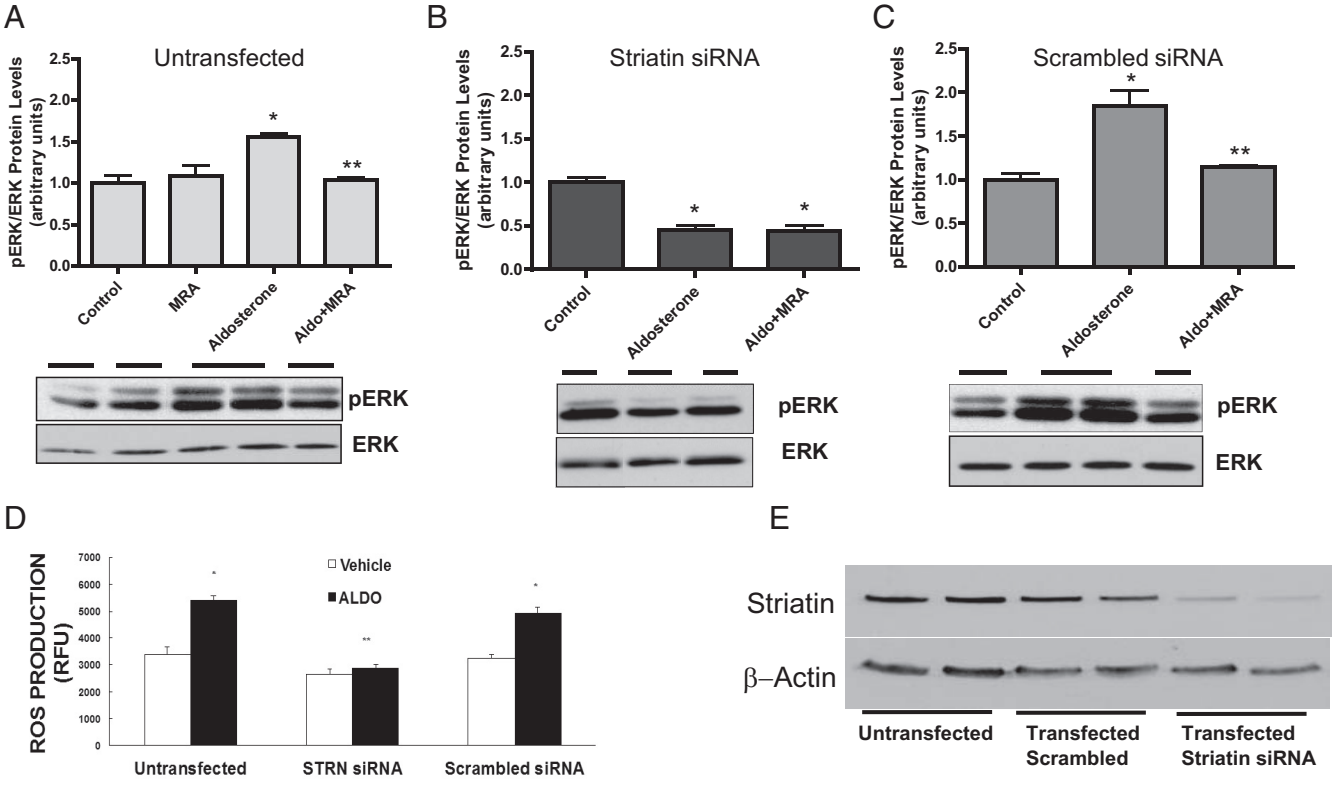

Figure 2. Striatin is essential in mediating MR-dependent rapid responses to ALDO. A, Untransfected EA.hy926 cells show an increase in the pERK/ERK ratio, after incubation with ALDO (10^{-8} mol/L) for 15 minutes (*, $P < .05$ vs control; **, $P < .05$ vs ALDO). B, The ALDO-mediated rapid increase in pERK levels is abolished when the EA.hy926 cells are transfected with striatin siRNA (*, $P < .01$ vs control). C, Transfection with scrambled siRNA does not block the ALDO-induced increase in the pERK/ERK ratio (*, $P < .05$ vs control; **, $P < .05$ vs ALDO). D, ROS production was measured as described in Materials and Methods, in the presence (black bars) or absence (vehicle [white bars]) of ALDO (10^{-8} mol/L) for 60 minutes at 37°C. Untransfected and scrambled siRNA-transfected EA.hy926 cells show an increase in ROS production after incubation with ALDO ($P < .05$ vs vehicle-treated cells). This ALDO-mediated increase in ROS production is abolished when EA.hy926 cells are transfected with striatin siRNA (*, $P <$ vs vehicle-treated cells; **, $P < .01$ vs untransfected ALDO-treated cells). Results shown are the averages ± SE of n = 9 independent experiments. E, Transfection of EA.hy926 cells with striatin siRNA but not scrambled leads to reduced striatin protein levels. siRNA transfections were performed as described above and proteins resolved by Western blotting. The Western blotting shown is a representative trace of 6 replicates. The membrane blot used to probe for striatin was stripped, washed, and reprobed for β-actin as loading control. RFU, relative fluorescence units.

