British Journal of Pharmacology (2001) 134, 224–232

www.nature.com/bjp

Influence of aldosterone on collagen synthesis and proliferation of rat cardiac fibroblasts

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1 Previous *in vivo* studies in men and experimental animal models have shown that hyperaldosteronemia is correlated with cardiac fibrosis due to increased total collagen synthesis. As yet, it is unclear whether aldosterone has direct pro-fibrogenic effect on cardiac fibroblasts, the fibrogenic effector cell in the myocardium, and if so which procollagens specifically are synthesized at higher rates.

2 The present study aims at establishing whether *de novo* collagen synthesis by cardiac fibroblasts is enhanced following exposure for 2×24 h to pharmacological (10^{-7}–10^{-8} M), near-physiological (10^{-9} M) or physiological (10^{-10}–10^{-11} M) aldosterone concentrations. During the last 24 h, cells were metabolically labelled with [^{35}S]-methionine/[^{35}S]-cysteine. Labelled procollagens were immunoprecipitated quantitatively using antibodies against specific procollagens. Contrary to expectations, 10^{-7} M aldosterone inhibited significantly *de novo* synthesis of procollagens type I and IV (−35% and −42%, respectively). For procollagen type III, only a tendency towards inhibition was observed. At lower concentrations of aldosterone (10^{-8}–10^{-10} M), synthesis of procollagens type I, III or IV was unaffected.

3 Cellular DNA synthesis under influence of aldosterone was evaluated by measuring BrdU incorporation. Cells were treated with aldosterone, while BrdU was added during the last 16 h of treatment. Aldosterone had no demonstrable effect on cellular proliferation.

4 Reverse transcription-polymerase chain reaction (RT–PCR) clearly demonstrated the presence of mineralocorticoid receptor mRNA in cardiac fibroblasts.

5 In spite of the expression of the mineralocorticoid receptor by cultured cardiac fibroblasts, the pro-fibrogenic effect of aldosterone as observed *in vivo*, is not likely to be due to a direct effect of this hormone in cardiac fibroblasts.

British Journal of Pharmacology (2001) **134,** 224–232

Keywords: Cardiac fibroblasts; aldosterone; cardiac fibrosis; collagen *de novo* synthesis; mineralocorticoid receptor

Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; TCA, tricholoroacetic acid

Introduction

The cardiac connective tissue is a complex meshwork of collagens and non-collagenous matrix components, in which cardiac myofibres and blood vessels are embedded. Two-thirds of cardiac cells are non-myocytes, the vast majority of which are fibroblasts. These fibroblasts are the principal extracellular matrix synthesizing cells of the diseased heart (Booz & Baker, 1995).

The literature concerning the influence of aldosterone on development of cardiac fibrosis is controversial. *In vivo* experiments in rat models of hyperaldosteronemia showed a pro-fibrogenic effect of aldosterone, whereas *in vitro* data of rat cultured cardiac fibroblasts provided conflicting results. Measuring ^{3}H-proline incorporation into collagenase sensitive proteins synthesized by cultured cardiac fibroblasts, showed a significant increase in total collagen following exposure to aldosterone. Not only total collagen synthesis changed under the influence of aldosterone but also the synthesis of collagen type I, the major fibrillar collagen in the heart (Brilla *et al*., 1994; Zhou *et al*., 1996). However, in other studies in which the same methodology was used, no statistically significant differences were found (Fullerton & Funder, 1994; Köhler *et al*., 1996). Therefore, it was uncertain whether the pro-fibrogenic effect of aldosterone as observed *in vivo*, was due to a direct effect of the hormone on cardiac fibroblasts, or was the result of indirect, e.g. hypertension-mediated, mechanisms.

In this study we measured *de novo* collagen synthesis by metabolic labelling and immunoprecipitation of collagens type I, III and IV in aldosterone-treated cardiac fibroblasts. This technique allowed the measurement of newly synthesized protein in the presence of a multitude of other proteins (Niki *et al*., 1999; Rombouts *et al*., 2001). Furthermore, the dynamic production of procollagens as well as their processed forms could be quantified.

Mineralocorticoid actions can be mediated by binding of aldosterone to the mineralocorticoid receptor, a member of the steroid/thyroid/retinoid super-family of ligand dependent

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transcription factors (Evans, 1988). This receptor is expressed at the greatest abundance in the sodium-transporting epithelia of the distal colon and distal nephron and at lower abundance in non-epithelial cells in a variety of other tissues such as the cardiovascular and the nervous system (Dekloet *et al*., 1998; Farman *et al*., 1991; Lombes *et al*., 1994). In this study we investigated the expression of the mineralocorticoid receptor by rat cultured cardiac fibroblasts, and the effect of aldosterone on *de novo* collagen synthesis and proliferation.

