Corticosteroids and Redox Potential Modulate Spontaneous Contractions in Isolated Rat Ventricular Cardiomyocytes

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Abstract—The mineralocorticoid receptor has been implicated in the development of several cardiac pathologies and could participate in the high incidence of lethal ventricular arrhythmias associated with hyperaldosteronism. We have observed previously that aldosterone markedly increases in vitro the rate of spontaneous contractions of isolated neonate rat ventricular myocytes, a putative proarrhythmogenic condition if occurring in vivo. In the present study, we investigated the effect of glucocorticoids, the involvement of the glucocorticoid receptor, and the modulation of their action by redox agents. Aldosterone and glucocorticoids exerted in vitro a similar, concentration-dependent chronotropic action on cardiomyocytes, which was mediated by both the mineralocorticoid and glucocorticoid receptors. However, the relative contribution of each receptor was different for each agonist, at each concentration. Angiotensin II induced a similar response that was entirely dependent on the activity of the glucocorticoid receptor. Corticosteroid action was modulated by the redox state of the cells, with oxidation increasing the response while reducing conditions partially preventing it. When only the mineralocorticoid receptor was functionally present in the cells, oxidation was necessary to reveal glucocorticoid action, but no obvious competition with mineralocorticoids was observed when both agonists were simultaneously present. In conclusion, corticosteroids exert a strong chronotropic action in ventricular cardiomyocytes, mediated by both the mineralocorticoid and glucocorticoid receptors and modulated by the redox state of the cell. This phenomenon is believed to be because of cell electric remodeling and could contribute in vivo to the deleterious consequence of inappropriate receptor activation, leading to increased susceptibility of patients to arrhythmias. (Hypertension. 2008;52:721-728.)

Key Words: corticosteroid ▪ cardiomyocyte ▪ redox potential ▪ contraction ▪ mineralocorticoid receptor ▪ glucocorticoid receptor ▪ angiotensin II ▪ arrhythmias

Aldosterone excess has been incriminated in the development of several cardiac dysfunctions, including heart hypertrophy, inflammation, fibrosis, apoptosis, and arrhythmias.1,2 Moreover, clinical trials, such as the Randomized Aldactone Evaluation Study and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study,3,4 have clearly demonstrated that low mineralocorticoid antagonist administration. Similarly, the threshold for ventricular fibrillation in experimental heart failure was significantly increased in rats by MR blockade with canrenone.5 Recently, transgenic mice conditionally overexpressing the human MR specifically in their cardiomyocytes exhibited a high rate of sudden death linked to severe ventricular arrhythmias, which were prevented by spironolactone.6

Several mechanisms have been proposed for explaining the relationship between a mineralocorticoid excess and the apparition of arrhythmias, such as aldosterone-induced hyperkalemia, cardiac fibrosis, or rise in blood pressure. The putative role of cardiomyocyte electric remodeling in the induction of arrhythmias has been also investigated both in vivo and in vitro.

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Indeed, we have shown that in vitro exposure of isolated neonatal rat ventricular cardiomyocytes to aldosterone for 24 hours markedly increases the rate of cell spontaneous contractions. Acceleration in the spontaneous beatings of individual ventricular myocytes can profoundly affect the synchronous contraction required for an optimal cardiac function, and the presence of a few cells contracting before the pacemaker signal could be a source of arrhythmias for the whole heart.

A well-known paradox in the field of mineralocorticoid physiology is the recognized ability of MR to bind glucocorticoids with similar affinity. Because free circulating concentrations of glucocorticoids are 10 to 100 times higher than those of aldosterone, we wondered how aldosterone overcomes MR occupancy by glucocorticoids to specifically activate the receptor. In epithelial mineralocorticoid target tissues, MR is protected against "illegitimate" occupancy by glucocorticoids, thanks to the presence of the 11β-hydroxysteroid dehydrogenase type 2 enzyme, which converts cortisol (or corticosterone in rodents) into their receptor-inactive 11-keto forms. In cardiomyocytes, however, this enzyme is absent, and cardiac MR is expected to be constantly occupied by glucocorticoids. Interestingly, transgenic mice overexpressing 11β-hydroxysteroid dehydrogenase type 2 enzyme in cardiomyocytes were normotensive but spontaneously developed cardiac hypertrophy, fibrosis, and heart failure and died prematurely. The MR antagonist eplerenone ameliorated this phenotype, revealing a possible tonic inhibitory role of glucocorticoids on MR that prevents such outcomes under physiological conditions.

