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Aldosterone Induces Cardiotrophin-1 Expression in HL-1 Adult Cardiomyocytes

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Aldosterone (ALDO) may induce cardiac hypertrophy by nonhemodynamic mechanisms that are not completely defined. Cardiotrophin-1 (CT-1) is a cytokine that exerts hypertrophic actions on isolated cardiomyocytes and promotes cardiac hypertrophy *in vivo*. We investigated whether ALDO induces CT-1 expression in HL-1 cardiomyocytes aiming at the possibility that the cytokine is involved in ALDO-induced cardiomyocyte hypertrophy. mRNA and protein expression were quantified by RT-PCR and Western blot. Cardiomyocyte area, as an index of hypertrophy, was assayed by image analysis in phalloidin-stained HL-1 cells. ALDO addition to adult HL-1 cardiomyocytes increased ($P < 0.01$) CT-1 mRNA and protein expression in a concentration-dependent manner. This effect was abrogated by actinomycin D, the mineralocorticoid and glucocorticoid receptor antagonists spironolactone and RU486, respectively, and the p38 MAPK blocker SB203580. CT-1 signaling pathway blockade with specific antibodies against the cytokine and its two receptor subunits avoided ($P < 0.01$) α-sarcomeric actin and c-*fos* protein overexpression as well as cell size increase induced by ALDO in HL-1 cells. *In vivo*, a single ALDO injection acutely increased ($P < 0.01$) the myocardial expression of CT-1 in C57BJ6 wild-type mice but not CT-1-null mice. The bolus of the mineralocorticoid increased ($P < 0.01$) ANP and c-*fos* mRNA expression in the myocardium of wild-type mice, whereas no changes were observed in CT-1-null mice. In summary, ALDO induces CT-1 expression in adult HL-1 cardiomyocytes via genomic and nongenomic mechanisms. CT-1 up-regulation could have relevance in the direct hypertrophic effects of ALDO in cardiomyocytes. (*Endocrinology* 149: 4970–4978, 2008)

ACCUMULATING CLINICAL and experimental evidences suggest that aldosterone (ALDO), independently from its hemodynamic effects, participates in the development of left ventricular hypertrophy (LVH) and myocardial remodeling present in cardiac diseases (1). In particular, LVH is specially overstated in subjects with primary hyperaldosteronism, compared with essential or renovascular hypertensive patients, and the presence of LVH is higher in patients with hyperaldosteronism than in essential or renovascular hypertensives, despite similar blood pressure values (2, 3). Plasma ALDO associates with LVH in patients with primary hyperaldosteronism (4, 5) and correlates with both left ventricular mass index and relative wall thickness in untreated essential hypertensives (6). In addition, an excess of plasma ALDO is associated with increased left ventricular wall thickness in normotensive subjects with familial hyperaldosteronism (7).

Studies in experimental models indicate that ALDO is locally implicated in the development of LVH and myocardial remodeling (8, 9). Transgenic mice overexpressing 11β-hydroxysteroid dehydrogenase type 2 in cardiomyocytes, which facilitates ALDO occupancy of mineralocorticoid receptor (MR), exhibit normal blood pressure values but spontaneously develop severe cardiac hypertrophy (10). Subcutaneous infusion of nonhypertensive doses of ALDO to normotensive rats leads to cardiac hypertrophy independently from blood pressure (11). Also, in normotensive rats, a high sodium intake for 8 wk produces LVH associated with increased cardiac ALDO production and ALDO synthase overactivity in the absence of blood pressure elevation (12). However, the molecular and cellular mechanisms underlying the direct hypertrophic effect of ALDO on the heart remain to be established.

Cardiotrophin-1 (CT-1), a cytokine belonging to the IL-6 family, is a 21.5-kDa protein capable of inducing hypertrophy in neonatal and adult cardiomyocytes via its membrane receptor, the heterodimer constituted by the leukemia inhibitory factor receptor (LIFR) and the glycoprotein 130 (gp130) (13–15). Intraperitoneal administration of recombinant CT-1 to normotensive mice increases left ventricular weight in a dose-dependent manner (16). Cardiac CT-1 expression is increased in several experimental models of hypertensive LVH (15, 17, 18). Furthermore, plasma levels of CT-1 are increased in hypertensive patients with LVH and decrease after antihypertensive therapy only in patients exhibiting LVH regression (19, 20). Of interest, it has been reported that some humoral factors classically involved in LVH development stimulate CT-1 expression (21–23).

We hypothesized that a mechanism for ALDO to induce cardiomyocyte hypertrophy may be the induction of CT-1 expression in these cells. To test this possibility, we first investigated whether ALDO was able to induce CT-1 expression and the intracellular pathways involved in this effect in HL-1 adult cardiomyocytes. Second, we analyzed the hypertrophic effects of ALDO in conditions of CT-1-pathway blockade in the same cell line. Finally, we approached the *in*

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Abbreviations: ALDO, Aldosterone; CT-1, cardiotrophin-1; gp130, glycoprotein 130; GR, glucocorticoid receptor; LIFR, leukemia inhibitory factor receptor; LVH, left ventricular hypertrophy; MR, mineralocorticoid receptor.

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vivo relevance of these data assaying the induction of cardiac CT-1 mRNA and hypertrophic genes in response to an acute ALDO overloading in C57BJ6 wild-type and CT-1-null mice.

Materials and Methods

Cell culture

HL-1, a cell line derived from adult mouse heart (24), were a gift from Dr. W. C. Claycomb (Louisiana State University, New Orleans, LA). Cardiomyocytes plated in flasks coated with fibronectin (Life Technologies, Inc., Gaithersburg, MD)-gelatin (Sigma, St. Louis, MO) were maintained in Complete Claycomb Medium (JRH Biosciences, Lenexa, KS) supplemented with 100 μmol/liter norepinephrine stock [consisting of 10 mmol/liter norepinephrine (Sigma) dissolved in 30 mmol/liter L-ascorbic acid (Sigma), 2 mmol/liter L-glutamine (Invitrogen, Paisley, UK) and 10% fetal bovine serum (JRH Biosciences)]. During culture, the medium was changed routinely every 48 h. For experiments, cells were serum starved 24 h before stimulation with ALDO (Fluka, Sigma) at 10^{-9} to 10^{-6} mol/liter for 3 and 24 h for mRNA and protein determination, respectively, except for time-response experiments. To investigate the intracellular pathways, the following inhibitors were added 30 min before ALDO addition: MR antagonist spironolactone (10^{-6} mol/liter; Sigma), glucocorticoid receptor (GR) antagonist RU486 (10^{-6} mol/liter; Sigma), RNA synthesis inhibitor actinomycin D (10^{-6} mol/liter; Calbiochem, EMD-Merck, Madison, WI), protein synthesis inhibitor cycloheximide (10^{-6} mol/liter; Calbiochem), p38 MAPK inhibitor SB203580 (10^{-5} mol/liter; Calbiochem), p42/44 MAPK inhibitor PD98059 (10^{-6} mol/liter; Calbiochem), calcium antagonist lercanidipine (10^{-6} mol/liter; a gift from Recordatti, Milan, Italy), and antibodies against LIFR and gp130 (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of cell size

HL-1 cardiomyocytes grown on glass coverslips were fixed in 4% formaldehyde, and stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Invitrogen) (1:50 dilution) for 30 min at 37 C to visualize F-actin. Cell surface area from at least 50 cardiomyocytes per condition was measured in three independent experiments using the automated image analysis system (Soft Imaging Analysis, Münster, Germany).

