Aldosterone and D-Glucose Stimulate the Proliferation of Human Cardiac Myofibroblasts In Vitro

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Abstract—The renin-angiotensin-aldosterone-system appears to be involved in the development of cardiac fibrosis in rodents, characterized by nonepithelial cell proliferation and changes in the extracellular matrix. The aim of our study was to investigate the effect of high aldosterone concentrations on the proliferation of human cardiac interstitial cells in vitro. In addition, the effect of D-glucose as another risk factor for fibrosis, eg, in the diabetic heart, was investigated. Human cardiac myofibroblast cultures were established, and growth rates were measured by WST-1 assay in fetal calf serum–free Dulbecco's modified Eagle's medium (DMEM). Cells in culture showed a significant increase in number between 24 to 72 hours of cultivation under basal conditions (DMEM, 10% fetal calf serum). Aldosterone at high concentrations (10^{-8} and 10^{-7} mol/L) significantly ($P<0.01$) increased the proliferation of cultured cardiac myofibroblasts. Comparable effects were observed after incubation of the cells with high D-glucose concentrations (15 and 25 mmol/L, $P<0.01$). No additive growth stimulation was evident when the cells were incubated in medium containing both aldosterone and D -glucose. These results suggest a role for aldosterone and glucose in mediating the cardiac fibrosis through stimulation of myofibroblast growth in patients with dysregulated renin-angiotensin-aldosterone-system (especially hyperaldosteronism) and impaired glucose homeostasis. **(*Hypertension*. 2002;39:756-760.)**

Key Words: aldosterone ■ glucose ■ cardiac myofibroblasts ■ human

Recent clinical studies demonstrate a reduction in cardiovascular mortality in patients with heart failure treated with ACE inhibitors, angiotensin II blockers, and aldosterone antagonists. These effects were also seen in patients with diabetes mellitus. Ramipril reduced not only cardiac failure and myocardial infarction but also diabetic complications and the incidence of new manifestations of diabetic disease.[1] In the RALES trial (Randomized Aldactone Evaluation Study), spironolactone added to standard therapy (ACE inhibitors, loop diuretics, digoxin, etc) had a beneficial effect on mortality in patients with congestive heart failure, with a 30% reduction in mortality seen in the spironolactone group.[2] This effect was only significant in patients with elevated serum levels of collagen synthesis markers, suggesting limitation of excessive extracellular matrix turnover as one mechanism contributing to the beneficial effect of spironolactone.[2] The molecular mechanisms of these therapeutic and possibly protective effects of such agents are not completely understood.

Angiotensin II and aldosterone are able to induce cardiac fibrosis characterized by enhanced accumulation of collagen and increased fibroblast proliferation in vivo.[3–5] In vitro, angiotensin II and aldosterone increase the synthesis of collagen by human cardiac fibroblasts in a dose-dependent manner.[6] Angiotensin II also stimulates the proliferation of human cardiac fibroblasts in vitro.[7] In contrast to angiotensin II, the role of aldosterone as a modulator or inducer of fibroblast proliferation is less well defined. Because cells of the fibroblast lineage are the active mediators of fibrosis, our study aims to test the hypothesis that aldosterone is able to stimulate the proliferation of human cardiac myofibroblasts.

The existence of a local cardiac renin-angiotensin-aldosterone-system comprising all components, including enzymes and receptors required for separate regulation, has been demonstrated.[8] In addition, local aldosterone production has been demonstrated in the hearts of 2-month-old rats[9] and in the fetal human, but not in the normal adult human heart.[10] Under pathological conditions, gene expression may switch back to a fetal pattern, as is known for certain peptide hormones.

Chronic hyperglycemia in humans is the main feature of diabetes mellitus and is responsible for pathologic changes in the cardiovascular system. Diffuse areas of fibrosis throughout the myocardium have been described, reflecting alterations in the microvascular system of the heart in diabetic patients, changes defined as diabetic cardiomyopathy. Post-

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mortem analyses of cardiac tissue from diabetic patients demonstrated interstitial and focal perivascular accumulation of collagen, indicating fibrosis.[11] We therefore hypothesized that aldosterone and glucose may have synergistic stimulatory effects on the proliferation of myofibroblasts which could contribute to the excess cardiovascular mortality in diabetics.