This finding supports a model proposed by Funder. The basic hypothesis is that, in contrast to aldosterone action, glucocorticoid-induced MR transactivation requires an oxidized environment. It was, therefore, proposed that the function of the cardiac MR, which is expected to be constantly occupied by glucocorticoids, is less to sense variations of circulating or locally produced aldosterone than to signal changes in the cellular redox state occurring under pathophysiological circumstances.

In the present study, we have investigated whether glucocorticoids prevent or mimic aldosterone action on spontaneous beatings of isolated neonatal rat cardiomyocytes; determined the relative function of MR and glucocorticoid receptor (GR) in these cells; and tested the influence of the cell redox state on the response to steroids. These results should help us to clarify why patients with primary aldosteronism have higher incidence of cardiovascular complications than those with essential hypertension and how mineralocorticoid antagonists can exert their protective action in patients with heart failure independent of blood pressure.

**Methods**

**Materials**

DMEM was obtained from Invitrogen. All of the other reagents were from Sigma unless otherwise indicated.

**Cell Culture**

Neonatal cardiac cells were isolated from 1- to 2-day-old Wistar rat ventricles by digestion with low trypsin-EDTA and type 2 collagenase, as described previously. Animals were euthanized in conformity with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication 85-23). Freshly isolated cells were seeded in plastic flasks to allow selective adhesion of cardiac fibroblasts. Thereafter, cardiomyocytes were decanted from the flasks and distributed in laminin-coated 90-mm Petri dishes or in 6-well culture plates. Cells from a same preparation were used for testing the various experimental conditions.

**Cell Contraction Frequency**

Spontaneously contracting cell monolayers were incubated for the indicated times with the appropriate concentration of agonist or vehicle in serum-free DMEM. Cell beating frequency was determined by counting, under light microscope, the number of contractions per time unit in 3 different locations of the dish.

In several preparations, the rate of contractions was also assessed by measuring the cytosolic free calcium fluctuations. In this case, spontaneous calcium transients were monitored by line scan mode of the LSM 5 Pascal confocal microscope (Zeiss). Cardiomyocytes cultured on laminin-coated coverslips were loaded for 20 minutes with the fluorescent calcium probe Fluo-4 AM (Molecular Probes, Invitrogen) in a medium containing (in mmol/L) the following: 140 NaCl, 5 KCl, 1.2 CaCl2, 1 MgCl2, 10 glucose, and 20 Hepes (pH 7.4) at room temperature. Fluorescence of a single line across the cell was then repeatedly measured for 1 minute at a frequency of 100 Hz.

**Statistics**

Results are expressed as the means±SEMs unless stated otherwise. The statistical significance of differences was assessed by 1- or 2-way ANOVA followed by posthoc tests between specific relevant groups. Bonferroni’s correction was applied when appropriate.

**Results**

**Steroids Accelerate Spontaneous Beatings of Isolated Rat Ventricular Cardiomyocytes**

Freshly isolated neonatal rat ventricular cardiomyocytes spontaneously contract in primary culture. To determine the rate of these contractions under basal and stimulated conditions, fluctuations of cytosolic free calcium concentrations were monitored in single cells with the calcium probe Fluo-4 by confocal microscopy in the line scan mode. Figure 1A shows cytosolic calcium transients corresponding with spontaneous cell contractions obtained in a control cell (Figure 1A, left, Ctrl) and in a cell from the same preparation exposed for 30 hours to 1000 nmol/L of aldosterone (Figure 1A, right, aldo). A marked increase of the rate of contractions was systematically observed on aldosterone stimulation. Aldosterone effect on the beating frequency was concentration dependent and mimicked by 2 glucocorticoids, corticosterone and dexamethasone (Figure 1B). The EC50 values were largely >1 nmol/L, for each steroid including aldosterone, suggesting the involvement, at least in part, of a low-affinity receptor like GR (see below).

The kinetics of the chronotropic response to aldosterone was also investigated (Figure 1C). No response was observed during the first 12 hours of incubation with aldosterone, whereas the acceleration was clearly observed between 18 and 48 hours, a time course supporting a genomic action of the agonist. Similar kinetics were observed with 1 μmol/L of corticosterone (data not shown).