Animals

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 82-23, revised in 1996). CT-1-null mice were a gift from Dr. M. Selzner (Zurich University Hospital, Zurich, Switzerland). Although cardiac characterization of this strain has not been reported, in our experience, at the age of 5 months, these mice do not spontaneously develop cardiac hypertrophy because they exhibit similar left ventricular morphometry (*i.e.* wall thickness and chamber diameter), similar cardiomyocyte diameter, and similar expression of ANP and c-*fos* genes than their wild-type background (our unpublished data). Male C57BJ6 wild-type mice (5 months old) and age-matched CT-1-null mice received one ip injection of ALDO 1 mg/kg or vehicle (150 mmol/liter NaCl, 5% ethanol) (n = 8, each group). Two hours after the injection, blood samples were obtained from the half of the animals (n = 4 each group) to measure plasma ALDO concentration by RIA (Diasorin, Saluggia, Vercelli, Italy). To avoid hemodynamic effects of the mineralocorticoid, animals were killed 6 h after the injection by cervical dislocation under 3% isoflorane anesthesia. Hearts were removed, weighed, and immediately frozen in liquid nitrogen for molecular studies.

Western blotting

Aliquots of 20 μg of proteins were size fractionated on 15% (for CT-1) or 10% (for c-*fos* and α-sarcomeric actin) polyacrylamide gels by electrophoresis and transferred to polyvinyl difluoride membranes. The following specific antibodies were used diluted at 1:500: CT-1 (Santa Cruz Biotechnology), p38, p38-P (Thr180/Tyr182; Cell Signaling Technology, Danvers, MA), MR, and GR (Abcam, Cambridge, MA). Western blots were also stained for β-actin (Sigma) diluted at 1:1000 to correct for protein loading. In all cases, bound antibody was detected by peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) and visualized using the ECL-Plus chemiluminescence detection system (Amersham Biosciences). After densitometry, OD values were expressed as arbitrary units. All Western blots were performed at least in triplicate for each animal or *in vitro* experimental condition.

Reverse transcription and real-time PCR

Total RNA was extracted from cells and hearts using Trizol (Invitrogen) and subsequently purified using RNeasy total RNA isolation kit (QIAGEN, Milden, Germany). Reverse transcription was performed with 500 ng of total RNA. Real-time PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems Inc., Foster City, CA) by using specific TaqMan MGB fluorescent probes (Applied Biosystems) for mice CT-1, ANP, and c-*fos*. All samples were assayed in triplicate and normalized on the basis of their constitutive 18S ribosomal RNA. To detect the expression of GR and MR in the HL-1 cell line, mRNA was retrotranscripted, and the following specific primers recently reported by Sierra *et al.* (25) were used: 5′-TGC TAT GCT TTG CTC CTG ATC TG-3′ and 5′-TGT CAG TTG ATA AAA CCG CTG CC-3′ for GR and 5′-GTG GAC AGT CCT TTC ACT ACC G-3′ and 5′-TGA CAC CCA GAA GCC TCA TCT C-3′ for MR.

Statistical analysis

Results are presented as mean ± SE and computed from the average measurements obtained from each experimental condition or from each group of animals. Normal distribution of data were checked by means of the Shapiro Wilks test. A Levene statistic test was performed to check the homogeneity of variances. The unpaired Student's *t* test or the Mann Whitney *U* tests were used to assess statistical differences between the two experimental groups. Differences among more than two experimental conditions were tested by the ANOVA one-way test followed by the Scheffé test to analyze differences between groups. $P < 0.05$ was considered significant.

Results

ALDO induces CT-1 up-regulation in HL-1 cardiomyocytes

Incubation of HL-1 with ALDO (10^{-9} to 10^{-6} mol/liter) for 3 or 24 h increased ($P < 0.01$) CT-1 mRNA and protein, respectively, in a concentration-dependent manner (Fig. 1, A and B). Time-course examination showed that early induction of CT-1 mRNA levels by ALDO peaked at 2.3-fold ($P < 0.01$) after 3 h. For longer periods, the increase in CT-1 mRNA was stable at around an 80%. The increase in CT-1 protein expression accounted progressively and later than mRNA increase (Fig. 1C). To evaluate whether CT-1 increase was a direct effect of ALDO or whether it involved the synthesis of intermediary proteins, we analyzed the effects of transcription and transduction inhibitors on ALDO-induced CT-1 up-regulation. The transcription inhibitor actinomycin D completely inhibited ($P < 0.01$) the increase of CT-1 mRNA and protein induced by ALDO, whereas the protein synthesis inhibitor cycloheximide did not affect CT-1 mRNA and completely blunted ($P < 0.01$) the protein induction (Fig. 2, A and B). Before investigating the involvement of MR and GR in this effect, we performed specific experiments to confirm that this cell type expressed the two steroid receptors. As shown in Fig. 2C, conventional PCR allowed the detection of MR and GR mRNA in HL-1 cardiomyocytes. Because ALDO binds MR and GR with different affinity, low hormone concentrations will bind MR and high concentrations will also bind GR. Moreover, receptor antagonists are usually used at