Methods

Subjects

Patients (4 women, 3 men; age, 66.5±2.8 years; body mass index, 25.7±2.2 kg/m^2; glycosylated hemoglobin, 5.7±0.2%) who underwent cardiac surgery for coronary artery bypass or valve replacement gave informed consent for biopsies from the right atrium. They were on comparable medications (diuretics, glycosides, ACE inhibitors, calcium-antagonists, β-blockers, nitrates). None of them had diabetes mellitus, although all had related conditions such as hypertension and dyslipidemia. The study was approved by the ethics committee of the medical faculty of the University of Leipzig.

Explanting Cardiac Biopsies

About 20 mg of human cardiac tissue was washed 5 times with 3 mL ice-cold PBS for 1 minute by gentle shaking. Fragments of 1 mm were placed on the bottom of dry cell culture flasks (75 cm^2 Greiner) at 37°C in humidified air with 5% CO_2 for 20 minutes. The tissue pieces were overlaid with 20 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in humidified air with 5% CO_2. The medium was changed every 72 hours. Subcultures were established from the cells grown in a 250-mL cell culture flask by overlaying 2 mL trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA in PBS). Trypsination was stopped after 2 to 3 minutes by the addition of 10 mL of DMEM supplemented with 10% FCS. Cells from third and fourth passages were used for experiments.

Cell Proliferation Assay

For estimation of the proliferation rate, the cells were quantified by the nonradioactive colorimetric assay WST-1 (Boehringer Mannheim), based on the cleavage of a tetrazolium salt, as recommended by the manufacturer.

Experiments

Two subconfluent cell cultures on 24-well plates were incubated with medium containing different potential growth modulators. To ensure reproducibility, cells were incubated with 5, 15, and 25 mmol/L D-glucose; 5 mmol/L D-glucose plus 10 nmol/L aldosterone; 5 mmol/L D-glucose plus 100 nmol/L aldosterone; and 25 mmol/L D-glucose plus 100 nmol/L aldosterone and with 25 mmol/L L-glucose as control for osmotic and glycosylation effects for 24, 48, or 72 hours. The medium was changed every 24 hours, and proliferation was measured by WST-1 assay. Each experiment was repeated 8 times.

Cell Culture Characterization

The origin of the cells was identified by fluorescence-activated cell sorter (FACS) analysis using fibroblast-specific monoclonal antibodies mAb-1 and mAb-2. The monocytes and endothelial cells appearing in the culture were characterized by CD-14, CD-11, and CD-31 antibodies. Indirect immunofluorescence analysis with monoclonal antibody against smooth muscle α-actin (SMAA; Progen) was performed as described elsewere.[12]

Measurement of Glucose Utilization and Lactate Production

D-Glucose and lactate concentrations of the medium were measured by glucose and lactate test kits (Sigma Diagnostics, Nos. 510-A and 826-UV) after incubation of the cells with 5 or 25 mmol/L D-glucose for 96 hours, and protein concentrations were measured according to the method of Bradford.[13]

Figure 1. Expression of SMAA in cultured myofibroblast-like cells (third passage) originating from heart explants. mAb against SMAA and Cy-3–conjugated anti-mouse IgG from goat. Bar, 50 μm.

Statistical Analysis

All data are expressed as mean±SEM. Repeated measurement ANOVA was used to compare time-dependent data. Analysis of >2 groups of data were performed by 2-way ANOVA and, when required, by Bonferroni adjustment. Values of $P<0.05$ were considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

FACS Analysis and Immunocytochemistry

The purity of the human cardiac myofibroblast cultures (2 to 3 passages) was confirmed by FACS analysis using the 2 fibroblast-specific antibodies, monoclonal antibody (mAb)-1 and mAb-2. FACS analyses of 2 cell lines from different patients were performed twice. The results showed high purity of the established cell cultures. Analysis of the worst case resulted in binding of 97.3% of the cells on fibroblast antibody mAb-1 and 99.7% on fibroblast antibody mAb-2 in comparison to low binding on the monocyte-specific antibodies CD-14 (0.5%) and CD-11 (0.4%), and the binding on the endothelial cell–specific antibody CD-31 (7.2%).