for the rapid effects of ALDO on pERK levels. Reduction in striatin may lead to altered PP2A function and in part explain lower pERK levels in ALDO-stimulated cells with reduced striatin that is MR independent. Alternatively, perhaps all membrane-initiated pERK activation requires the presence of striatin. We performed additional control experiments to characterize the effect of reduced striatin on epidermal growth factor (EGF)-stimulated EA.hy926 ECs on pERK levels. We studied the effect of EGF on pERK levels in 2 stably transfected EA.hy926 striatin knockdown cell lines and an empty vector control cell line that were recently characterized (25). Four separate cultures derived from 2 independent clones for each cell line were performed. In contrast to striatin's role in ALDO and estrogen's effects on phosphorylation of ERK and endothelial nitric oxide synthase (eNOS), respectively, knocking down striatin does not block phosphorylation of ERK in response to EGF, a nonsteroidal agonist (Supplemental Figure 4). Indeed, if anything the response to EGF is enhanced. Thus, not all membrane-initiated ERK activation pathways require the presence of striatin. However, further studies are needed to clarify this issue.

Because ALDO has been previously shown to rapidly stimulate ROS production in vitro (5), EA.hy926 cells were transfected as above with siRNA for striatin and subsequently stimulated with ALDO and ROS production measured as detailed above in Materials and Methods (Figure 2D). ALDO caused a significant increase in ROS production in these cells. The transfection process itself did not modify baseline levels of ROS significantly, as assessed in cells transfected with scrambled siRNA and untransfected cells, which had nearly identical ROS levels, basally and in response to ALDO. However, when striatin levels were substantially reduced by treatment with siRNA (Figure 2E), the ALDO-stimulated ROS response was abrogated, supporting the concept that striatin is necessary for the rapid effects of ALDO. Striatin protein levels were measured 48 hours after addition of striatin siRNA (Figure 2E).

Striatin is not essential in mediating MR-dependent genomic effects of ALDO

To determine whether reducing striatin levels would have any effect on some of ALDO's classical genomic actions, EA.hy926 cells were stimulated with ALDO for different time periods, and the mRNA levels of known ALDO-responsive genes (26, 27) were quantified using real-time RT-qPCR analyses (Figure 3, A and B) as described by us (7). Our results show that, consistent with previous reports, mRNA levels for both WNK4 and SGK1 were elevated at 5 hours but not at 15 minutes of ALDO treatment. Similar results were observed when cells were transfected with siRNA for striatin, thus suggesting that reducing striatin levels does not alter ALDO's genomic effects as exemplified by the lack of an effect on WNK4 and SGK1 expression.

Pretreatment with ALDO enhances estrogen signaling in ECs

As we recently reported (7), incubating ECs with ALDO for 5 hours significantly increases striatin levels. Because striatin is involved in estrogen's rapid signaling, we determined whether estrogen regulates striatin levels. ALDO, but not estrogen, significantly increases striatin levels (Figure 4A). These results raise the possibility that ALDO could influence estrogen's rapid effects by increasing striatin levels. To test our hypothesis, EA.hy926 cells were preincubated with ALDO or placebo for 5 hours followed by the addition of estrogen (5×10^{-8} mol/L) for different time periods. Consistent with other reports (6, 25), Figure 4B shows that there is an elevation of the phosphorylated eNOS (peNOS)/eNOS ratio after 15 minutes of estrogen treatment. The increases in the peNOS/eNOS ratio occur more rapidly and to a greater level when the cells are preincubated with ALDO, suggestive of a functional interplay between the 2 steroid receptors, whereby ALDO can modulate estrogen function. To test whether estrogen can modulate the responses to ALDO, we studied whether estrogen pretreatment for 5 hours could influence ALDO's effect on the pERK/ERK ratio. Results show that estrogen preincubation does not significantly affect ALDO's rapid signaling (Figure 4C). Because ERα has been shown to interact with striatin, we transfected the cells with striatin siRNA and as-