Methods

Isolation and culture of cardiac fibroblasts

Eight-week-old male Wistar rats (Catholic University Leuven, Experimental Animal Centre, Belgium) were used to obtain cultured adult cardiac fibroblasts as described previously with minor modifications (Crabos *et al*., 1994; Eghbali *et al*., 1991). Rats received a standard diet and water *ad libitum*, and were treated according to the medical sciences guidelines for the care and use of laboratory animals of the Council for International Organisations of Medical Sciences (CIOMS), as required by the Belgian National Fund for Scientific Research. Each experiment was performed on cells derived from at least three rats.

In brief, rats were heparinized (200 units 100 g^{-1} body weight), anaesthetized, and their hearts removed under sterile conditions and placed immediately in sterile Joklik's medium (Gibco, Paisley, Scotland) at 4°C. Using a catheter (size 14 G) and ligature, the hearts were perfused for 5 min *via* the ascending aorta with Joklik's medium (37°C). After this wash-out period, the perfusate was changed to a re-circulating in Ca^{2+}-Joklik's solution containing of 0.1% collagenase (Boehringer, Mannheim, Germany)/2% bovine serum albumin (BSA) (Sigma, St. Louis, MO, U.S.A.). Re-circulation was allowed for 20 min at 5 ml min^{-1}. Thereafter, the heart was disconnected from the apparatus and atria and vessels removed. All ventricular tissue was digested by a 0.05% collagenase/1% BSA solution, under constant shaking at 37°C. After a centrifugation of 10 min, 400 × *g* at 20°C (Merck EuroLab, Leuven, Belgium), the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, U.K.) supplemented with 10% foetal calf serum, 250 μg ml^{-1} amphotericin B and 7 μg ml^{-1} gentamicin (Gibco). Cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. After 2 h, supernatant was aspirated and the adhering cells, mainly fibroblasts, were washed twice with DMEM. This procedure removed non-fibroblast cells that did not adhere so quickly.

Complete medium was replaced every 2–3 days and cells were washed with versene buffer (Gibco). Cells were grown to confluency and were routinely split at a 1 : 3 ratio using 15 ml culture flasks. Cultures were frozen at various passages with 10% dimethylsulphoxide (DMSO), 10% foetal calf serum and stored in liquid nitrogen. For this study cardiac fibroblasts at passage 7–8 were used for immunoprecipitation and proliferation assay. RT–PCR was performed on cardiac fibroblasts of passage 5 and more activated cardiac fibroblasts of passage 10.

Immunocytochemistry of cardiac fibroblasts

Cells were characterized using phase-contrast microscopy and anti α-smooth muscle actin (α-SMA) immunocytochemistry (Adams, 1981). Additional immunocytochemistry of collagens I, III and IV was performed on cells cultured in Chamber Slides Lab-Tek™ (Nunc, Life Technologies Ltd. Paisley, U.K.) as described previously (Geerts *et al*., 1998). Controls consisted of substitution of the primary antibody by normal mouse IgG and omission of the primary antibody. They were invariably negative. Table 1 summarizes the characteristics of the antibodies used in this study.

Treatment of cardiac fibroblasts with aldosterone

Cardiac fibroblasts were exposed to various concentrations of aldosterone ranging from 10^{-7}–10^{-11} M (Brilla *et al*., 1994; Zhou *et al*., 1996) for 24 h in the presence of 50 μg ml^{-1} sodium ascorbate (Merck, Darmstadt, Germany) and 64 μg ml^{-1} β-aminopropionitrile (Sigma, Bornem, Belgium).

Metabolic labelling and immunoprecipitation

After 24 h of exposure to aldosterone or vehicle only, cardiac fibroblasts were metabolically labelled for another 24 h with 25 μCi ml^{-1} Trans ^{35}S-label™ (ICN Biomedicals, CA, U.S.A.) (70% [^{35}S]-methionine, 15% [^{35}S]-cysteine) with or without 10^{-7}–10^{-11} M aldosterone. The labelling medium consisted of methionine-free DMEM supplemented with 10% foetal calf serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} sodium ascorbate and 64 μg ml^{-1}

Table 1 Antibodies used for immunocytochemistry (ICC) and for immunoprecipitations (IP)

	Type	*Source*	*ICC*	*IP*
Primary antibody				
α-SMA	Monoclonal.IgG	Sigma[1]	1/1000	/
Collagen type I	Goat polycl. AP[2]	SBA[3]	1/50	1/40
Collagen type III	Rabbit polycl. AS[4]	(Schuppan *et al*., 1986)	1/100	1/75
Collagen type IV	Goat polycl. AP	SBA	1/50	1/75
Secondary antibody				
Rabbit IgG	Goat polycl. AP	Jackson[5]	1/500	1/500
Goat IgG	Rabbit polycl. AS	Jackson	1/500	1/100
Mouse IgG	Goat polycl. AP	Amersham[6]	1/100	1/100

[1]Sigma Chemie, Bornem, Belgium; [2]affinity purified; [3]Southern Biotechnology Associates; [4]antiserum; [5]Jackson, West grove (PE) U.S.A.; [6]Amersham, Little Chalfont, U.K.