Under the light microscope the cells grown on the coverslips were heterogeneous in size and morphology. To establish the origin of these cells, mAb against SMAA, the marker of myofibroblastic differentiation, was used.[12] Strong staining for SMAA was seen throughout the cytoplasm of the cells (Figure 1). The majority of the cells possessed filopodium- and lamellipodium-like structures projecting out of flat soma. Also, spindle-shaped cells and those with rodlike morphology could be observed.

Glucose Utilization and Lactate production

Glucose utilization and lactate production by human myofibroblasts (10 000 cells=0.6 μg protein) were measured after incubation with DMEM containing 5 or 25 mmol/L D-glucose on a 24-well plate containing 1 mL of the medium in each well. After 96 hours of incubation, a range of D-glucose utilization of 1.07±0.03 nmol/μg protein was observed. For

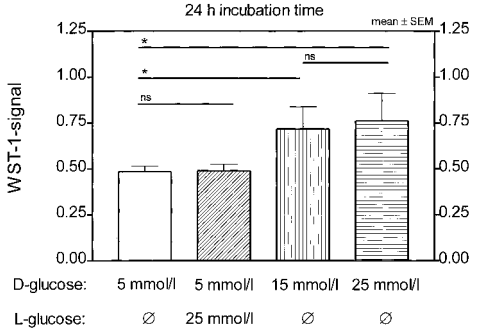

Figure 2. Effect of D-glucose (15 and 25 mmol/L) on proliferation of human cardiac fibroblasts from nondiabetic patients (n=16, passages 2 and 3). Cells were incubated in serum-free DMEM with and without D-glucose for 24 hours. *$P<0.05$.

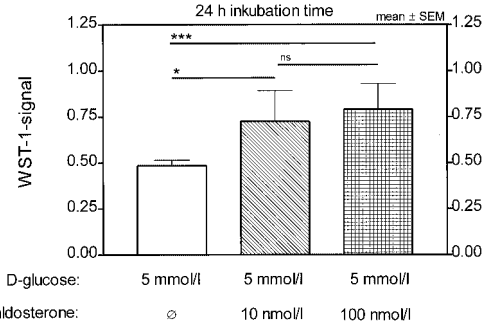

Figure 3. Effect of aldosterone (10 and 100 nmol/L) on proliferation of human cardiac fibroblasts from nondiabetic patients (n=16, passages 2 and 3). Cells were incubated in serum-free DMEM with and without aldosterone for 24 hours. *$P<0.05$; ***$P<0.001$.

25 mmol/L D-glucose, glucose utilization was nearly double (2.30±0.06 nmol/μg protein). This represents a reduction of initial D-glucose concentration of <1% within 96 hours for both glucose concentrations. Lactate production after incubation of the cells in 5 mmol/L D-glucose containing DMEM for 96 hours was 5.41±0.28 nmol/μg protein, whereas in the medium containing 25 mmol/L D-glucose, lactate production was 6.99±0.21 nmol/μg.

Proliferation of Human Cardiac Myofibroblasts

To evaluate the proliferation of human cardiac myofibroblast cultures under basal conditions, cell numbers of 7 human cardiac fibroblast lines were measured after 24, 48, 72, and 96 hours by the WST-1 proliferation assay in DMEM containing 5% D-glucose and 10% FCS. The mean of the WST-1 signals from all observed cell lines showed a significant increase in cell number between 24 and 48 hours ($P<0.0001$) and a further increase from 48 to 72 hours ($P<0.0001$). No change in cell number was seen between 72 and 96 hours ($P>0.05$). The maximum increase in cell number was seen between 48 and 72 hours, with a doubling time of >70 hours.

Proliferation of Human Cardiac Myofibroblasts Stimulated by D-Glucose

Supplementing FCS-free DMEM with additional glucose (15 and 25 mmol/L) increased human cardiac myofibroblast proliferation after 24 hours compared with those incubated in 5 mmol/L D-glucose medium. This effect was absent when cells were incubated in FCS-free DMEM with L-glucose (25 mmol/L). The stimulatory effect of D-glucose was seen after 24 hours (Figure 2) and at 48 and 72 hours (data not shown) of incubation time. There was no significant difference in proliferation of cells incubated with either 15 or 25 mmol/L D-glucose (Figure 2).