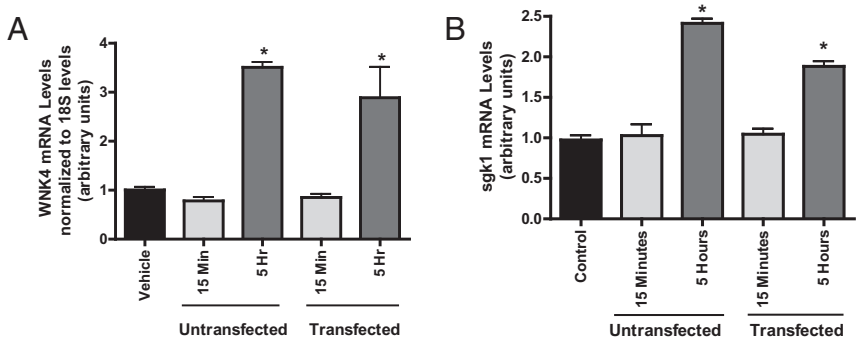

Figure 3. Striatin is nonessential in mediating the MR-dependent genomic responses to ALDO. EA.hy926 cells, transfected with striatin siRNA or controls (untransfected), were treated with ALDO (10−8 mol/L for 15 min and 5 h), and mRNA levels of WNK4 (A) and SGK1 (B) were determined. ALDO increased WNK4 (*, $P < .05$ vs control) and SGK1 mRNA levels (*, $P < .001$ vs control) and these genomic responses were not blocked by siRNA to striatin.