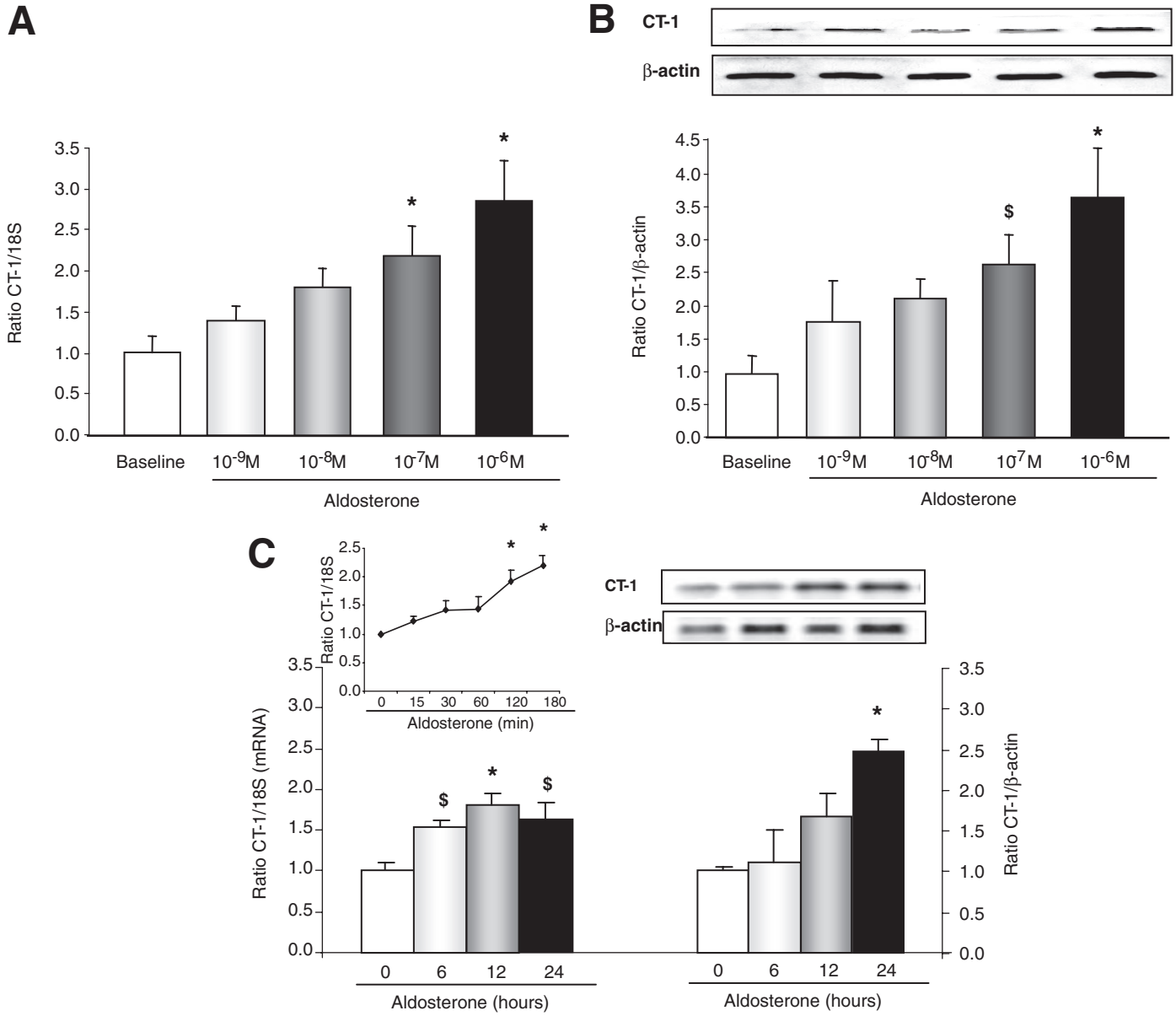

FIG. 1. Aldosterone induces CT-1 expression in a concentration- and time-dependent manner. HL-1 cardiomyocytes were incubated for 3 or 24 h with ALDO at the indicated concentrations to assay mRNA (A) or protein (B) expression, respectively. Western blot is representative of three independent experiments. C, Time-response curve obtained incubating HL-1 cardiomyocytes with ALDO (10^{-6} M) for 15–180 min (*upper left graph*), histogram bars showing CT-1 mRNA (*left panel*) and protein (*right panel*) expression at the indicated time points and a representative Western blot of CT-1 protein expression at the same time points (*upper-right panel*). All histogram bars represent the mean ± SE of the three independent experiments. *, $P < 0.01$ *vs.* baseline; $, $P < 0.05$ *vs.* baseline.

10- to 100-fold excess to ensure an effective blockade. Thus, to investigate the role of MR and GR, cells were preincubated for 30 min with spironolactone and/or RU486 at 10^{-6} mol/liter, and ALDO was added at 10^{-8} or 10^{-7} mol/liter. Prior addition of the specific MR and/or the GR blocker to HL-1 culture abolished ($P < 0.01$) ALDO-induced CT-1 up-regulation (Fig. 2, C and D), indicating that both receptors may mediate CT-1 induction. In additional experiments, we confirmed that the GR agonist dexamethasone used at 10^{-6} mol/liter was able to induce 2-fold the synthesis of CT-1 protein (data not shown). None of the chemical inhibitors tested in these experiments affected CT-1 expression when incubated alone (data not shown).

Once established that CT-1 up-regulation was a genomic effect of ALDO, we investigated the potential participation of other intracellular cascades that ALDO activates in cardiac cells, by the use of specific chemical inhibitors. As illustrated in Fig. 3, the p38 MAPK inhibitor SB203580 completely abolished ($P < 0.01$) the induction of CT-1 expression, whereas the p42/44 MAPK inhibitor PD98059 and the calcium L-type channel antagonist lercanidipine did not modify CT-1 expression induced by ALDO (Fig. 3, A and B). None of the

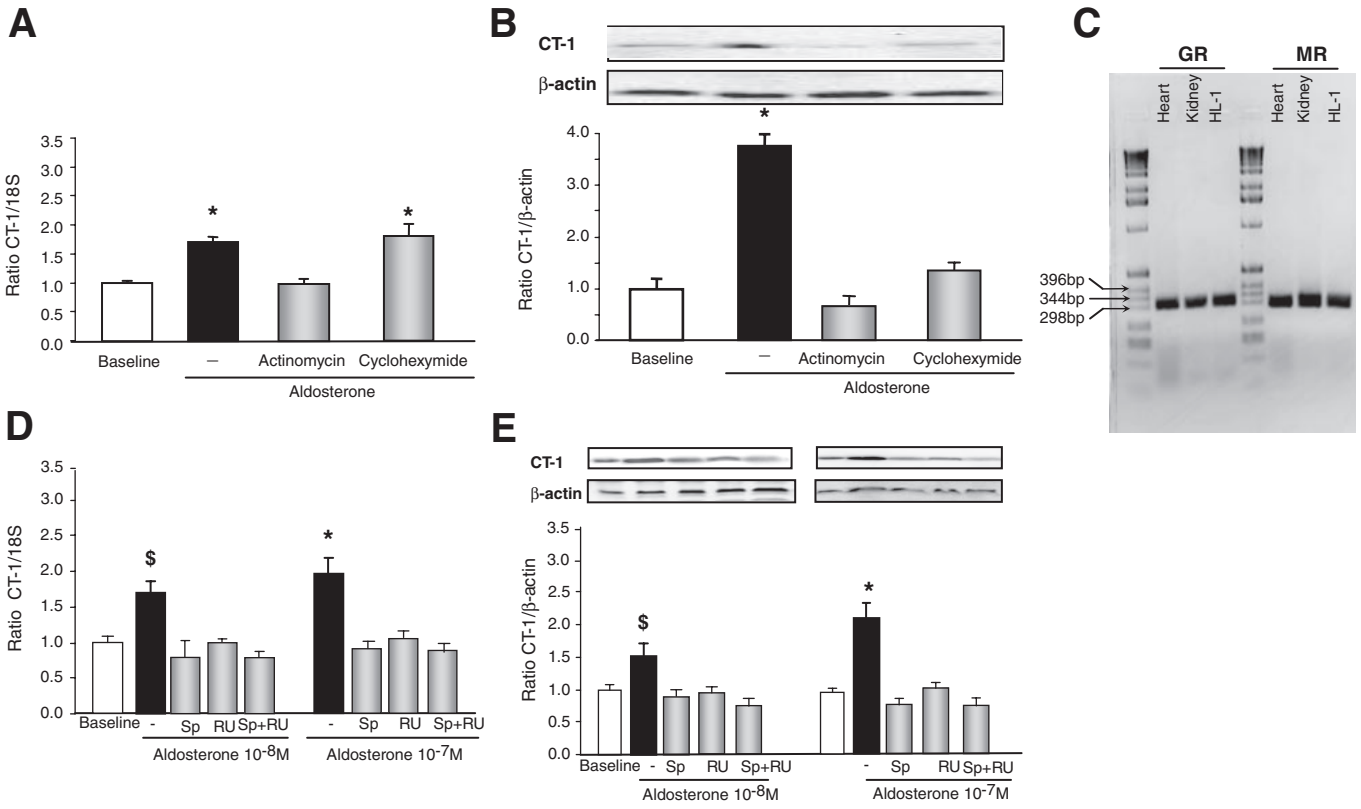

FIG. 2. Aldosterone-induced CT-1 expression is mediated by MR and GR and requires mRNA and protein synthesis. A and B, HL-1 cardiomyocytes were incubated with ALDO (10^{-6} M) for 3 or 24 h to assay mRNA and protein expression, respectively, in the absence or presence of actinomycin D (10^{-6} M) or cycloheximide (10^{-6} M). C, Single band obtained after GR and MR cDNA amplification by conventional nonquantitative PCR in mice heart and kidney and HL-1 cardiomyocytes. The two amplicon sizes expected were 299 bp for GR and 286 bp for MR. D, For the assessment of MRs and GRs involvement, cells were incubated with ALDO at 10^{-7} or 10^{-8} M with or without spironolactone, RU-486, or both at 10^{-6} M. Western blots in B and D are representative of triplicates. All histogram bars represent the mean ± SE of three independent experiments. *, $P < 0.01$ *vs.* baseline and ALDO with inhibitors; $, $P < 0.05$ *vs.* baseline and ALDO with inhibitors.