Proliferation of Human Cardiac Myofibroblasts Stimulated by Aldosterone

Addition of high aldosterone concentrations (10^{-8} or 10^{-7} mol/L) to FCS-free medium for human cardiac myofibroblast cultures resulted in significantly higher numbers of cells compared with those incubated without aldosterone. The stimulatory effect of aldosterone was seen after 24 (Figure 3), 48, and 72 hours (data not shown) of incubation. There was no significant difference in proliferation between cells incubated with either 10^{-8} or 10^{-7} mol/L aldosterone (Figure 3).

Proliferation Stimulated by Coincubation of D-Glucose and Aldosterone

Simultaneous incubation of human cardiac myofibroblasts with D-glucose (25 mmol/L) and aldosterone (10^{-7} mol/L) in FCS-free DMEM resulted in a significant increase in cell number after 24 hours of incubation compared with those incubated in normal D-glucose medium (5 mmol/L) without aldosterone. There was no synergistic effect of coincubation with 25 mmol/L D-glucose plus 10^{-7} mol/L aldosterone compared with incubation with equimolar concentrations of aldosterone and D-glucose alone (Figure 4).

Discussion

Cardiac remodeling leading to changes in the extracellular matrix, and in some cases to cardiac fibrosis, is a process involving myofibroblasts as an active component. We established primary cultures of myofibroblasts from human cardiac explants. The results of FACS analysis and the immunocytochemical investigations clearly demonstrate the myofibroblastic origin of the cells growing out of heart explants. Evidence for this was by (1) strong staining for SMAA, the main myofibroblastic marker protein of perivascular cells,[12] and (2) morphological features, ie, the well-defined leading-edge filopodium-

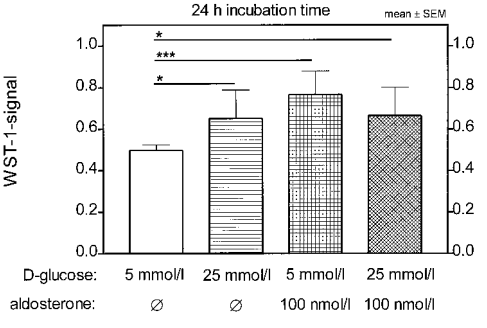

Figure 4. Effect of the incubation of aldosterone (100 nmol/L) and D-glucose (25 mmol/L) on proliferation of human cardiac fibroblasts (n=16, passages 2 and 3). Cells were incubated in serum-free DMEM with and without aldosterone, and/or D-glucose for 24 hours. *$P<0.05$; ***$P<0.001$.

and lamellipodium-like structures, typical for activated myofibroblasts, the main producers of extracellular matrix. Although the outgrowth technique may lead to a selection of cardiac cells, the morphology of our myofibroblasts is very similar to fibroblasts obtained from human heart tissue treated by trypsin and collagenase digestion.[7] Although in vivo studies strongly suggest direct aldosterone effects on fibroblasts, there is a surprising lack of information about the effect of aldosterone on cell proliferation. It has been reported that aldosterone increases the colony formation/proliferation of granulocyte-macrophage cells and diminishes the same parameters for erythroid cells.[14] Recent experiments have shown a downregulation of the mRNAs for proliferative proto-oncogenes by aldosterone.[15] In contrast, aldosterone may mediate angiotensin II–stimulated vascular smooth muscle cell proliferation in vitro.[16]