The role of MR and GR in the chronotropic response to each steroid was studied using the receptor-respective antagonists, spironolactone and RU-486. The addition of increas-
ing concentrations of spironolactone during the 24-hour incubation markedly reduced the response to 100 nmol/L of aldosterone down to basal values (Figure 2A, left), whereas the MR antagonist had no effect on the response to corticosterone (1 μmol/L). In contrast, high concentrations of RU-486 completely abolished the chronotropic action of corticosterone and showed a tendency to decrease aldosterone action, without reaching a statistical significance. These results suggest that corticosterone action on the myocyte contractions is essentially mediated by GR, whereas both MR and GR are involved in the response to high, supraphysiological concentrations of aldosterone.

This assumption was confirmed by results obtained when incubating cells with increasing concentrations of steroids in the presence of a fixed amount of antagonists (Figure 2B). Although the responses to corticosterone (Figure 2B, middle) or dexamethasone (Figure 2B, right) were completely prevented by 10 μmol/L of RU-486, spironolactone, at the same concentration, did not affect the response to dexamethasone at all and only had a slight inhibitory effect on the response to 100 nmol/L of corticosterone. In contrast, both MR and GR antagonists significantly affected the response to aldosterone, but with clearly distinct properties (Figure 2B, left). Indeed, spironolactone was very efficient to inhibit the response to aldosterone up to 10 nmol/L but failed to maintain a low beating frequency at aldosterone concentrations high enough to activate GR. The exact opposite pattern was observed with RU-486, which was completely inefficient up to 10 nmol/L of aldosterone and started to exert its inhibition only above this concentration.

Angiotensin II Mimics the Action of Corticosteroids

Because angiotensin II (Ang II) is known to share some deleterious effects with aldosterone on the cardiac function, we determined whether this hormone mimics the chronotropic action of corticosteroids. As shown in Figure 3A, incubation of cardiomyocytes for 24 hours with Ang II (100 nmol/L) led to an increase of spontaneous beating frequency similar to that elicited by aldosterone. This effect of Ang II was mediated by the Ang II type 1 receptor, because it was almost entirely prevented by losartan (100 μmol/L). As expected, spironolactone selectively reduced the response to aldosterone without affecting that of Ang II; however, RU-486 completely abolished the response to Ang II, suggesting that GR could be involved in the chronotropic response to Ang II. Finally, combining spironolactone and RU-486 logically abolished the response to each agonist.
To further characterize the action of Ang II, several cellular messenger pathways, classically activated by this hormone, were pharmacologically tested. Figure 3B shows that 10 μmol/L of UO126, a p42/44 mitogen-activated protein kinase inhibitor; 10 μmol/L of SB203580, an inhibitor of p38 mitogen-activated protein kinase; 100 nmol/L of wortmannin or 20 μmol/L of LY294002, 2 independent blockers of the phosphatidylinositol 3-kinase/Akt pathway; and 10 μmol/L of cyclosporine A, a calcineurin inhibitor, were not able to prevent the chronotropic response to Ang II and aldosterone. Moreover, the effects of these 2 agonists were not additive (data not shown). The signaling pathway putatively linking the Ang II type 1 receptor to the rate of contractions remains, therefore, to be elucidated.

Redox State of the Cells Modulates the Chronotropic Response to Steroids

Because the cardiomyocyte response to MR activation has been proposed to depend on the cell redox state,9,15 we have measured the effect of aldosterone and corticosterone on beating frequency under oxidative and reducing conditions. For this purpose, the 24-hour incubation with steroids was performed in the presence or absence of L-buthionine sulfoximine (L-BSO, 5 mmol/L), an oxidative agent interfering with glutathione metabolism within the cell,16 or sodium dithionite (5 mmol/L), a reducing agent. As shown in Figure 4, the chronotropic response to both aldosterone (Figure 4A) and corticosterone (Figure 4B) was modulated by these redox agents, positively with L-BSO and negatively with dithionite. Importantly, the redox effect on the spontaneous beating frequency was observed only in the presence of steroids.