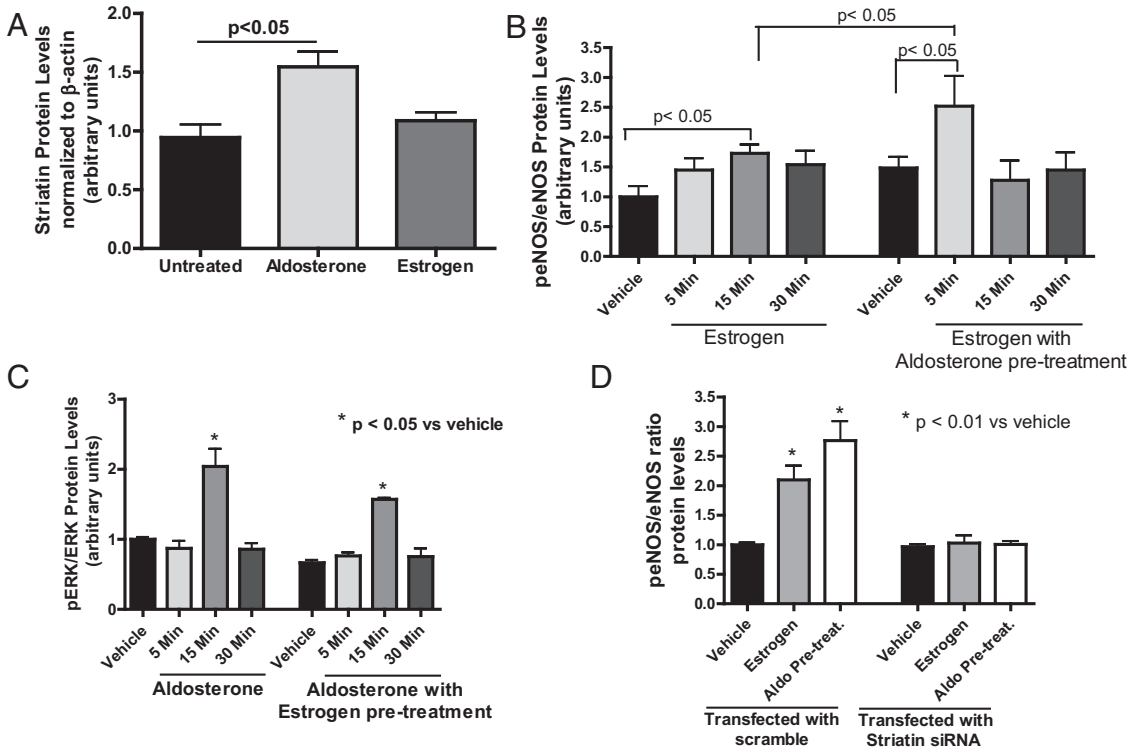

Figure 4. Pretreatment with ALDO increases estrogen's nongenomic response. A, EA.hy926 cells were treated with either 10^{-8} mol/L ALDO or 5×10^{-8} mol/L estrogen for 5 hours. ALDO but not estrogen treatment increases striatin protein levels. B, EA.hy926 cells were pretreated with or without ALDO (10^{-8} mol/L) for 5 hours. Then, peNOS/eNOS levels in response to estrogen (5×10^{-8} mol/L) were measured at 5, 15, and 30 minutes. Pretreatment with ALDO significantly increased the peak peNOS/eNOS response to estrogen and shifted the response to an earlier time point. C, EA.hy926 cells were transfected with scrambled (left) or striatin siRNA (right) and pretreated with (white bars) or without ALDO (10^{-8} mol/L) for 5 hours (black and gray bars). Then, peNOS/eNOS levels in response to estrogen (5×10^{-8} mol/L) were measured at 5 minutes (white bars) and 15 minutes (gray bars), relative to vehicle (black bars). (*, $P < .05$ vs vehicle) D, EA.hy926 cells were pretreated with or without estrogen (5×10^{-8} mol/L) for 5 hours. Then, pERK/ERK levels in response to ALDO (10^{-8} mol/L) were measured at 5, 15, and 30 minutes. Pretreatment with estrogen did not alter either the magnitude or time course of ALDO's nongenomic response (*, $P < .01$ vs vehicle).

sessed the responses to estrogen (Figure 4D). As observed with ALDO, reducing striatin levels abrogates estrogen's rapid effects on peNOS (Figure 4D). It is important to note that in these cells, ALDO did not significantly affect peNOS/eNOS ratios as described in Supplemental Figure 5; results that are consistent with reports in HUVEC cells (28).

Striatin is not essential in mediating ERα's genomic effects of estrogen

EA.hy926 cells were stimulated with estrogen for different time periods, and the mRNA levels of 2 known estrogen-responsive genes (29) were quantified using real-time RT-qPCR analyses (Figure 5). Our results show that, consistent with previous reports, the mRNA levels for both PTGIS and PTGS1 were elevated at 5 hours of estrogen treatment, and this response was not modified in cells transfected with siRNA for striatin, leading us to propose that striatin is involved in the nongenomic, but not the genomic, actions of estrogen.

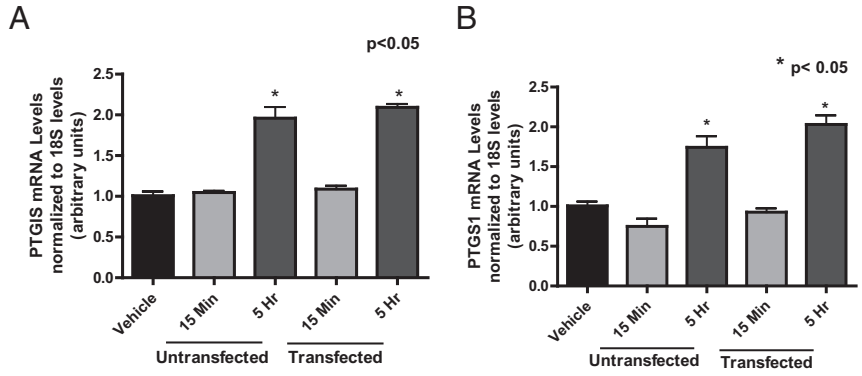

Figure 5. Striatin siRNA does not modify genomic responses to estrogen. EA.hy926 cells, transfected with striatin siRNA or controls (untransfected), were treated with estrogen (5×10^{-8} mol/L), and mRNA levels of PTGIS (A) and PTGS1 (B) were determined. Results show no significant difference in the genomic response after 5 hours of estrogen stimulation in the transfected and control cells (*, $P < .05$ vs vehicle).