chemicals tested affected CT-1 expression when incubated alone (data not shown). Because p38 MAPK is one of the signaling pathways used by ALDO to exert rapid nongenomic effects (26, 27), we next studied whether p38 MAPK activation occurred independently or downstream from ALDO-MR binding. A time-response analysis of ALDO-induced p38 MAPK activation demonstrated a significant 2- to 3.5-fold activation ($P < 0.01$) from 30 min to 3 h of incubation. Additionally, whereas p38 phosphorylation induced by ALDO was not affected by spironolactone at the first 30 min, the MR blocker inhibited this effect up to 3 h (Fig. 3C). These findings indicate that ALDO-induced p38 MAPK activation in HL-1 involves early MR-independent mechanisms followed by MR-dependent pathways.

CT-1 is involved in HL-1 hypertrophy induced by ALDO

We next investigated the hypertrophic effect of ALDO on HL-1 cardiomyocytes and the potential involvement of CT-1 in this effect. As shown in Fig. 4, ALDO addition to HL-1 induced a significant increase ($P < 0.01$) in α-sarcomeric actin and c-*fos* protein expression in 24 h and promoted a significant ($P < 0.01$) cell area enlargement in 48 h. Prior addition of specific antibodies against each of the two CT-1 receptor subunits, gp130 and LIFR, or against CT-1 to the culture medium abolished ($P < 0.01$) the increase in cell area and the overexpression of hypertrophic proteins induced by the mineralocorticoid. None of the three antibodies exerted any effect when incubated alone (data not shown).

ALDO acutely induces the expression of cardiac hypertrophic genes in wild-type but not in CT-1-null mice

To investigate whether our observations in HL-1 cardiomyocytes also accounted *in vivo*, we performed an experiment of ALDO acute overloading in mice to analyze cardiac mRNA expression of CT-1 and hypertrophic genes. In wild-type mice C57BJ6, a single ip injection of ALDO (1 mg/kg) resulted in a significant increase ($P < 0.01$) of myocardial CT-1 mRNA expression, compared with mice injected with vehicle. As expected, this response was not observed in CT-1-null mice (Fig. 5A). Interestingly, the single bolus of the mineralocorticoid resulted in a significant ($P < 0.01$) cardiac overexpression of ANP and c-*fos* genes in wild-type mice, compared with those that received vehicle, whereas no changes in the expression of these genes were observed in the myocardium of CT-1-null mice (Fig. 5, B and C). The degree of elevation in serum ALDO concentration after the hormone injection was similar in the two strains of mice (× 940 in wild-type and × 1145 in CT-null mice).

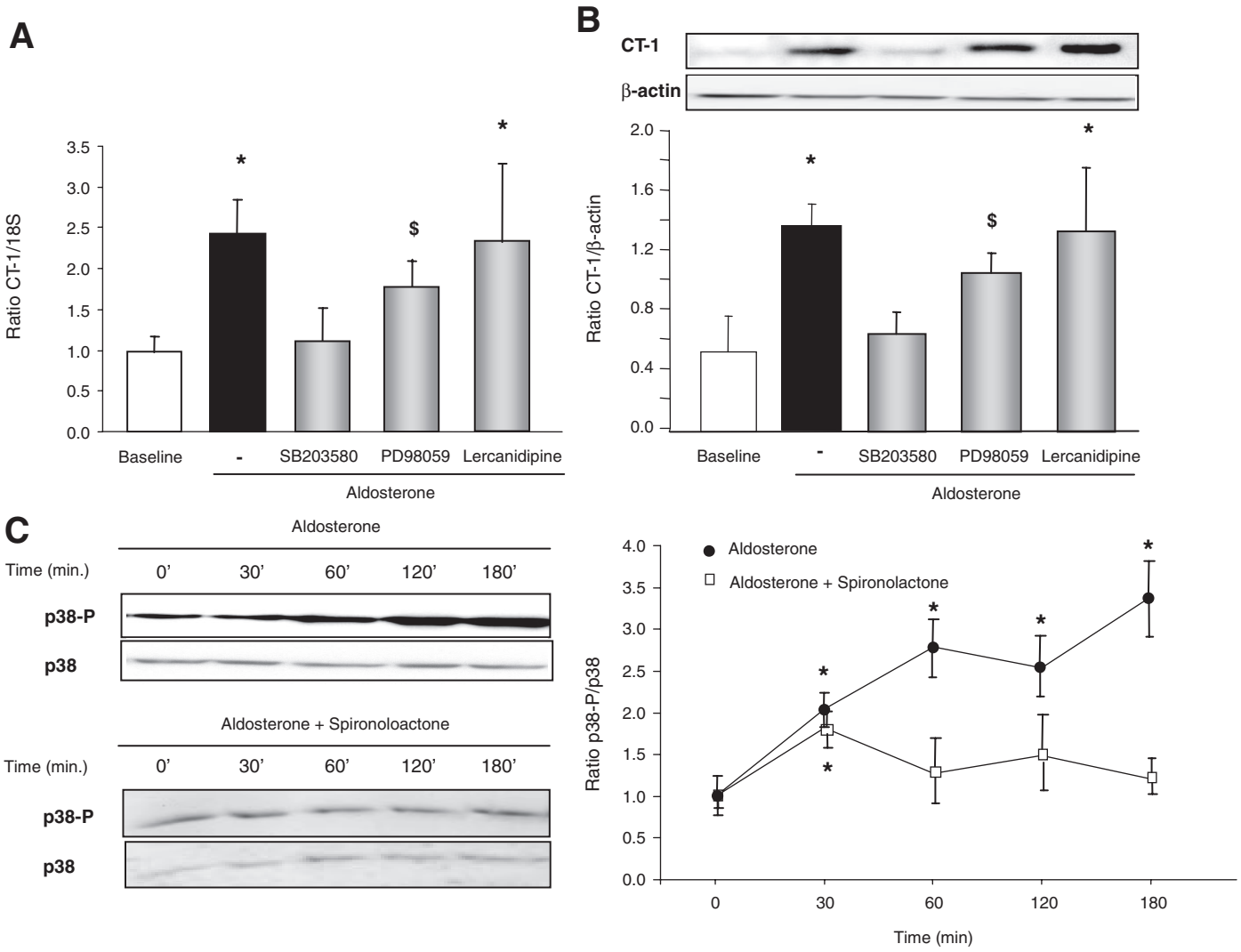

FIG. 3. Aldosterone-induced CT-1 expression is mediated by p38 MAPK. HL-1 cardiomyocytes were incubated with ALDO (10^{-6} M) for 3 or 24 h to assay CT-1 mRNA (A) and protein (B) expression, respectively, in the absence or presence of the p38 MAPK inhibitor SB203580 (10^{-5} M), the p42/44 MAPK inhibitor PD98059 (10^{-6} M), or the L-type calcium channel inhibitor lercadinipine (10^{-6} M). All histogram bars represent the mean ± SE of three independent experiments. C, HL-1 cardiomyocytes were incubated with ALDO (10^{-6} M) or ALDO and spironolactone (10^{-6} M) for the indicated times and p38 phosphorylation was investigated. Western blots are representative of triplicates. The diagram in D shows the ratio phosphorylated to nonphosphorylated p38 recorded at the indicated time points. All experiments were performed in triplicate. *, $P <$ 0.01 *vs.* baseline.