To distinguish between an effect of redox on MR and GR, the same experiment was performed in the presence of either spironolactone (Figure 4C) or RU-486 (Figure 4D). When only GR was active (Figure 4C), L-BSO had no more effect, and dithionite was much less efficient. In contrast, the chronotropic response to corticosterone mediated by MR (Figure 4D) absolutely required oxidized conditions. The next series of experiments was systematically performed in the presence of 10 μmol/L of RU-486 (Figure 5). As shown previously, under these conditions no effect of corticosterone alone (1000 nmol/L) was observed (Figure 5A). Nevertheless, a significant chronotropic response was elicited by the glucocorticoid under oxidized conditions (L-BSO). This response was clearly mediated by MR because it was completely abolished with spironolactone. Similar results were then obtained when using dexamethasone (1000 nmol/L) instead of corticosterone, speaking against the formation of a hypothetical metabolite of corticosterone with mineralocorticoid activity under oxidized conditions.
Aldosterone (100 nmol/L), LY294 (10 nmol/L) exhibited a significant response even in the presence of losartan (100 nmol/L), spironolactone (10 μmol/L), RU-486 (10 μmol/L), or a combination of spironolactone and RU-486. Basal frequency was determined in each experiment in the absence of agonist. B, Cells were stimulated as in A, but in the presence of UO126 (10 μmol/L), SB203580 (10 μmol/L), wortmannin (100 nmol/L), LY294 (10 μmol/L), or cyclosporine A (10 μmol/L). Data are the mean of 4 to 7 experiments performed in duplicate. Differences vs the corresponding controls were tested. *P<0.05, **P<0.01, or ***P<0.005.

According to the model proposed by Funder,9 glucocorticoids would freely bind cardiac MR under reducing conditions but not activate the receptor. If true, glucocorticoids should, therefore, compete with aldosterone, acting as MR antagonists. We directly tested this hypothesis, as illustrated in Figure 5B. As shown previously, aldosterone alone (10 nmol/L) exhibited a significant response even in the presence of RU-486. However, this response was not affected by the presence of either corticosterone or dexamethasone (1 μmol/L each), in spite of a glucocorticoid over mineralocorticoid ratio (100:1) favoring glucocorticoid binding on MR and, therefore, speaking against a competition between glucocorticoids and mineralocorticoids for MR occupancy. Alternatively, aldosterone by itself could have modified the redox state of the cardiomyocytes in such a way that glucocorticoids would now act as agonists. We, therefore, repeated the same experiment in the presence of dithionite. Under such reducing conditions, aldosterone action was reduced significantly, as observed previously, but not prevented by either corticosterone or dexamethasone. Finally, the complete inhibition of the residual chronotropic responses by spironolactone confirmed the role of MR in this process.

**Discussion**

In the present study, we found that glucocorticoids in vitro markedly increase the rate of spontaneous contractions of isolated neonate rat ventricular cardiomyocytes, like that observed previously with aldosterone.11 This chronotropic response to corticosteroids involves the activation of both MR and GR, but the relative contribution of each receptor depends on the nature and concentration of the agonist, as well as on the redox state of the cell. This chronotropic action of steroids on cardiomyocytes is believed to represent a substantial risk in vivo to develop ventricular arrhythmias.

Mineralocorticoid antagonists reduce morbidity and mortality in patients with heart failure,3,17 and this beneficial effect is in great part attributed to a significant reduction in the occurrence of sudden cardiac death, suggesting that inappropriate activation of the cardiac MR could be proarrhythmic.6,7 Moreover, patients with primary aldosteronism not only are more likely to have left ventricular hypertrophy and stroke than patients with essential hypertension18 but can also display a higher incidence of ventricular arrhythmias, fibrillations, or sudden death.19–21 These observations have contributed to the consideration of MR antagonists as efficient antiarrhythmic drugs.1,22

The mechanisms linking MR activation to the incidence of arrhythmias can be multiple, including changes in electrolyte excretion (leading to hypokalemia), induction of hypertension, or myocardial fibrosis. However, MR antagonist concentrations used in the Randomized Aldactone Evaluation Study and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study had little effect on these parameters, and their contributions were taken into account.3,17 Moreover, conditional cardiac-specific overexpression of the human MR in transgenic mice has been shown to increase the susceptibility of these animals to severe ventricular arrhythmias, a phenotype associated with ionic channel remodeling but without any development of cardiac fibrosis.10

The in vitro experimental approach that we have used in the present study, consisting of measuring the chronotropic action of corticosteroids on isolated cardiomyocytes, a rather robust phenotype, allows for evaluating the risk of arrhythmias associated with MR and GR activation without any confounding systemic factor, such as blood pressure, hypokalemia, or fibrosis. An increase of the rate of spontaneous ventricular cell contractions represents a risk of ventricular arrhythmias if occurring in vivo. Indeed, the pacemaker cells of the sino-atrial node control the rate and synergy of cardiac contractions by imposing their own frequency to ventricular cardiomyocytes. This is possible only if the pacemaker sends its signal at a higher frequency than that of ventricular cell spontaneous contractions. If not, ventricular cells contracting...
too early would be a source of electric perturbation possibly leading to arrhythmias.