Rapid responses to ALDO and estrogen in mouse aortic ECs

Early passages of mouse aortic ECs were stimulated with 10^{-8} mol/L ALDO (Figure 6A) or 5×10^{-8} mol/L estrogen (Figure 6B) and

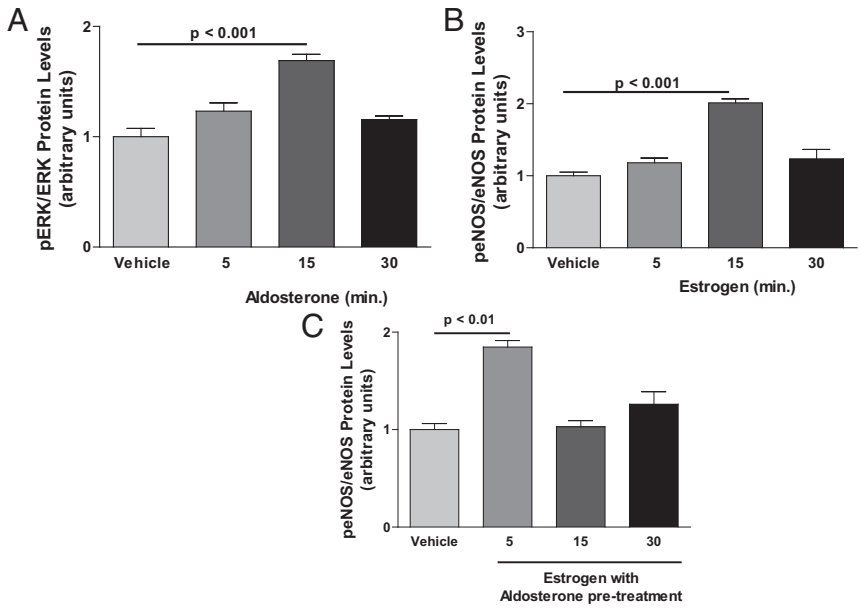

Figure 6. Effect of ALDO and estrogen incubation in early passages of mouse aortic ECs. A, ALDO (10^{-8} mol/L) leads to a rapid increase in pERK/ERK ratio. B, Estrogen (5×10^{-8} mol/L) leads to a rapid increase in peNOS/eNOS ratio. C, ECs were pretreated with or without ALDO (10^{-8} mol/L) for 5 hours. Then, peNOS/eNOS levels in response to estrogen (5×10^{-8} mol/L) were measured at various times. Pretreatment with ALDO resulted in a more rapid estrogen-mediated increase in peNOS/eNOS ratio.

the levels of pERK/ERK and peNOS/eNOS ratios measured. Our results show that, consistent with our data in EA.hy926 cells, there is a peak in the pERK/ERK ratio at 15 minutes when the cells are stimulated with ALDO. We show that the peNOS/eNOS ratio increases at 15 minutes when the cells are stimulated with estrogen. Similar to the human ECs, preincubating mouse aortic ECs with ALDO leads to a more rapid response to estrogen (Figure 6C).

Interaction of MR and CAV_1 with striatin

CAV_1 and MR have been shown to interact in vascular tissue and regulate ALDO function. Because striatin contains a CAV_1 binding domain, we used IP assays to clarify interactions between MR, striatin, and CAV_1 following protocols previously described by us (7) and detailed in Materials and Methods. The presence of CAV_1 protein was confirmed by Western blot analysis (Figure 7A). Subsequent IP studies show that both MR and CAV_1 interact with striatin (Figure 7, B–E) in EA.hy926 cells.

To determine whether CAV_1 was necessary for the interaction of MR with striatin, IP studies were performed in cardiac tissues from CAV_1 knockout (KO) mice. In contrast to the results in wild-type mice, there was no discernable interaction between MR and striatin in tissues from CAV_1 KO mice (Figure 7, F and G). These observations suggest the existence of a complex between MR, striatin, and CAV_1, where CAV_1 is an obligate component. Importantly, no striatin was observed when either a nonspecific antibody (IgG) or water was used for the IP (Figure 7H).

Caveolae are membrane invaginations that disappear in cells in culture and are composed mainly of CAV_1 (30–32). They are considered platforms for different signaling pathways and are linked to molecules such as eNOS and ERK (33–35). We confirmed the presence of caveolae in the cells used in these studies. Cells were fixed and subjected to EM analysis that documented the presence of caveolae in both EA.hy926 and aortic EC cells from wild-type mice (Supplemental Figure 2, A–C) but not in ECs from CAV_1 KO mice or HEPG2 cells (Supplemental Figure 2, A and D).