Discussion

The major findings presented in this study are: 1) ALDO induces CT-1 expression in HL-1 adult cardiomyocytes via MR and GR and through the activation of p38-MAPK signaling pathway, 2) CT-1 pathway blockade with specific antibodies avoids ALDO-induced hypertrophy in this cell line, 3) exogenously administrated ALDO induces myocardial CT-1 expression and a genetic pattern of hypertrophy in C57BJ6 wild-type mice, and 4) this genetic pattern is absent in CT-1-null mice subjected to the same maneuver.

Although a number of findings suggest a role for ALDO in LVH, it is difficult to distinguish between the direct actions of the hormone on the myocardium and those secondary to blood pressure elevation induced by the mineralocorticoid. Only two studies have reported direct hypertrophic effects of ALDO on cardiomyocytes (28, 29). The two molecular mechanisms proposed to explain ALDO-induced cardiomyocyte hypertrophy agree in the involvement of MR and differ in the downstream intracellular intermediates that are involved. Whereas Na^+/H^+ exchanger type 1 activation seems to be crucial to cell-size increase promoted by ALDO in neonatal ventricular cardiomyocytes (29), activation of the intracellular intermediates protein kinase C-α, p42/44, and c-Jun N-terminal kinase MAPKs precedes the increase of the sarcomeric proteins α- and β-myosin in the same model (28). Present data showing that ALDO induces CT-1 expression in HL-1 cardiomyocytes, together with the observation that CT-1 pathway blockade avoids both α-sarcomeric actin and c-*fos* protein overexpression, and the cell area enlargement induced by the hormone, suggest a new molecular mechanism to explain ALDO-induced hypertrophy in adult cardiomyocytes. The *in vivo* relevance for this mechanism is sup-

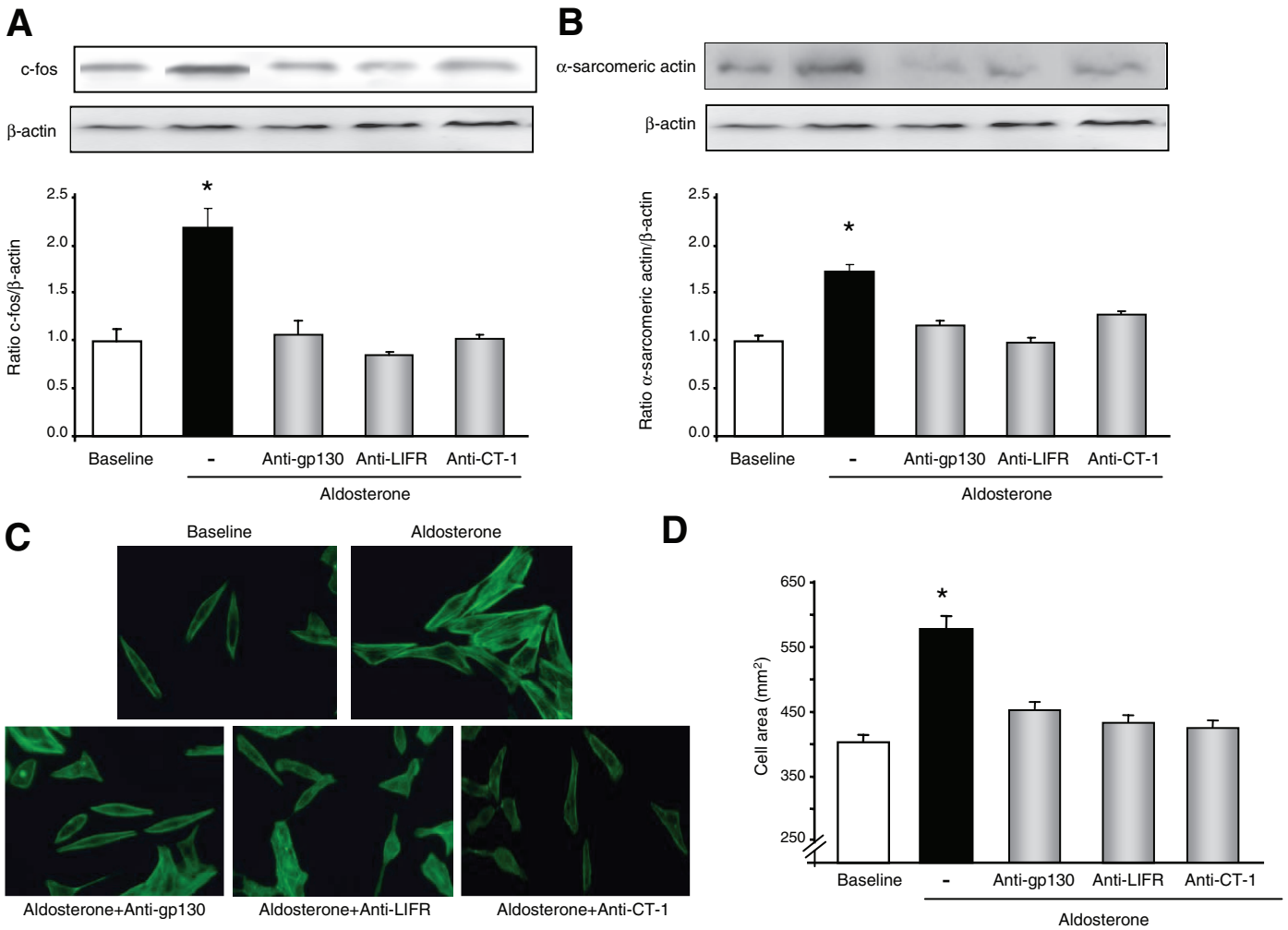

FIG. 4. CT-1 pathway blockade inhibits aldosterone-induced hypertrophy. HL-1 cardiomyocytes were incubated with ALDO (10^{-6} M) in the absence or presence of antibodies against gp130 (1 μg/ml), LIFR (1 μg/ml), or CT-1 (1 μg/ml) for 24 h to measure the expression of c-*fos* protein and α-sarcomeric actin (A and B) and for 48 h to assay cell area (C and D). *Bars* represent mean ± SE of three independent experiments. For cell area determination, at least 50 cardiomyocytes per experimental condition were measured. C, magnification was ×40. *, $P < 0.01$ *vs.* baseline and treatment with antibodies.

ported by the finding that CT-1-null mice are unable to acutely increase ANP and c-*fos* myocardial expression in response to acute ALDO overloading as observed in the wild-type mice. Of importance, our observations in mice do not allow to exclude the possibility that both myocardial cell types, cardiomyocytes and cardiac fibroblasts, participate in cardiac CT-1 up-regulation observed *in vivo*. In this regard, preliminary data from our laboratory revealed that ALDO induces mRNA and protein expression of CT-1 in adult cardiomyocytes and fibroblasts freshly isolated from adult Wistar rats (our unpublished data). Ongoing studies will clarify the real role of this autocrine/paracrine mechanism in ALDO-associated cardiac hypertrophy that accounts in pathological conditions.

