Aldosterone rapidly activates Src kinase in M-1 cells involving the mineralocorticoid receptor and HSP84

Sabine Braun, Ralf Lösel, Martin Wehling, Brigitte Boldyreff*

Department of Clinical Pharmacology, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Received 25 March 2004; revised 7 June 2004; accepted 8 June 2004

Available online 22 June 2004

Edited by Richard Marais

Abstract We investigated the effect of aldosterone on Src kinase. In the kidney cell line, M-1 aldosterone leads to a >2-fold transient activation of Src kinase seen as early as 2 min after aldosterone administration. Maximal Src kinase activation was measured at an aldosterone concentration of 1 nM. In parallel to activation, autophosphorylation at Tyr-416 of Src kinase increased. Src kinase activation was blocked by spironolactone. Aldosterone led to increased association of Src with HSP84. Furthermore, rapamycin blocked aldosterone-induced Src activation. We conclude that Src activation by aldosterone is mediated through the mineralocorticoid receptor and HSP84.
© 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Aldosterone; Signaling; Non-genomic; Src kinase; Spironolactone; Heat shock protein

1. Introduction

Like all steroid hormones, aldosterone has a dual mechanism of action. It affects the transcriptional modulation of target genes via the intracellular mineralocorticoid receptor, but also elicits rapid non-genomic effects on second messenger systems and signaling cascades similar to those initiated by peptide hormones or catecholamines (for review see [1]). However, the signaling cascade and in particular the initiating step of rapid aldosterone signaling is rather unclear. A large body of evidence points to a membrane aldosterone receptor different from the mineralocorticoid receptor, since most described rapid effects could not be blocked by the MR inhibitor spironolactone. In the case of other steroid hormones, most reports indicate that the classical intracellular hormone receptors are responsible for rapid signaling. Rapid progesterone, androgen, estrogen and vitamin D signaling starts with activation of Src kinase, which is achieved by direct interaction of the steroid–steroid hormone receptor-complex with Src [2–4]. Whether such a mechanism also applies to rapid aldosterone signaling is so far unknown. The only report dealing with Src kinase and rapid mineralocorticoid signaling stems from Krug et al. [5]. The authors show indirect proofs for Src kinase activation in Chinese hamster ovary cells which became aldosterone responsive upon transfection with the epidermal growth factor receptor.

In this study, we wanted to know whether Src kinase is involved in rapid aldosterone signaling in the kidney cell line M-1. M-1 cells were chosen, since they serve as a model system for cortical collecting duct cells which are aldosterone target cells [6]. There is increasing evidence that aldosterone exerts rapid effects in the kidney playing a role in modulation of ion channel and transporter activity and thereby influencing renal transport (for review see [7]). In M-1 cells, aldosterone produced a rapid increase in intracellular Ca^{2+} concentrations [8]. In a cell line with properties similar to M-1 cells, Madin–Darby canine kidney cells (MDCK), Gekle et al. demonstrated rapid activation of Na^+/H^+ exchange which required Ca^{2+} and rapid increase of plasma membrane conductance by aldosterone [9] and that aldosterone uses the EGF-R-ERK1/2 signaling cascade to elicit its rapid effects in MDCK cells [10]. Furthermore, as already mentioned proofs exist that Src kinase is involved in aldosterone caused EGF-R activation [5], i.e., inhibition of Src prevented the effect of aldosterone on downstream signaling. Since aldosterone was shown to induce a left-shift in the dose–response curve of EGF, which is known to be a highly potent cellular proliferation factor, not only ion transport, but also cell proliferation might be modulated by aldosterone.

We show that aldosterone rapidly leads to Src kinase activation in M-1 cells, analyze the time course and dose–response of this effect and characterize the mechanism involved by checking the phosphorylation status of Src, identifying Src-associated proteins and examining the effect of spironolactone and rapamycin.

2. Materials and methods

2.1. Tissue culture

The M-1 cell line (European Collection of Cell Cultures, Salisbury) was cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (Gibco), supplemented with 10% FCS, 0.5% penicillin/streptomycin and 2 mM L-glutamine. Before treatment with various substances, cells were grown to subconfluence and serum-starved for 2 h in PSS (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES and 5.5 mM glucose, pH 7.4). Aldosterone (Fluka), 17β-estradiol (Sigma) or EGF (Sigma) was directly added to PSS and incubated for 5 min when not otherwise indicated. Control treatment was done with the solvent ethanol (J.T. Baker) for aldosterone and 17β-estradiol, or with DMEM/F-12 for EGF. For inhibitor treatment, the inhibitors were directly added to PSS 15 min prior to the addition of aldosterone (PP2 and PP3, Calbiochem, dissolved in DMSO; spironolactone, Sigma, dissolved in ethanol) or added simultaneously with aldosterone

* Corresponding author. Present address: KinaseDetect ApS, Stationsvej 80, DK-5792 Aarslev, Denmark. Fax: +45-65-99-26-40.
E-mail address: boldy@kinasedetect.dk (B. Boldyreff).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.031

(rapamycin, Sigma, dissolved in ETOH). Concentrations of the different substances added are given in the figure legends.