Discussion

Our data support the hypothesis assessed in this study. First, we show that striatin is critical in ALDO-mediated increases in pERK and ROS production, because reduction of striatin levels prevented the ALDO-stimulated rise in these rapid nongenomic responses. However, reducing striatin levels did not prevent 2 well-characterized genomic responses to ALDO. We confirmed that the effect of striatin on several previously characterized nongenomic and genomic responses to estrogen is as previously reported and similar to its role in mediating ALDO's nongenomic effect (6, 13). Importantly, we confirmed (Figure 4A) that ALDO but not estrogen increases striatin's mRNA and protein levels (7). Second, when the EC is pretreated with ALDO, the nongenomic response to estrogen (peNOS/eNOS ratio) was significantly increased and associated with ALDO-mediated increases in striatin. Importantly, the opposite interaction does not occur, when ECs were pretreated with estrogen, the nongenomic responses to ALDO were not significantly affected.

In 2000, the Pallas group demonstrated that striatin family members are physically associated with PP2A and may represent a new class of PP2A regulatory subunits (36). Other investigators have suggested that calmodulin and phocein also interact with members of the striatin family. We expand on these observations and show a relationship of striatin with pERK and ROS production. We observed that cells transfected with scrambled siRNA and untransfected cells had nearly identical pERK/ERK and