Classic genomic effects of ALDO are characterized by a latency of onset and their sensitivity to mRNA and protein synthesis inhibitors. Our results showing the time course of CT-1 mRNA increase and the inhibition exerted by actinomycin D on mRNA and protein increase clearly demonstrate that the induction of CT-1 expression is a new genomic effect of the mineralocorticoid in cardiomyocytes. Moreover, the observation that the transduction blocker cycloheximide does not affect the CT-1 mRNA increase induced by ALDO indicates that this is a direct primary effect that does not require the synthesis of intermediate proteins.

Regarding the receptor involved in this effect, our results indicate that both MR and GR are able to mediate the CT-1 up-regulation induced by ALDO. Previous *in vitro* studies have reported ALDO primary genomic effects partially or completely mediated by GR in epithelial (30) and nonepithelial cells (31, 32). ALDO, as well as glucocorticoids, is able to bind GR and MR with the affinity being 100-fold higher for MR than GR (33). It is well established that the two types of steroid receptors share structural and functional homology, and both are transcription factors in cardiomyocytes (34–36). In this study, homology searchers of the CT-1 promoter region for transcription factors binding elements by means of the Transfact Produktion-Planung-Steurung (PPS) software (Transfact Industrial Software & Engineering, Heikendorf, Germany) led us to identify at least two putative

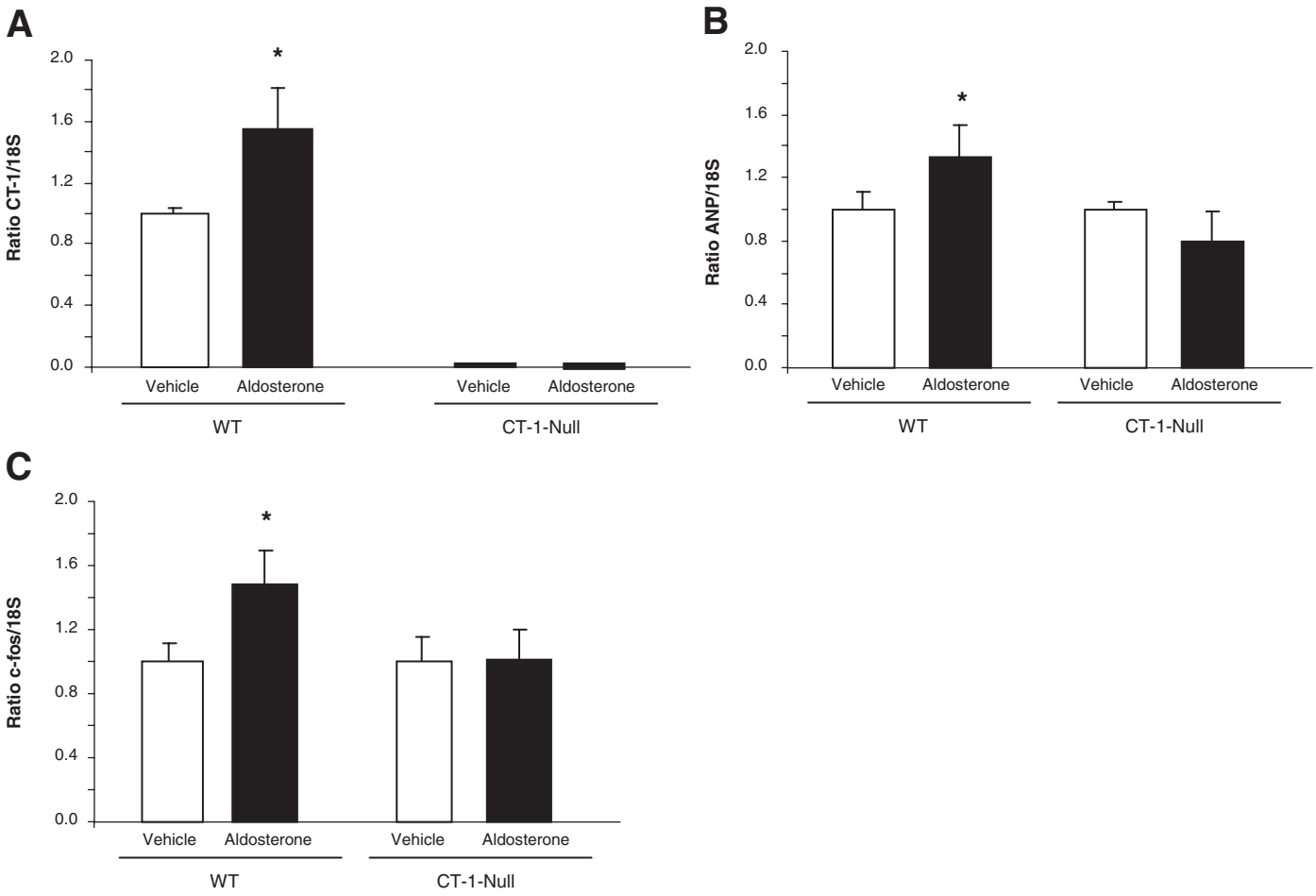

FIG. 5. Aldosterone induces myocardial expression of CT-1, ANP, and c-*fos* in wild-type but not CT-1-null mice. Myocardial expression of CT-1 (A) c-*fos* and ANP mRNA in wild-type (n = 8) and CT-1-null mice (n = 8) 6 h after one ip injection of ALDO (10^{-6} M) or vehicle. Histogram bars represent the mean ± SE of each group of animals. *, $P < 0.01$ *vs.* vehicle.

steroid response elements located at around 0.9 and 1.3 kb upstream from the transcription start site. Thus, it is reasonable to hypothesize that, at least *in vitro*, either MR- or GR-ligand complexes might bind these target sites to induce CT-1 expression. Concerning the *in vivo* context, recent studies in the field of MR *vs.* GR specificity in cardiovascular corticosteroids actions indicate that the importance of MR signaling is controlled at two levels. First, it is controlled by prereceptor mechanisms, *i.e.* mineralocorticoids/glucocorticoids proportion, which is mainly determined by the expression 11β-hydroxysteroid dehydrogenase 2 and the endogenous synthesis of ALDO in cardiovascular tissues (37). Second, it has been recently proposed that the intracellular context, *i.e.* redox state of the cell, may resolve activation of MR-dependent signals by glucocorticoids (38, 39). Hence, further studies are required to determine the precise corticosteroid signaling pathway that may intervene in this response in pathophysiological conditions.