Well-established effects of aldosterone on the heart have been shown in vivo.[3–5] Rats receiving either aldosterone or angiotensin II developed hypertension, left ventricular hypertrophy, and increased collagen volume fraction and [^{3}H]-proline incorporation in both ventricles. In contrast to these in vivo effects, exposure of neonatal rat fibroblasts to 10^{-8} mol/L aldosterone was without effect on incorporation of [^{3}H]-proline into collagen,[17] raising questions about the existence of mineralocorticoid receptors (MRs) in these cells. The presence of MRs and the 11β-hydroxysteroid-dehydrogenase in rat and human heart homogenates and myocytes[8,10,18–20] was found to be a prerequisite for selective aldosterone effects.[21] Evidence that MR and 11β-hydroxysteroid-dehydrogenase do exist in rat cardiac fibroblasts was provided by binding studies, which later were confirmed by immunohistochemical and biochemical studies; thus, molecular biological experiments demonstrate the expression of MR-mRNA.[22,23] Myofibroblasts, preferentially found in areas of active collagen deposition, can synthesize collagen type I and III and contain SMAA.[12] In addition, we observed that these cells have contractile features, characteristic of myofibroblasts when stimulated with angiotensin II (G.H. Scholz and S. Neumann, 1994, unpublished observations). The concept that these cardiac myofibroblasts may be the target of aldosterone action in vivo is supported by the recent finding of intramyocardial sodium-dependent aldosterone synthesis, contributing to cardiac hypertrophy and fibrosis in rats.[9,24] Recently the expression of CYP11B2 (aldosterone synthase)-mRNA was detected in the failing but not in the normal human heart,[25] supporting, on the molecular level, previous studies on activated intracardiac aldosterone production in humans with left ventricular systolic dysfunction.[26]

It has been reported that the myocardial aldosterone concentration in rats is about 1.6×10^{-8} mol/L.[9] This is 16-fold higher than the mean plasma aldosterone concentration in this animal. The myofibroblast cultures used in our experiments showed a proliferation stimulating activity of aldosterone at concentrations of 10^{-7} and 10^{-8} mol/L. That the lower aldosterone concentration has already maximum stimulatory effect on cell proliferation supports the involvement of classical MR effects. Intermediate early genes as of sgk (serum- and glucocorticoid-regulated kinase) are induced at 10^{-8} mol/L aldosterone and can be blocked by ZK91587, a specific MR antagonist.[27] This concentration also approaches that achieved in patients with primary and secondary hyperaldosteronism. Therefore, it seems reasonable to assume that there are at least 2 possibilities to explain the effect of aldosterone on the development of cardiac fibrosis in humans. First, such an effect may reflect enhanced synthesis of connective tissue components (collagen, fibronectin) by the individual fibroblast or myofibroblast; in addition, aldosterone may increase the number of myofibroblasts by promoting the proliferation of these cells.

In our experiments, high D-glucose concentrations stimulated the proliferation of myofibroblasts. This effect was not related to changes in osmolarity or to production of advanced glycosylation end products, because equimolar concentrations of nonmetabolizable L-glucose did not influence proliferation. A stimulatory effect of high glucose (10 to 30 mmol/L) was found in human dermal fibroblasts,[28] arterial smooth muscle cells,[28] and human renal cortical fibroblasts.[29] Other groups reported an inhibitory or a toxic effect of high D-glucose on the proliferation of human dermal fibroblasts,[30] which was prevented by aldose reductase inhibitors. A biphasic effect of enhanced D-glucose concentrations on the proliferation of mesangial cells was found, possibly related to a time-dependent change in the level and activity of paracrine factors.[31] Recently published data have also shown a stimulating effect of high glucose concentrations on cellular proliferation of vascular smooth muscle cells, suggesting that transforming growth factor-β and protein kinase C (PKC) play an important role in this process.[32] It seems possible that glucose, by activation of PKC, enhances the expression of *c*-fos and *c*-jun, which as components of the activator protein-1 (AP-1) complex are known to interact with the AP-1 binding site on the transforming growth factor-β receptor type II promotor.[33] Apart from the potential role of paracrine factors, the experimental design may contribute to alternative results. A critical factor may be the time of incubation with glucose, which in confluent cultures can lead to substrate exhaustion and growth retardation. This is unlikely to be the case in our experiments; even in the presence of maximally growth stimulating 10% FCS, glucose utilization was low and the medium was changed every 24 hours. Because the experiments with high and normal D-glucose were done in FCS-free medium, even lower glucose utilization can be assumed. Taken together, the available data suggest that hyperglycemia might be able to induce fibrosis in the heart in different ways: via temporary or sustained stimulation of cells of the fibroblast lineage or via an increase in extracellular matrix production of these cells. To examine a possible synergistic effect of aldosterone and glucose on proliferation, we coincubated myofibroblasts with aldosterone and glucose. A stimulatory effect, and in some experiments even a synergistic effect, of aldosterone and glucose was observed.[34] Later it was shown in rat neonatal cardiomyocytes that indeed high glucose is able to modulate aldosterone-induced protein synthesis and that one of the possible key regulators of glucose-mediated proliferation, PKC, is also a target of aldosterone action.[35] In this study on human cardiac myofibroblasts in primary culture, we have demonstrated that high D-glucose concentrations, as well as subglucocorticoid but near-maximal aldosterone concentra-