β-aminopropionitrile. Cell layer and medium were collected separately as described previously (Niki *et al*., 1996). The total incorporation of Trans ^{35}S-label™ into protein was determined by the hot trichloroacetic acid (TCA) precipitation method (Niki *et al*., 1996). Labelled medium and cell layer were then subjected to immunoprecipitation as described previously, using antibodies as summarized in Table 1. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis was performed at 150 V constant voltage using a Mini PROTEIN II Electrophoresis Cell System (BioRad, Hercules, CA, U.S.A.). Gels were immersed in Amplify (Amersham, Little Chalfort, U.K.), dried and exposed at −70°C to preflashed Hyper-film MP (Amersham). The results were quantified by Phosphor Imaging technology using Molecular Analyst™ software (BioRad) (Niki *et al*., 1996; Rombouts *et al*., 2001).

Proliferation assay

To investigate whether aldosterone has an effect on DNA synthesis of cardiac fibroblasts we performed an immunoassay, based on the measurement of BrdU into DNA synthesis (Boehringer, Mannheim, Germany). Preliminary experiments showed that a cell density of 6000 cells/well, with a BrdU-labelling time of 10 h was the optimal condition to examine the effect of aldosterone on the proliferation rate of cardiac fibroblasts. Subconfluent cultures of 6000 cells/well were treated with different concentrations of aldosterone for 24 h in the presence of 10% FCS. Foetal calf serum was required for survival and proliferation of cardiac fibroblasts. After 24 h of aldosterone treatment, cells were incubated for 10 h with BrdU in the presence of aldosterone. BrdU incorporation was measured performing BrdU ELISA. Colour development was measured using a UV-VIS 3550 microplate reader (450–690 nm) (Bio-Rad Laboratories, Nazareth, Belgium).

Detection of the mineralocorticoid receptor

Sample preparation RNA was extracted from cultured rat cardiac fibroblasts (passage 5) and more activated cardiac fibroblasts (passage 10) using RNeasy Mini Kit (Qiagen, Hilden, Germany). As positive controls we used RNA extracted from tissue homogenates of brain and kidney (Köhler *et al*., 1996).

Primer selection Primers for mineralocorticoid receptor cDNA amplification were selected from the published sequence for rat mineralocorticoid receptor (Köhler *et al*., 1996). Selected primers were located near the 3′-end of the coding region (5′-end primer cctgctctccacgctcaaccg and 3′-end primer gcagctggtcggtgatgatctcc). The primers yielded a 631-bp-long PCR product from both rat mineralocorticoid receptor cDNA (EMBL database M3607 (Patel *et al*., 1989) and human mineralocorticoid (EMBL database M16801 (Arriza *et al*., 1987)). To check for contamination with chromosomal DNA in the cDNA preparation, β actin was amplified using 5′-primer agagccaccaatccacacaga and 3′-primer actatcggcaatgagcggttc (EMBL database V01217 and J00691). These primers recognized sequences in different exons of the actin gene.

PCR amplifications RT–PCR was performed according to the manufacturer's manual (Perkin Elmer, New Jersey, U.S.A.). All PCR reactions were performed in a total volume of 25 μl. The cDNA was first denatured at 94°C for 5 min, followed by PCR for 35 cycles, each consisting of a denaturation step (94°C, 1 min), and annealing step (60°C, 1 min) and a primer extension step (72°C, 1 min). At the end of the 35th cycle, the reaction mixture was kept at 70°C for 10 min for final elongation.

Statistics

The ratio of [^{35}S]-methionine/[^{35}S]-cysteine incorporation into protein of treated cells over control cells – hereafter referred to as treated/control ratio – was calculated for each concentration of aldosterone and expressed as mean ± s.e.mean. At least three independent observations (cells of three different rats) were used to calculate the mean. The results are presented as bar graphs with the control values normalized at 100. Immunoprecipitation was performed on an aliquot of medium containing 5.0×10^5 or 1.0×10^6 c.p.m. This aliquot was a known fraction of the radioactivity in medium.

To determine the statistical significance of the difference between control and aldosterone-treated cells, 95% confidence intervals were calculated. The effect was considered statistically significant, when 100 (normalized control value) did not belong to the 95% confidence interval of the treated/control ratio. We also calculated the 90% confidence interval for each treated/control ratio. If 100 did not belong to the 90% interval, we considered this ratio to show a tendency towards change (Niki *et al*., 1999; Rombouts *et al*., 2001).

Results

Morphological characterization of cardiac fibroblasts

Cells were characterized by phase contrast microscopy and immunocytochemistry. Cultured cells had the typical characteristics of cardiac fibroblasts. They were spindle-shaped with centrally located well defined nucleus, growing in monolayer (Figure 1a). These observations excluded the presence of vascular smooth muscle cells that are bigger, growing in multiple layers, and endothelial cells which grow in a 'cobblestone' pattern (Figure 1b). The cardiac fibroblast origin of cultured cells was further demonstrated by the expression of α-SMA (Figure 2a).