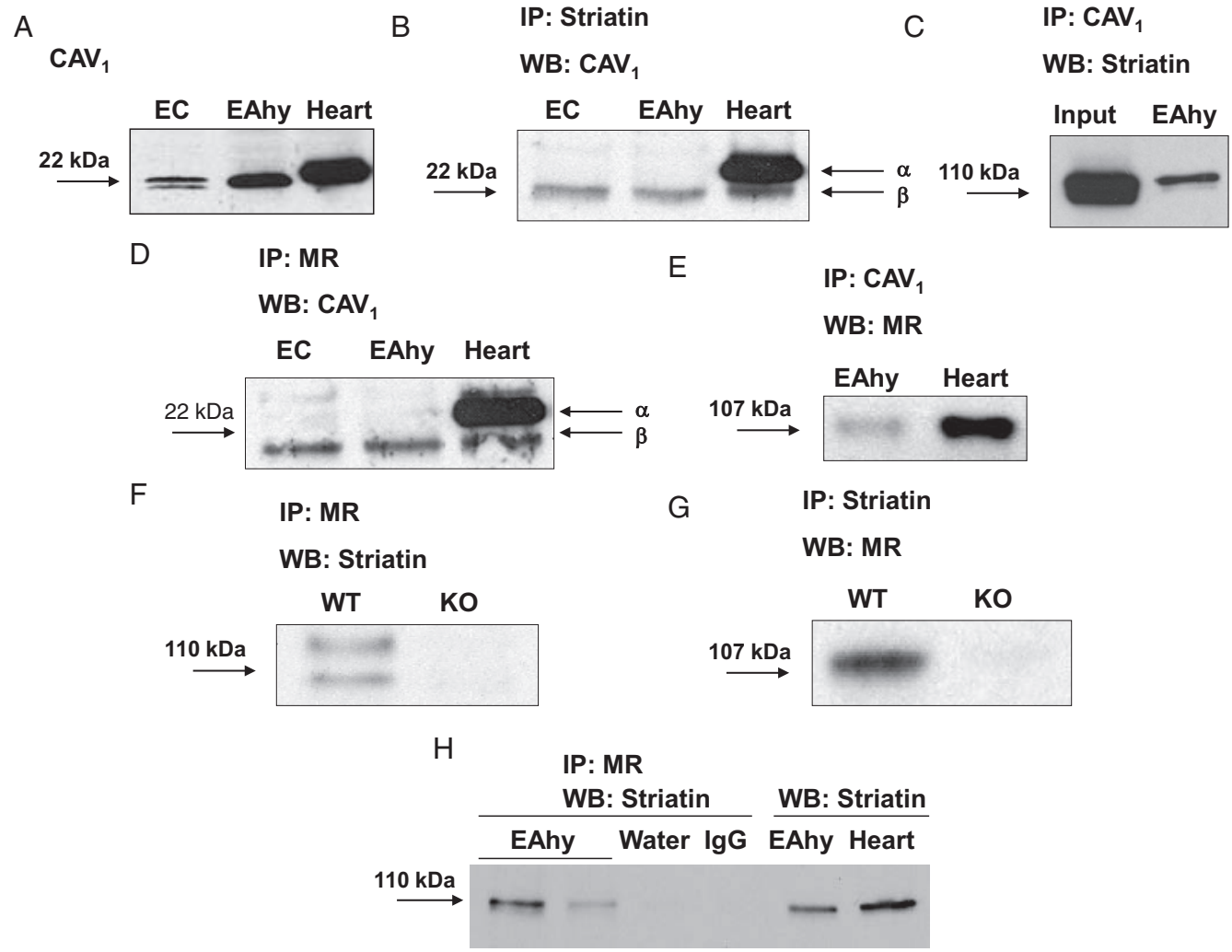

Figure 7. MR, CAV_1, and striatin coprecipitate. A, Western blot showing the presence of CAV_1 in ECs, EA.hy926 cells (EAhy) and mouse heart. B–G, IP studies. Striatin coprecipitates with CAV_1 (B); CAV_1 coprecipitates with striatin, input represents the starting material before IP (C); MR coprecipitates with CAV_1 (D); and CAV_1 coprecipitates with MR (E). F and G, In heart tissue from CAV_1 wild-type (WT) and KO animals, MR only interacts with striatin in WT animals. H, MR coprecipitation with striatin, water, or irrelevant IgG, vs unprecipitated striatin in EA.hy926 cells and mouse heart. WB, Western blot.

ROS levels, basally and in response to ALDO. Therefore, the transfection process itself did not modify the levels. Thus, striatin-siRNA treatment not only prevents phosphorylation of ERK and ROS production in response to ALDO but also inhibits endogenous ERK phosphorylation at baseline. Furthermore, our data suggest that not all agonist-induced phosphorylation of ERK or eNOS requires striatin, because only estrogen's and ALDO's but not EGF's effects were modified when striatin was knocked down.

Information concerning the cardiovascular roles of striatin family members is beginning to emerge regarding their roles in rapid estrogen signaling (25) and MR activation. We have recently shown increased striatin levels in heart and aortas from mouse and rat models of MR activation, eg, with sodium restriction (7, 21). In addition, we studied 2 previously described mouse models of increased ALDO levels: 1) ip ALDO administration and 2) a model of chronic ALDO-mediated cardiovascular damage after treatment with N(G)-nitro-L-arginine methyl ester plus angiotensin II. In both models, we observed increased abundance of striatin protein in heart tissue. These results further support the contention that striatin is a component of MR activation in the vasculature.

MR has been identified in cardiac and vascular tissues, where its interaction with ALDO affects cardiac and vascular function in animals (37) and ROS production (5, 38, 39). Recent data from our group demonstrate that CAV_1 deficiency (CAV_1 KO mice) is associated with reduced MR expression in the aorta and heart (40). These results suggest a potential interaction between MR and CAV_1. The evidence for this interaction is further supported by our findings and reports that CAV_1 may be involved in mediating the effects of steroid hormones on their receptors

(41). MR are found in the caveolae, which are reported to function in signaling events through the compartmentalization of signaling molecules and receptors such as ERα (42). Caveolin proteins function both to maintain the structure of the caveolae and are involved in the modulation of cell signaling (43). For example, binding to caveolin through the scaffolding domain is sufficient to repress the kinase activity of c-Src or to maintain the inactive conformation of G proteins (44). A relationship between the Ras-p42/44 MAPK pathway (mitogen-activated protein kinase kinase 1/2 and ERK1/2) and CAV_1 has also been established (45). In addition, CAV_1 and striatin directly interact (6, 46). Our data extends these observations to suggest that there is a 3-way interaction between MR-CAV_1-striatin and that upon MR activation by ALDO a change occurs within MR (47) that may affect CAV_1 and lead to increased levels of pERK and ROS production. Importantly, in CAV_1 KO animals, there was no evidence for an association between cardiac MR and striatin.