Increasing evidence indicates that, besides the classical MR-dependent genomic actions, ALDO exerts a set of rapid nongenomic effects via MR-dependent and/or MR-independent mechanisms (40, 41). Furthermore, despite details of the mineralocorticoid signaling are missing, available data indicate that a cross talk between genomic and nongenomic activities may be the key for understanding the real significance of mineralocorticoids in the cardiovascular system (41, 42). Hence, rapid activation of protein kinase-C induced by ALDO is necessary for a subsequent genomic effect of the hormone, *i.e.* induction of Na^+/K^+-ATPase expression, in renal cells (43). Similarly, activation of c-Src tyrosine kinase and endothelial growth factor receptor transactivation are two rapid MR-mediated effects that support the following ALDO-induced endothelial growth factor receptor overexpression in several cell lines (44). Callera *et al.* (26) reported that ALDO-induced activation of c-Src and succeeding p38 MAPK phosphorylation are two nongenomic events mediating the subsequent fibrogenic effect of the mineralocorticoid in freshly isolated aortic vascular smooth muscle cells. Here we tested the involvement of nongenomic mechanisms in the increase of CT-1 mRNA by analyzing the three rapid signaling pathways that ALDO activates in cardiac cells: p38 and p42/44 MAPK, and L-type calcium channels (28, 29, 45, 46). Among the chemical blockers tested, only the p38 MAPK inhibitor abrogated ALDO-induced CT-1 expression (Fig. 3), suggesting a determinant role for this MAPK in this genomic effect of ALDO. This finding is in accordance with results previously reported demonstrating the involvement of p38 MAPK on ALDO-induced connective tissue growth factor

up-regulation in cardiac cells (46). Furthermore, several data presented here reinforce the possibility that ALDO-induction of CT-1 is mediated by the cross talk of early nongenomic mechanisms that support following genomic pathways. On the one hand, ALDO increases p38 MAPK phosphorylation via MR-independent and –dependent pathways without modifying the expression of nonphosphorylated p38 MAPK, On the other hand, the time-course analysis of p38 phosphorylation and CT-1 mRNA up-regulation suggests that the first precedes the increase of cytokine expression induced by ALDO. Thus, regarding the stage of the knowledge in mineralocorticoid signaling, this study adds to a novel example of cross talk between nongenomic and genomic signals that may clarify the mechanisms by which ALDO induces hypertrophy in cardiac cells.

In summary, findings here presented suggest that cardiac CT-1 may be up-regulated by ALDO through mechanisms that involve MR and GR and the cross talk between rapid nongenomic and genomic mechanisms. Furthermore, ALDO-induced CT-1 up-regulation seems to play a role in the ability of the mineralocorticoid to produce cardiomyocyte growth, and, as a result, cardiac hypertrophy. The potential clinical relevance of these findings is based on the association between increased CT-1 and LVH observed in essential hypertensives (19, 20) as well as the association of increased CT-1 and left ventricular dysfunction reported in patients with heart failure (47–49), thus setting the stage for studies aimed to test the effects of MR antagonists on CT-1.

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References

1. **Marney AM, Brown NJ** 2007 Aldosterone and end-organ damage. Clin Sci (Lond) 113:267–278
2. **Matsumura K, Fujii K, Oniki H, Oka M, Iida M** 2006 Role of aldosterone in left ventricular hypertrophy in hypertension. Am J Hypertens 19:13–18
3. **Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ** 2005 Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. J Am Coll Cardiol 45:1243–1248
4. **Rossi GP, Sacchetto A, Visentin P, Canali C, Graniero GR, Palatini P, Pessina AC** 1996 Changes in left ventricular anatomy and function in hypertension and primary aldosteronism. Hypertension 27:1039–1045
5. **Rossi GP, Di Bello V, Ganzaroli C, Sacchetto A, Cesari M, Bertini A, Giorgi D, Scognamiglio R, Mariani M, Pessina AC** 2002 Excess aldosterone is associated with alterations of myocardial texture in primary aldosteronism. Hypertension 40:23–27
6. **Nakahara T, Takata Y, Hirayama Y, Asano K, Adachi H, Shiokawa G, Sumi T, Ogawa T, Yamashina A** 2007 Left ventricular hypertrophy and geometry in untreated essential hypertension is associated with blood levels of aldosterone and procollagen type III amino-terminal peptide. Circ J 71:716–721
7. **Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D, Marwick TH** 2005 Evidence for abnormal left ventricular structure and function in normotensive individuals with familial hyperaldosteronism type I. J Clin Endocrinol Metab 90:5070–5076
8. **Beggah AT, Escoubet B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, Bocchi B, Peuchmaur M, Delcayre C, Farman N, Jaisser F** 2002 Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. Proc Natl Acad Sci USA 99:7160–7165
9. **Tsukamoto O, Minamino T, Sanada S, Okada K, Hirata A, Fujita M, Shintani Y, Yulin L, Asano Y, Takashima S, Yamasaki S, Tomoike H, Hori M, Kitakaze M** 2006 The antagonism of aldosterone receptor prevents the development of hypertensive heart failure induced by chronic inhibition of nitric oxide synthesis in rats. Cardiovasc Drugs Ther 20:93–102
10. **Qin W, Rudolph AE, Bond BR, Rocha R, Blomme EA, Goellner JJ, Funder JW, McMahon EG** 2003 Transgenic model of aldosterone-driven cardiac hypertrophy and heart failure. Circ Res 93:69–76
11. **Yoshida K, Kim-Mitsuyama S, Wake R, Izumiya Y, Izumi Y, Yukimura T, Ueda M, Yoshiyama M, Iwao H** 2005 Excess aldosterone under normal salt diet induces cardiac hypertrophy and infiltration via oxidative stress. Hypertens Res 28:447–455
12. **Takeda Y, Yoneda T, Demura M, Miyamori I, Mabuchi H** 2000 Sodium-induced cardiac aldosterone synthesis causes cardiac hypertrophy. Endocrinology 141:1901–1904
13. **Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, Darbonne WC, Knutzon DS, Yen R, Chien KR, Barker JB, Wi W** 1995 Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. Proc Natl Acad Sci USA 92:1142–1146
14. **Wollert KC, Taga T, Saito M, Narazaki M, Kishimoto T, Glembotski CC, Vernallis AB, Heath JK, Pennica D, Wood WI, Chien KR** 1996 Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series via gp130/leukemia inhibitory factor receptor-dependent pathways. J Biol Chem 271:9535–9545
15. **López N, Díez J, Fortuño MA** 2006 Differential hypertrophic effects of cardiotrophin-1 on adult cardiomyocytes from normotensive and spontaneously hypertensive rats. J Mol Cell Cardiol 41:902–913
16. **Jin H, Yang R, Keller GA, Ryan A, Ko A, Finkle D, Swanson TA, Li W, Pennica D, Wood WI, Paoni NF** 1996 *In vivo* effects of cardiotrophin-1. Cytokine 8:920–926
17. **Ishikawa M, Saito Y, Miyamoto Y, Harada M, Kuwahara K, Ogawa E, Nakagawa O, Hamanaka I, Kajiyama N, Takahashi N, Masuda I, Hashimoto T, Sakai O, Hosoya T, Nakao K** 1999 A heart-specific increase in cardiotrophin-1 gene expression precedes the establishment of ventricular hypertrophy in genetically hypertensive rats. J Hypertens 17:807–816
18. **Pemberton CJ, Raudsepp SD, Yandle TG, Cameron VA, Richards AM** 2005 Plasma cardiotrophin-1 is elevated in human hypertension and stimulated by ventricular stretch. Cardiovasc Res 68:109–117
19. **López B, Gonzalez A, Lasarte JJ, Sarobe P, Borras F, Diaz A, Barba J, Tomas L, Lozano E, Serrano M, Varo N, Beloqui O, Fortuño MA, Díez J** 2005 Is plasma cardiotrophin-1 a marker of hypertensive heart disease? J Hypertens 23:625–632
20. **Gonzalez A, López B, Martín-Raymondi D, Lozano E, Varo N, Barba J, Serrano M, Díez J** 2005 Usefulness of plasma cardiotrophin-1 in assessment of left ventricular hypertrophy regression in hypertensive patients. J Hypertens 23:2293–2300
21. **Funamoto M, Hishinuma S, Fujio Y, Matsuda Y, Kunisada K, Oh H, Negoro S, Tone E, Kishimoto T, Yamauchi-Takihara K** 2000 Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation. J Mol Cell Cardiol 32:1275–1284
22. **Sano M, Fukuda K, Kodama H, Takahashi T, Kato T, Hakuno D, Sato T, Manabe T, Tahara S, Ogawa S** 2000 Autocrine/paracrine secretion of IL-6 family cytokines causes angiotensin II-induced delayed STAT3 activation. Biochem Biophys Res Commun 269:798–802
23. **Sano M, Fukuda K, Kodama H, Pan J, Saito M, Matsuzaki J, Takahashi T, Makino S, Kato T, Ogawa S** 2000 Interleukin-6 family of cytokines mediate angiotensin II-induced cardiac hypertrophy in rodent cardiomyocytes. J Biol Chem 275:29717–29723
24. **Claycomb WC, Lanson Jr NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo Jr NJ** 1998 HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 95:2979–2984
25. **Sierra A, Gottfried-Blackmore A, Milner TA, McEwen BS, Bulloch K** 2008 Steroid hormone receptor expression and function in microglia. Glia 56:659–674
26. **Callera GE, Montezano AC, Yogi A, Tostes RC, He Y, Schiffrin EL, Touyz RM** 2005 c-Src-dependent nongenomic signaling responses to aldosterone are increased in vascular myocytes from spontaneously hypertensive rats. Hypertension 46:1032–1038
27. **Callera GE, Touyz RM, Tostes RC, Yogi A, He Y, Malkinson S, Schiffrin EL**