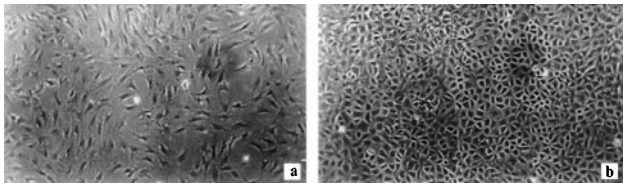

Figure 1 Phase contrast microscopy of cardiac fibroblasts. (a) Cultured cardiac fibroblasts are spindle-like shaped and grow in a typical monolayer. (b) Culture of cardiac endothelial cells. The cell population exhibits the typical 'cobblestone' appearance. Phase contrast microscopy shows a clear morphological difference between the two cell types. Magnification ×150.

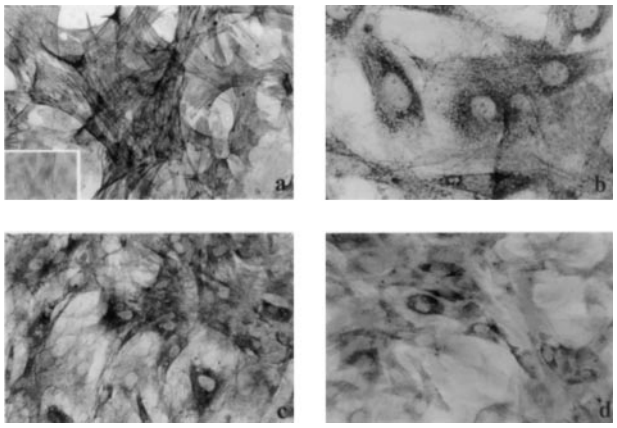

Figure 2 Immunocytochemistry of cardiac fibroblasts at passage 7. (a) Cultured rat cardiac fibroblasts after several passages have become strongly positive for α-SMA which is a sign of activation. α-SMA is present in bundles of microfilaments. The insert of (a) shows negative control cells. (b–d) Presence of collagen type I (b), collagen III (c) and collagen IV (d) in cardiac fibroblast cultured on plastic. Most of the staining for collagens I, III and IV was concentrated around the nucleus in vesicular structures. Magnification ×200.

Immunocytochemistry with antibodies to collagens type I, III or IV revealed that cardiac fibroblasts contained these proteins (Figure 2b–d). Immunoreactive collagens were present in small vesicles concentrated in the perinuclear cytoplasm. This pattern was compatible with collagens being synthesized in the rough endoplasmic reticulum, and processed in the Golgi apparatus. The majority of cardiac fibroblasts stained positive for collagens type I and III (Figure 2b,c). Some cells, however, were only faintly labelled. Collagen type IV immunoreactivity was less prominent (Figure 2d).

Immunoprecipitations

Cultured cells were exposed to aldosterone in pharmacological (10^{-7}–10^{-8} M), near-physiological (10^{-9} M) or physiological (10^{-10}–10^{-11} M) concentrations for 2×24 h prior to analysis. During the last 24 h of treatment, the cells were metabolically labelled with [^{35}S]-methionine/[^{35}S]-cysteine. Preliminary experiments on cardiac fibroblasts confirmed that 80–90% of *de novo* synthesized procollagens were secreted into the cell culture media, similar to previous experiments in hepatic stellate cells (data not shown) (Niki *et al*., 1999; Rombouts *et al*., 2001). Therefore, conditioned media were collected, processed and subjected to immunoprecipitations with specific antibodies to procollagens type I, III or IV. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and quantified by phosphor imaging.

Representative polyacrylamide gels of immunoprecipitates are shown in Figure 3. Procollagen type I was detected as several bands, i.e. chains of procollagen α1(I) and α2(I), and the N-terminally processed pCα1(I) and pCα2(I) (Figure 3a). In the medium, intact procollagen was predominant and only little fully processed collagen was present. Visual inspection of the procollagen type I immunoprecipitates showed a modestly suppressed signal in 10^{-7} M aldosterone-treated cells as compared to control cells. However, in cells exposed to aldosterone at concentrations ranging from 10^{-8}–10^{-11} M, a slightly elevated signal was observed (Figure 3a). Procollagen type III was visible as proα1(III) and the C-terminally processed pNα1(III) (Figure 3b). Due to some co-reaction from fibronectin, the antiserum to procollagen III precipitated an extra band of fibronectin (upper band). The latter did not disturb the interpretation of the data. Signals of procollagen type III immunoprecipitates showed a similar tendency i.e. suppression at 10^{-7} M aldosterone and stimulation at lower concentrations (Figure 3b). The immunoprecipitates of procollagen type IV consisted of two closely spaced chains: proα1(IV) and proα2(IV). Again, incubation of cells with 10^{-7} M aldosterone led to a decrease of procollagen IV immunoprecipitable counts. Lower concentrations of aldosterone had no effect (Figure 3c).