Electric automaticity in any type of cell requires the presence of particular conductances, responsible for progressively depolarizing the cell membrane during the diastolic phase and bringing the voltage to the threshold for the release of the next action potential. The amplitude of the current responsible for this slow diastolic depolarization will directly control the duration of the period between 2 consecutive action potentials and, therefore, their frequency. Two distinct classes of ionic channels are putatively responsible for this inward current: the hyperpolarization-activated cyclic nucleotide-gated channels,23 responsible for the so called “funny” current (if) observed in pacemaker cells, and low-threshold voltage-activated, T-type Ca$^{2+}$/H$^{+}$11001 channels.24 Interestingly, both channels are normally poorly expressed in adult ventricular myocytes but strongly re-expressed or upregulated in these cells on pathological conditions such as cardiac infarction, failure, or hypertrophy.25–27 Because of this “fetal” re-expression program on pathological conditions, embryonic or neonatal cardiomyocytes appear to us as a relevant model for this kind of study.

We have shown previously that aldosterone in vitro induces the expression of the α1H isoform of T-type channels in ventricular myocytes and that selective inhibition of these channels reduces the rate of stimulated contractions to control values.11 Similarly, we are currently investigating the relative contribution of T and hyperpolarization-activated cyclic nucleotide-gated channels in the chronotropic response to glucocorticoids. In this context, the fact that Ang II also affects the rate of spontaneous contractions, apparently through GR activation but independent of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, or calcineurin pathways (Figure 3), makes these investigations particularly relevant. Although the increase of the expression of channels involved in pacemaking, like T-type calcium channels or hyperpolarization-activated cyclic nucleotide-gated channels, is theoretically sufficient for explaining the chronotropic action of aldosterone, we cannot formally exclude additional mechanisms, such as steroid-induced calcium sensitization, involving, eg, a change of the ryanodine receptor activity.

Steroids, including aldosterone, are known to exert both genomic and nongenomic actions.28 The responses observed in the present study are clearly genomic, because beating acceleration in response to aldosterone or glucocorticoids is delayed by >12 hours (Figure 1C) and is dependent on the activation of nuclear receptors (Figure 2). Moreover, we have shown previously that the chronotropic response to aldosterone is completely abolished by actinomycin D, a blocker of gene transcription.11

Glucocorticoids are generally considered clinically as cardioprotective hormones, a property that could be attributed to their ability to antagonize aldosterone-induced activation of the MR by competitive binding on the receptor in nonepithelial tissues devoid of the 11β-hydroxysteroid dehydrogenase type 2 enzyme.7,13,29 The action of glucocorticoids observed in the present study appears, therefore, quite paradoxical. Indeed, glucocorticoids mimicked (rather than antagonized)
the aldosterone action on the acceleration of contractions (Figure 1B) and could, thus, be expected to also increase the risk of arrhythmias in vivo. In fact, deleterious effects of glucocorticoids on the cardiac function have been also reported. For example, higher levels of serum cortisol are independent predictors of increased mortality risk in patients with systolic heart failure. Moreover, the beneficial action of MR antagonists on cardiac hypertrophy and failure is also observed in hypertensive rats with low aldosterone, suggesting that, in this particular situation, MR is not activated by aldosterone itself but through glucocorticoid binding. The possibility that changing the cellular redox state transforms glucocorticoids from antagonists to agonists for MR, as proposed by Funder, was, therefore, tested.

A major finding of this study is the fact that cell oxidation sensitizes, whereas reducing conditions partially prevent the chronotropic response to both aldosterone and corticosterone (Figure 4). In fact, on control redox conditions, only aldosterone can induce a chronotropic response through MR (Figure 5), and glucocorticoid action was revealed only on cell oxidation. Moreover, the absence of antagonistic action of glucocorticoids when both glucocorticoids and mineralocorticoids are present together suggests that the redox state also affects steroid binding properties on MR and not only the receptor activation.

Perspectives
Oxidative stress can be experienced by cardiomyocytes on several pathophysiological situations, such as a postischemic reperfusion, eg. Under these circumstances, glucocorticoids would start to bind and activate MR, and their action on GR will be potentiated. Both receptors will contribute to electric remodeling of ventricular cardiomyocytes, making these cells more prone to spontaneous contractions, therefore increasing the risk of ventricular arrhythmias and sudden death. This relatively simplistic pathophysiological hypothesis will need to be confirmed in the future, and our in vitro experimental approach will provide a quite robust way to evaluate several aspects of this model, including new putative therapeutic drugs.

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