2005 Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src. Hypertension 45:773–779
28. **Karmazyn M, Liu Q, Gan XT, Brix BJ, Fliegel L** 2003 Aldosterone increases NHE-1 expression and induces NHE-1-dependent hypertrophy in neonatal rat ventricular myocytes. Hypertension 42:1171–1176
29. **Okoshi MP, Yan X, Okoshi K, Nakayama M, Schuldt AJ, O'Connell TD, Simpson PC, Lorell BH** 2004 Aldosterone directly stimulates cardiac myocyte hypertrophy. J Card Fail 10:511–518
30. **Gaeggeler HP, Gonzalez-Rodriguez E, Jaeger NF, Loffing-Cueni D, Norregaard R, Loffing J, Horisberger JD, Rossier BC** 2005 Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. J Am Soc Nephrol 16:878–891
31. **Sugiyama T, Yoshimoto T, Tsuchiya K, Gochou N, Hirono Y, Tateno T, Fukai N, Shichiri M, Hirata Y** 2005 Aldosterone induces angiotensin converting enzyme gene expression via a JAK2-dependent pathway in rat endothelial cells. Endocrinology 146:3900–3906
32. **Gauer S, Segitz V, Goppelt-Struebe M** 2007 Aldosterone induces CTGF in mesangial cells by activation of the glucocorticoid receptor. Nephrol Dial Transplant 22:3154–3159
33. **Fuller PJ, Lim-Tio SS, Brennan FE** 2000 Specificity in mineralocorticoid versus glucocorticoid action. Kidney Int 57:1256–1264
34. **Benitah JP, Vassort G** 1999 Aldosterone upregulates Ca(2+) current in adult rat cardiomyocytes. Circ Res 85:1139–1145
35. **Whitehurst Jr RM, Zhang M, Bhattacharjee A, Li M** 1999 Dexamethasone-induced hypertrophy in rat neonatal cardiac myocytes involves an elevated L-type Ca(2+)current. J Mol Cell Cardiol 31:1551–1558
36. **Muller O, Pradervand S, Berger S, Centeno G, Milet A, Nicod P, Pedrazzini T, Tronche F, Schutz G, Chien K, Rossier BC, Firsov D** 2007 Identification of corticosteroid-regulated genes in cardiomyocytes by serial analysis of gene expression. Genomics 89:370–377
37. **Funder JW** 2006 Mineralocorticoid receptors and cardiovascular damage: it's not just aldosterone. Hypertension 47:634–635
38. **Funder JW** 2007 Mineralocorticoid receptor activation and oxidative stress. Hypertension 50:840–841
39. **Rickard AJ, Funder JW, Morgan J, Fuller PJ, Young MJ** 2007 Does glucocorticoid receptor blockade exacerbate tissue damage after mineralocorticoid/salt administration? Endocrinology 148:4829–4835
40. **Funder JW** 2006 Minireview: aldosterone and the cardiovascular system: genomic and nongenomic effects. Endocrinology 147:5564–5567
41. **Grossmann C, Gekle M** 2007 Non-classical actions of the mineralocorticoid receptor: misuse of EGF receptors? Mol Cell Endocrinol 277:6–12
42. **Hammes SR** 2003 The further redefining of steroid-mediated signaling. Proc Natl Acad Sci USA 100:2168–2170
43. **Le Moellic C, Ouvrard-Pascaud A, Capurro C, Cluzeaud F, Fay M, Jaisser F, Farman N, Blot-Chabaud M** 2004 Early nongenomic events in aldosterone action in renal collecting duct cells: PKCα activation, mineralocorticoid receptor phosphorylation, and cross-talk with the genomic response. J Am Soc Nephrol 15:1145–1160
44. **Grossmann C, Benesic A, Krug AW, Freudinger R, Mildenberger S, Gassner B, Gekle M** 2005 Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. Mol Endocrinol 19:1697–1710
45. **Perrier R, Richard S, Sainte-Marie Y, Rossier BC, Jaisser F, Hummler E, Benitah JP** 2005 A direct relationship between plasma aldosterone and cardiac L-type Ca2+ current in mice. J Physiol 569:153–162
46. **Lee YS, Kim JA, Kim KL, Jang HS, Kim JM, Lee JY, Shin IS, Lee JS, Suh W, Choi JH, Jeon ES, Byun J, Kim DK** 2004 Aldosterone upregulates connective tissue growth factor gene expression via p38 MAPK pathway and mineralocorticoid receptor in ventricular myocytes. J Korean Med Sci 19:805–811
47. **Talwar S, Squire IB, Downie PF, O'Brien RJ, Davies JE, Ng LL** 2000 Elevated circulating cardiotrophin-1 in heart failure: relationship with parameters of left ventricular systolic dysfunction. Clin Sci (Colch) 99:83–88
48. **Zolk O, Ng LL, O'Brien RJ, Weyand M, Eschenhagen T** 2002 Augmented expression of cardiotrophin-1 in failing human hearts is accompanied by diminished glycoprotein 130 receptor protein abundance. Circulation 106: 1442–1446
49. **Gonzalez A, Ravassa S, Loperena I, Lopez B, Beaumont J, Querejeta R, Larman M, Diez J** 2007 Association of depressed cardiac gp130-mediated antiapoptotic pathways with stimulated cardiomyocyte apoptosis in hypertensive patients with heart failure. J Hypertens 25:2148–2157

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