In keeping with our visual impression, quantification of the radioactivity in the immunoprecipitated procollagens by phosphor imaging and calculation of the immunoprecipitable counts per 10^6 cells showed that procollagen type I synthesis was inhibited by 35% at 10^{-7} M aldosterone ($P<0.05$). Lower aldosterone concentrations, immunoprecipitable counts were slightly increased (Figure 3d) ($P<0.05$). 10^{-7} M aldosterone slightly decreased immunoprecipitable procollagen type III (11%) ($P<0.1$). At lower concentrations of aldosterone (10^{-8}–10^{-10} M) expression of procollagen type III was increased (16–51%) ($P<0.1$) (Figure 3e). The amount of immunoprecipitable counts of procollagen type IV was inhibited by 42% ($P<0.05$) at 10^{-7} M aldosterone. Lower concentrations of aldosterone had no effect (Figure 3f).

DNA synthesis

In order to investigate the effect of aldosterone on cellular proliferation of cardiac fibroblasts, cells were treated with different concentrations of aldosterone for 24 h, followed by incubation with BrdU in the presence of aldosterone for 10 h. Longer incubation of the cells with BrdU did not change the outcome of the results. The amount of BrdU incorporation was measured by ELISA. Figure 4 summarized the obtained results. Data were given as mean±s.e.mean of each concentration of aldosterone. Aldosterone had no demonstrable effect on cellular proliferation.

Detection of the mineralocorticoid receptor

Since collagen synthesis by cardiac fibroblasts appeared to be moderately sensitive to aldosterone we investigated whether these cells expressed the mineralocorticoid receptor. Following RT–PCR experiments, we concluded that cardiac fibroblasts (passage 5 and 10) express the mineralocorticoid receptor (Figure 5a). These results were obtained when 35 PCR cycles were applied. All amplicons showed the correct length (631 bp). By sequencing we confirmed the identity of the amplicons (rat mineralocorticoid receptor; EMBL database: Acc. No. M36074). cDNA samples were also subjected to PCR using β actin specific primers selected in different exons of the β actin gene. We obtained amplicons of the expected length of 300 bp, indicating that these products originated solely from cDNA (Figure 5b). If genomic DNA had been present, amplicons of 500 bp should also have been formed. The latter was not the case.