2.2. Src kinase immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF and 1 Complete Minitablet EDTA free, Boehringer–Mannheim, per 10 ml) by rotating at 4 °C and centrifuged (15 min, $20\,800 \times g$, 4 °C). Lysates (1 μg/μl) were precleared with 100 μl protein G agarose (Boehringer–Mannheim)/ml. Supernatants were incubated with anti-src, clone GD11, agarose (Upstate Biotechnology) or protein G-agarose at a concentration of 4 μg/150 μg protein lysate overnight at 4 °C. Immunoprecipitates were washed three times with PBS.

2.3. Identification of Src-associated proteins

25 mg lysate obtained from 25×10 cm petri dishes of cells treated with aldosterone (10^{-9} M, 5 min) was, after preclearing, immunoprecipitated with 50 μg anti-src, clone GD11, agarose, separated by SDS–PAGE and stained with Coomassie blue. Bands were excised and reduced/alkylated by sequential treatment with DTT and iodoacetamide. Proteolysis was performed using modified trypsin (Promega) at 37 °C for 4 h with final cleanup on C18 ZipTips (Millipore). The digests were spotted on a stainless steel target in cyanohydroxycinnamic acid as matrix. Peptide mass spectra were collected on a BiflexIII (Bruker) in the reflector mode. Protein identification was done with the Mascot search engine (www.matrixscience.com).

2.4. Western blotting analysis

Immunoprecipitates from 250 μg lysate were separated by 10% SDS–PAGE and blotted. Western blots were incubated with the following antibodies: anti-c-src (Santa Cruz), anti-phospho-src (Tyr416), anti-phospho-src (Tyr527) (Cell Signaling) and anti-HSP84 (Dianova). Detection was done using Western blotting detection reagent (Amersham).

2.5. Src kinase assay

Immunoprecipitates from 150 μg lysate were washed twice with Src kinase reaction buffer (100 mM Tris–HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.25 mM sodium orthovanadate and 2 mM DTT) and reactions were carried out using the Src Kinase Assay Kit (Upstate Biotechnology). For inhibitor studies, PP2 or PP3 was added to the immunoprecipitates and incubated 15 min prior to Src kinase assay.

2.6. Data evaluation

Mean values and standard deviations of Src kinase activities were calculated from at least three independent experiments. Data were analyzed by an unpaired *t*-test, one-sampled *t*-test or ANOVA where appropriate.

3. Results and discussion

3.1. Src kinase is activated upon treatment of M-1 cells with aldosterone

M-1 cells, a model system for aldosterone responsive cells of the cortical collecting duct, were treated with pharmacological doses of aldosterone. Src kinase was immunoprecipitated from total cell lysates and assayed for kinase activity (Fig. 1A). Control treated cells displayed a basal Src activity of 0.37 pmol/min per 150 μg immunoprecipitated lysate. Aldosterone treatment led to a stimulation of Src kinase activity by a factor of 2.42 ± 0.69, which was statistically highly significant ($P = 0.003$). In cell lysates incubated with protein G, neither Src kinase was precipitated nor activity was measurable and in kinase reactions without added specific substrate negligible activity was measured. These results indicate the specificity of the used anti-Src antibody and the Src kinase activity test.

Further proof that indeed Src kinase activity was measured came from experiments with the Src kinase inhibitor PP2 (Fig. 1B). When immunoprecipitates from aldosterone stimulated cells were pretreated with PP2 no activation of Src kinase was measured, whereas in reactions carried out with the unactive compound PP3 the stimulatory aldosterone effect was

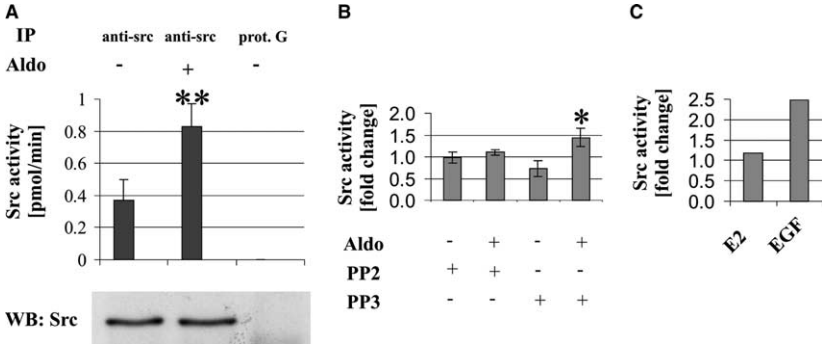

Fig. 1. (A) Effect of aldosterone (10^{-9} M) on Src kinase activity. Src was immunoprecipitated and activity tests were performed. Control immunoprecipitation was done using protein G agarose. In parallel, Src immunoprecipitates were analyzed by Western blotting using an anti-Src antibody. (B) Effect of PP2 and PP3 (10^{-7} M) on aldosterone-induced Src kinase activity. (C) Effect of 17β-estradiol and EGF (both 10^{-9} M) on Src kinase activity. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