To activate the MR in our studies, we have used doses of ALDO up to 10 nmol/L to characterize the in vitro effects of ALDO in human and mouse ECs. The dose range used by us is similar to what has been recently reported by other investigators doing in vitro studies (48, 49). In addition, our group, over the past 30 years, has collected data on subjects (HyperPATH cohort) that have participated in numerous dietary sodium restriction studies. We observed that in 828 hypertensive subjects, the circulating ALDO levels ranged between 0.05 and 11.53 nmol/L (mean ± SD, 1.37 ± 0.94 nmol/L; median, 1.13 nmol/L). In addition, ALDO levels in patients with primary aldosteronism secondary to tumors of the adrenal gland can range as high as 3–3.5 nmol/L (50). Furthermore, ALDO levels over 100 nmol/L have been observed in heart failure patients (51, 52). To activate the ER in our studies, we used doses of estradiol up to 50 nmol/L to characterize its in vitro effects in ECs. These doses are similar to what has been recently reported by other investigators using estradiol in ECs (6, 25). In addition, there are reports showing maximal circulating mouse estradiol of about 1.4 nmol/L at peak estrus and rising to 6.6 nmol/L during pregnancy (53). In humans, circulating estradiol levels during pregnancy can reach as high as 26 nmol/L and about 1 nmol/L at peak estrus.

Our data supports the contention that striatin plays a central role in the interaction between estrogen and ERα (6). Both MR and ERα require striatin for their rapid effects on ERK or eNOS phosphorylation, respectively. However, ALDO's genomic effects on WNK4 and SGK1 were unaffected by the absence of striatin. We do not know whether loss of striatin can modify other rapid/nongenomic or genomic effects of ALDO. In addition, our study does not address the issue that other nongenomic or genomic effects of ALDO might actually be the mechanism for the up-regulation of estradiol effects on eNOS activity rather than striatin increases. Limitations on the interpretation of our results need to be considered. First, are the results that we observe actually due to striatin? Previous reports have suggested that striatin is not present in ECs and that the antibodies that have suggested its presence cross-react with other members of the striatin family, eg, SG2NA (54). However, our studies support its presence in both EA.hy926 and early cultures of mouse aortic ECs. Furthermore, we used striatin-specific siRNA oligonucleotides that were tested to ensure that they did not modify other members of the striatin family, eg, SG2NA and zinedin, and observed reduced striatin mRNA and protein levels. Second, the specificity of striatin's role in mediating the rapid/nongenomic vs the classical genomic effects of ALDO and estrogen is supported by the following data. 1) In the presence of striatin siRNA, only the rapid/nongenomic pathways were modified not the genomic at a time that striatin protein levels were reduced. 2) Lu et al (6), using a different technique to block estrogen interaction with striatin, reported a similar specificity: only the rapid/nongenomic not genomic pathway was inhibited. However, additional studies need to be performed to clarify the nature of the triple complex-striatin/CAV_1/MR and its relevance to ALDO/MR and estrogen/ERα signaling. The absence of an animal model lacking striatin hampers our ability to assess the physiological relevance of our findings.

We have assumed that the mechanism that we observed is mediated by the classical MR. We have not defined the ligand that potentially could be mediating this effect in vivo, because both ALDO and cortisol/corticosterone can interact with MR in the experimental paradigm used in this study. In addition, our data do not specifically exclude a role for the glucocorticoid receptor (GR) in this process. However, our results and data from the literature suggest that such an effect is unlikely. 1) ALDO increased the pERK/ERK ratios and ROS production in our system; ALDO has limited, if any, ability to interact with the GR. 2) Canrenoic acid, a specific inhibitor of MR, blocks the effect of ALDO. 3) Meltser et al (23) reported that in vivo in response to acoustic trauma, pERK is reduced in the cochlea, a reduction that is reversed when corticosterone synthesis is inhibited; these results imply that if anything, corticosterone reduces not increases pERK. 4) There is in vitro evidence that both cortisol and dexamethasone reduced ERK activation in oocytes (24) and cortisol in synovial fibroblasts; these results could be secondary to actions mediated by GR to reduce the phosphorylation of ERK in contrast to the increase in this action when MR is

activated. 5) We have not excluded the possibility that ALDO is interacting with a recently proposed nongenomic regulator of ALDO function, G protein-coupled estrogen receptor 1 (3).

In summary, in ECs, we describe a role for striatin as a critical factor of the nongenomic effects of steroids, documented here for ALDO and confirmed for estrogen. Furthermore, we provide evidence for a striatin-mediated, synergistic effect between MR and ERα: ALDO increases striatin levels and enhances the EC's response to estrogen. Thus, our results suggest a unique level of interactions between steroids on the cardiovascular system that may have broad application for understanding steroid function.

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