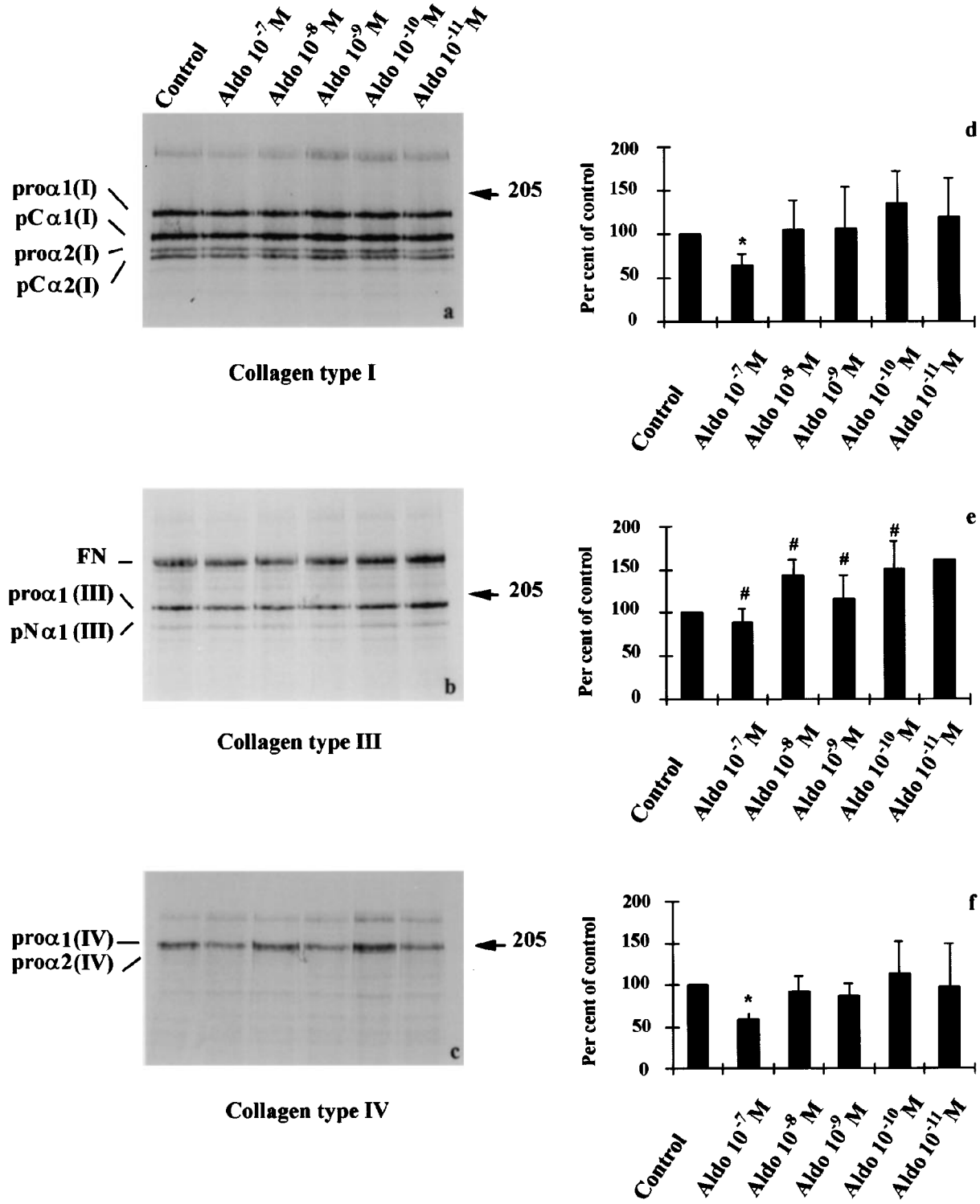

Figure 3 The effect of aldosterone on *de novo* synthesis by cardiac fibroblasts. Cells were treated with different concentrations of aldosterone ($10^{-7}-10^{-11}$ M) and metabolically labelled with [^{35}S]-methionine/[^{35}S]-cysteine. Each protein was analysed by immunoprecipitation with adequate amounts of specific antibodies. (a–c) Show autoradiographs of representative polyacrylamide gels containing immunoprecipitates. (d,f) Show the relative immunoprecipitable radioactivity per 10^6 cells in control and aldosterone treated cells. Radioactivity in the immunoprecipitates was quantified by Phosphor Imaging (Bio-Rad). Within each experiment, the control values were normalized and set at 100. The other data were recalculated accordingly. The ratios of immunoprecipitated radioactivity in treated samples over immunoprecipitated radioactivity in control samples were calculated for each concentration of aldosterone. The bars represent mean values ± s.e.mean of three separate experiments. The arrowhead to the right of the panels shows the position of the molecular weight marker (myosin, 205 kD). Asterisks indicate when immunoprecipitable counts per 10^6 cells of aldosterone-treated cultures differ significantly from those of control cultures: $*P<0.05$. If 100 did not belong to the 90% confidence interval, but belonged to the 95% confidence interval, we considered this ratio to show a tendency towards change: $\#P<0.1$.

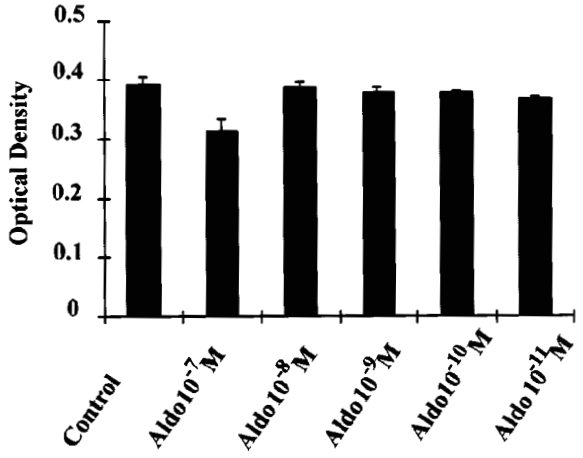

Figure 4 Cultured cardiac fibroblasts (6000 cells/well) were incubated with different concentrations of aldosterone ($10^{-7}-10^{-11}$ M) for 24 h. Cells were treated for an additional 10 h with aldosterone in the presence of BrdU. After performing a BrdU ELISA, optical density was measured. We found no effect of aldosterone on the proliferation activity of cardiac fibroblasts. Results represented the mean of three independent experiments. Each experimental condition was measured in triplicate.

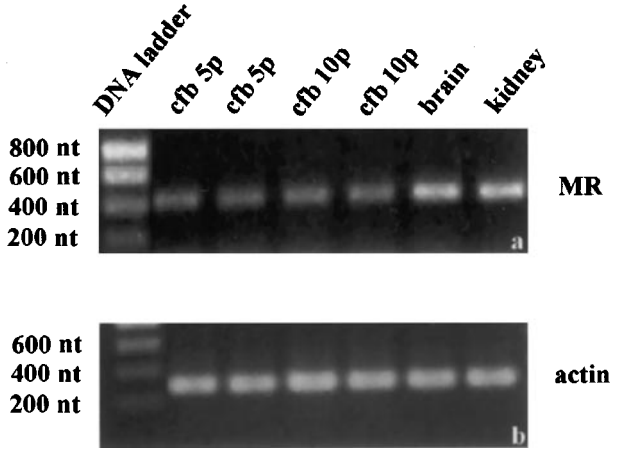

Figure 5 Amplification of mineralocorticoid receptor mRNA by RT–PCR. cDNA derived from cardiac fibroblasts passage 5 (p5) and more activated cardiac fibroblasts (p10), brain and kidney was subjected to PCR using primers specific for the mineralocorticoid receptor and β actin. (a) Shows presence of mineralocorticoid receptor (MR) expression by cardiac fibroblasts. Brain and kidney were used as positive controls. cDNA amplification yielded 631 bp amplicons. (b) Shows no contamination of chromosomal DNA in the cDNA preparation. If chromosomal DNA had been present, amplicons of 500 bp should also have been formed; the latter was not the case since only amplicons of 300 bp were observed by performing PCR for β actin.