tions, stimulate cell proliferation compared with that of the normal plasma concentration. This effect can also be seen in coincubation experiments, although we cannot exclude the possibility that the supramaximal concentrations of both stimuli used in our experiments may have masked other additive, synergistic, or even antagonistic effects occurring at different stimulus thresholds. Especially in regard to the much lower aldosterone concentrations necessary for non-genomic aldosterone effects on IP3, intracellular calcium, and PKC occurring at 10^{-11} to 10^{-10} mol/L,[36] which can also be activated by glucose.[31] This kind of possible interaction has to be investigated in the future.

In summary cardiac fibrosis may be induced or aggravated by high D-glucose and aldosterone concentrations in vivo, which alone or in combination may stimulate myofibroblast proliferation and extracellular matrix production.

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References

1. HOPE Study Investigators. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *N Engl J Med*. 2000;342:145–153.
2. Zannad F, Alla F, Dousset B, Perez A, Pitt B. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with CHF: insight from the randomized aldactone evaluation study (RALES). *Circulation*. 2000;102:2700–2706.
3. Brilla CG, Weber KT. Mineralocorticoid excess, dietary sodium, and myocardial fibrosis. *J Lab Clin Med*. 1992;120:893–901.
4. McEwan PE, Gray GA, Sherry L, Webb DJ, Kenyon CJ. Differential effects of angiotensin II on cardiac cell proliferation and intramyocardial perivascular fibrosis in vivo. *Circulation*. 1998;98:2765–2773.
5. Young M, Head G, Funder JW. Determinants of cardiac fibrosis in experimental hypermineralocorticoid states. *Am J Physiol*. 1995;269: E657–E662.
6. Funck RC, Wilke A, Rupp H, Brilla CG. Regulation and role of myocardial collagen matrix remodeling in hypertensive heart disease. *Adv Exp Med Biol*. 1997;432:35–44.
7. Agocha A, Hyeon-Woo L, Mahboubeh E-W. Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: effects of TGF-β, thyroid hormone, angiotensin II and basic FGF. *J Mol Cell Cardiol*. 1997;29:2233–2244.
8. Lombes M, Alfaidy N, Eugene E, Lessana A, Farman N, Bonvalet J-P. Prerequisite for cardiac aldosterone action: mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase in the human heart. *Circulation*. 1995;92:175–182.
9. Silvestre J-S, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic J-M, Swynghedauw B, Delcayre C. Myocardial production of aldosterone and corticosterone in the rat. *J Biol Chem*. 1998;273:4883–4891.
10. Kayes-Wandover KM, White PC. Steroidogenic enzyme gene expression in the human heart. *J Clin Endocrinol Metab*. 2000;85:2519–2525.
11. Factor SM, Okun EM, Minase T. Capillary microaneurysms in the human diabetic heart. *N Engl J Med*. 1980;302:384–388.
12. Buniatian GH, Gebhardt R, Mecke D, Traub P, Wiesinger H. Common myofibroblastic features of newborn rat astrocytes and cirrhotic rat liver stellate cells in early cultures and in vivo. *Neurochem Int*. 1999;35: 317–327.
13. Bradford M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1975;72:248–254.
14. Grafte-Faure S, Leveque C, Vasse M, Soria C, Norris V, Vannier JP. Effects of glucocorticoids and mineralocorticoids on proliferation and maturation of human peripheral blood stem cells. *Am J Hematol*. 1999; 62:65–73.
15. Verrey F, Pearce D, Pfeiffer R, Spindler B, Mastroberardino L, Summa V, Zeevic M. Pleiotropic action of aldosterone in epithelia mediated by transcription and post-transcription mechanisms. *Kidney Int*. 2000;57: 1277–1282.