Cytotoxicity

To examine a possible cytotoxic effect of aldosterone on cardiac fibroblasts, we measured *de novo* synthesis of total secreted proteins by hot tricholoroacetic acid (TCA) precipitation (Hellemans *et al*., 1999; Niki *et al*., 1999; Rombouts *et al*., 2001). Cells were treated for 2×24 h with different concentrations of aldosterone and incubated with [^{35}S]-methionine/[^{35}S]-cysteine in the last 24 h. Figure 6 shows

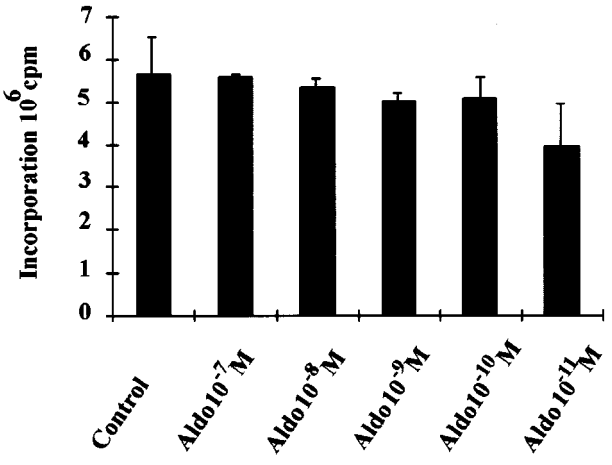

Figure 6 Cardiac fibroblasts were treated with different concentrations of aldosterone for 24 h. Cells were treated for an additional 24 h with aldosterone and metabolically labelled with [^{35}S]-methionine/[^{35}S]-cysteine. Subsequently, media were collected and TCA precipitation was performed to measure incorporation of [^{35}S]-labelled amino acids into total secreted protein, a dynamic parameter used to evaluate potential cytotoxicity of added compounds. Exposure to aldosterone did not significantly alter incorporation of labelled amino acids into total secreted protein.

that incorporation of [^{35}S]-methionine/[^{35}S]-cysteine into secreted protein was not altered significantly, excluding major differences in synthesis due to cytotoxicity.

Discussion

Hyperaldosteronemia has been shown to exert a pro-fibrogenic effect on the heart (Brilla *et al*., 1993; Weber *et al*., 1994). Prolonged elevated levels of aldosterone lead to cardiac fibrosis in patients and in experimental animals (Funck *et al*., 1997; Young *et al*., 1994). How aldosterone brings about these pro-fibrogenic effects is still uncertain.

Previous *in vitro* reports on the influence of aldosterone on collagen synthesis by cardiac fibroblasts gave rise to conflicting results (Fullerton & Funder, 1994; Köhler *et al*., 1996; Zhou *et al*., 1996). Possibly, these divergent results were due to the method used. Measuring incorporation of ^{3}H-proline into collagenase sensitive proteins (Takahashi & Mao-Jung, 1987) has three major disadvantages: (1) this method does not provide information on individual collagen types, (2) several non-collagenous proteins such as collectins (Hansen & Holmskov, 1998), complement factors C3 and C1q (Stuart *et al*., 1997), and acetylcholinesterase (Colley & Baenziger, 1987; Eghbali *et al*., 1988) contain short collagenous stretches that will also be degraded by collagenase, (3) minor contamination of the collagenase used with other proteinases may yield falsely high collagen amounts.

In this study we used a more specific method to measure *de novo* synthesis of single procollagens (Niki *et al*., 1999; Rombouts *et al*., 2001) based on labelling cells with [^{35}S]-methionine/[^{35}S]-cysteine, followed by quantitative immunoprecipitation of the protein under investigation and measuring radioactivity in the immunoprecipitate by phosphor imaging. Using this method, we have reinvestigated whether exposure of cultured cardiac fibroblasts to aldosterone led to increased synthesis of collagens that are predominant in

fibrotic heart muscle (Weber & Brilla, 1991; Zeydel *et al*., 1991). Surprisingly, we found that 10^{-7} M aldosterone inhibited significantly synthesis of procollagens type I and IV with a tendency towards inhibition for procollagen type III. In contrast, lower concentrations of aldosterone (10^{-8} M – 10^{-10} M) showed a tendency to enhance procollagen type III synthesis.

A possible explanation for decreased collagen synthesis at 10^{-7} M aldosterone could be binding of the hormone at high concentrations to the glucocorticoid as well as the mineralocorticoid receptor. It is well known that activation of the glucocorticoid receptor in skin fibroblasts by corticosterone or dexamethasone inhibits collagen synthesis (Verbruggen & Salomon, 1980; Verbruggen & Abe, 1982; Verbruggen *et al*., 1983). However, alternative mechanisms should also be considered because Fullerton & Funder (1994) have shown that 10^{-7} M aldosterone did not cross-react with the glucocorticoid receptor in cardiac fibroblasts. Aldosterone might also induce non-genomic effects, i.e. effects unrelated to the mineralocorticoid receptor (Christ *et al*., 1993; Trapp & Holsboer, 1996; Wehling *et al*., 1991). These effects have been well documented in four different types of studies: (1) canrenone, known as a specific mineralocorticoid receptor antagonist, was unable to inhibit these non-genomic effects of aldosterone (Wehling *et al*., 1991), (2) Haseroth *et al*. (1999) showed in mineralocorticoid-receptor-knockout mice the presence of aldosterone short-term effects, (3) recent studies showed the existence of putative membrane receptors with a high affinity for steroids (Falkenstein *et al*., 1996; Schmidt *et al*., 1998), (4) one of the best described aldosterone short-term cellular responses is the activation of the Na^+/H^+ exchanger in cultured vascular smooth muscle cells and human mononuclear leukocytes.