16. Xiao F, Puddefoot JR, Vinson GP. Aldosterone mediates angiotensin II–stimulated rat vascular smooth muscle cell proliferation. *J Endocrinol*. 2000;165:533–536.
17. Fullerton MJ, Funder JW. Aldosterone and cardiac fibrosis: in vitro studies. *Cardiovasc Res*. 1994;28:1863–1867.
18. Pearce P, Funder JW. High-affinity aldosterone binding sites (type I receptors) in rat heart. *Clin Exp Pharmacol Physiol*. 1987;14:859–866.
19. Slight S, Ganjam VK, Gomes-Sanchez CE, Zhou MY, Weber KT. High-affinity NAD^+-dependent 11β-hydroxysteroid dehydrogenase in the human heart. *J Mol Cell Cardiol*. 1996;28:781–787.
20. Bonvalet JP, Alfaidy N, Farman N, Lombes M. Aldosterone: intracellular receptors in human heart. *Eur Heart J*. 1995;16:92–97.
21. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor mediated. *Science*. 1988; 242:583–585.
22. Kohler E, Bertschin S, Woodtli T, Resink T, Erne P. Does aldosterone-induced cardiac fibrosis involve direct effects on cardiac fibroblasts? *J Vasc Res*. 1996;33:315–326.
23. Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circ Res*. 1992;71:503–510.
24. Takeda Y, Yoneda T, Demura M, Miyamori I, Mabuchi H. Sodium-induced cardiac aldosterone synthesis causes cardiac hypertrophy. *Endocrinology*. 2000;141:1901–1904.
25. Young MJ, Clyne CD, Cole TJ, Funder JW. Cardiac steroidogenesis in the normal and failing heart. *J Clin Endocrinol Metab*. 2001;86: 5121–5126.
26. Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, Harada E, Nakayama M, Nakamura S, Ito T, Shimasaki Y, Saito Y, Nakao K. Aldosterone production is activated in failing ventricle in humans. *Circulation*. 2001;103:72–77.
27. Naray-Fejes-Tóth A, Fejes-Tóth G. The sgk, an aldosterone-induced gene in mineralocorticoid target cells, regulates the epithelial sodium channel. *Kidney Int*. 2000;57:1290–1294.
28. Turner JL, Biermann EL. Effects of glucose and sorbitol on proliferation of cultured human skin fibroblasts and arterial smooth-muscle cells. *Diabetes*. 1978;27:583–588.
29. Jones SC, Saunders HJ, Pollock CA. High glucose increase growth and collagen synthesis in cultured human tubulointerstitial cells. *Diabet Med*. 1999;16:932–938.
30. Sibbitt WL Jr, Mills RG, Bigler CF, Eaton RP, Griffey RH, Vander Jagt DL. Glucose inhibition of human fibroblast proliferation and response to growth factors is prevented by inhibitors of aldose reductase. *Mech Ageing Dev*. 1989;47:265–279.
31. Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadeh FN. High glucose induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int*. 1992;42:647–656.
32. Yasuda Y, Nakmura J, Hamada Y, Nakayama M, Chaya S, Naruse K, Nakashima E, Kato K, Kamiya H, Hotta N. Role of PKC and TGF-β receptor in glucose-induced proliferation of smooth muscle cells. *Biochem Biophys Res Comm*. 2001;281:71–77.
33. Wilmer WA, Cosio FG. DNA binding of activator protein-1 is increased in human mesangial cells cultured in high glucose concentrations. *Kidney Int*. 1998;53:1172–1181.
34. Scholz GH, Huse K, Neumann S. Effects of aldosterone and glucose on the proliferation and collagenase secretion of cultured human cardiac fibroblasts. The Endocrine Society 77th Annual Meeting Proceedings. Washington, DC, Endocrine Society Press, 1995;263. Abstract.
35. Sato A, Funder JW. High glucose stimulates aldosterone-induced hypertrophy via type I mineralocorticoid receptors in neonatal rat cardiomyocytes. *Endocrinology*. 1996;137:4145–4153.
36. Christ M, Günther A, Heck M, Schmidt BMW, Falkenstein E, Wehling M. Aldosterone, not estradiol, is the physiological agonist for rapid increases in cAMP in vascular smooth muscle cells. *Circulation*. 1999; 99:1485–1491.