The tendency towards increased procollagen type III synthesis at 10^{-8} – 10^{-10} M aldosterone, might be due to the activation of the Na^+/H^+ exchanger. In human mononuclear leukocytes and vascular smooth muscle cells, aldosterone-induced activation of the Na^+/H^+ exchanger leads to changes of sodium, potassium and calcium concentrations, and cell swelling (Wehling *et al*., 1991). These observations proved that aldosterone can lead to mineralocorticoid receptor-unrelated functional changes. Svegliati Baroni and colleagues have demonstrated that in rat hepatic stellate cells, activation of the Na^+/H^+ exchanger induces a decrease in intracellular pH which leads to increased proliferation and collagen synthesis. This induction could be inhibited by the selective inhibition of Na^+/H^+ exchange by amiloride (Di Sario *et al*., 1997; 1999; Svegliati-Baroni *et al*., 1999). It is conceivable that aldosterone stimulates the Na^+/H^+ exchanger in cardiac fibroblasts which is followed by the observed slight increase of procollagen synthesis. The observed stimulation of procollagen type III synthesis at 10^{-8} – 10^{-10} M, but its tendency towards inhibition at 10^{-7} M, indicates that two or more counteracting effects could occur simultaneously with each of these effects having a maximal effect at different concentrations. The involvement of matrix metalloproteinase 1 (MMP 1) in the observed tendency to increase collagen synthesis is unlikely, for it is known that aldosterone has no effect on cardiac fibroblast-mediated collagen degradation through this MMP 1 (Funck *et al*., 1997). The effect of aldosterone on other MMP and on the tissue inhibitors of metalloproteinases (TIMP) needs further investigation.

We performed RT – PCR to establish whether the mineralocorticoid receptor was expressed in cardiac fibroblasts. In cardiac fibroblasts at passages 5 and 10, we demonstrated clearly the presence of the mineralocorticoid receptor mRNA. Our data confirm those obtained by Köhler *et al*. (1996).

The observed tendency to stimulation of procollagen type III synthesis by 10^{-8} – 10^{-10} M aldosterone (16% – 51 %) is modest in comparison to the induction of extracellular matrix synthesis by pro-fibrogenic factors such as TGF-β_1. When treating cardiac fibroblasts with 10 – 15 ng/ml TGF-β_1 the mRNA steady state levels of procollagen chains of proα2(I) and proα1(III) collagens were increased by 112% and 97% respectively (Eghbali *et al*., 1991). Similarly, TGF-β_1 increased procollagen type I mRNA steady state levels by 60 – 450% in skin fibroblasts (Raghow *et al*., 1987; Reed *et al*., 1994) and 3 – 6 fold in cultured hepatic stellate cells (Garcia-Trevijano *et al*., 1999; Weiner *et al*., 1989).

In this study we also investigated the possible mitogenic effect of aldosterone on cardiac fibroblasts. Our data confirm those obtained by Köhler *et al*. (1996) and Brilla *et al*. (1994) who found no stimulatory effect of aldosterone on cardiac fibroblasts DNA synthesis.

In conclusion, contrary to expectation, aldosterone at high concentration (10^{-7} M) suppresses procollagen I, III and IV synthesis by cultured rat cardiac fibroblasts. At 10^{-8} M or lower, aldosterone shows a tendency to increase synthesis of procollagen type III but synthesis of procollagens I and IV are unaltered. Because in injured heart tissue activated fibroblasts are the principal cellular sources of extracellular matrix, it is not likely that the pro-fibrogenic effect of aldosterone in hyperaldosteronemia *in vivo*, can be explained by a direct fibrogenic effect on the cardiac fibroblasts.

We are grateful to Kit Van Den Berg and J.M. Lazou (Laboratory for Molecular Liver Cell Biology, Faculty of Medicine and Pharmacy, Free University of Brussels, Belgium) for their technical assistance. Krista Rombouts is a research fellow of the 'Vlaams Instituut voor bevordering van het wetenschappelijk onderzoek in de industrie' (IWT, Contract 96.13.30). This study was supported by FWO-V (Fonds voor Wetenschappelijk Onderzoek Vlaanderen) Grants no. G. 004496 and 1.5.618.98 and by Searle Continental Pharma Inc. Brussels, Belgium.

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(Received February 13, 2001
Revised May 11, 2001
Accepted June 21, 2001)