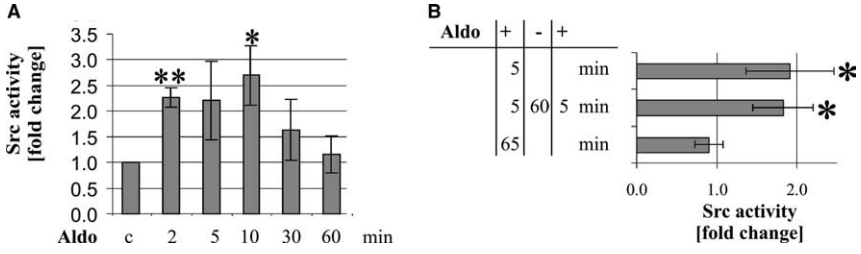

Fig. 2. (A) Time dependency of aldosterone (10^{-9} M)-induced Src kinase activation. (B) Sensitivity of aldosterone-induced Src kinase activation towards repeated stimulation. Aldosterone was added for 5 min, 2×5 min with a wash out phase of 60 min, and 65 min, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

not inhibited. The same results were obtained when cells instead of immunoprecipitates were pretreated with PP2 or PP3 prior to aldosterone administration (not shown), i.e., the inhibitory effect of PP2 in vitro was also obtained in vivo.

To determine the specificity of the effect of aldosterone, we assessed the effect of 17β-estradiol (Fig. 1C). 17β-Estradiol could not activate Src kinase. On the other hand, EGF, a growth hormone known to act on cortical collecting duct cells via tyrosine kinases [11], did cause an activation.

The data are the first direct proof that aldosterone leads to Src kinase activation in a cellular system, known to be responsive to aldosterone. The approximately 2-fold Src kinase activation after treatment of cells with 1 nM aldosterone found here is comparable to what can be achieved by most other steroid hormones, however, at a 10 times higher concentration [2,3].

3.2. Time dependence of aldosterone-induced Src kinase activation

Significant changes of Src kinase activity were obtained upon addition of aldosterone over the range of 2–60 min (overall $P = 0.0068$, ANOVA). The result is consistent with a non-genomic activation of Src by aldosterone. Although we did not test whether inhibitors of transcription or translation affect the aldosterone stimulated Src activation, the rapidity of the activation, seen already 2 min after aldosterone administration, excludes a genomic mechanism. The activation is only transient, as seen similarly for other rapid steroid hormone effects as well [12]. However, when aldosterone was washed out after administration of 5 min and applied a second time after a 60-min recovery period, cells become again sensitive (Fig. 2B).

3.3. Aldosterone leads to Src activation in a dose-dependent manner by increasing its autophosphorylation

The aldosterone effect on Src kinase activity was dose-dependent (Fig. 3). It was maximal at an aldosterone concentration of 10^{-9} M (2.56-fold ± 0.74). Higher concentrations led to the same or slightly reduced increase in Src kinase activity (Fig. 3). The dose dependency of the aldosterone-induced rapid Src kinase activation is similar to dose dependency of other rapid aldosterone effects [13] and the half maximal effect occurred at physiological aldosterone concentrations of 0.1–1 M aldosterone [14].

We further analyzed the phosphorylation state of Src at the two tyrosine residues at positions 416 and 527. Phosphorylation at Tyr-416 is required for enzymatic activity. Phosphorylation at Tyr-527 causes adoption of an inactive conformation [15]. Src phosphorylated at Tyr-416 increased in parallel to the activity with increasing aldosterone concentrations, whereas the phosphorylation status of Tyr-527 did virtually not change (Fig. 2B). Hence, Src activated by aldosterone treatment is phosphorylated at both tyrosine residues, Tyr-416 and Tyr-527. According to the model of Src activation developed by Superti-Furga [15], association of Src with other proteins overcomes the inactive conformation despite a phosphorylated Tyr-527. Our results indicate therefore that aldosterone induces phosphorylation of Tyr-416 and association of Src with other proteins, resulting in an active Src kinase.

3.4. Aldosterone-induced Src kinase activation is consistent with mineralocorticoid receptor participation

Next, we asked the question whether it is a direct interaction between Src kinase and the mineralocorticoid receptor which

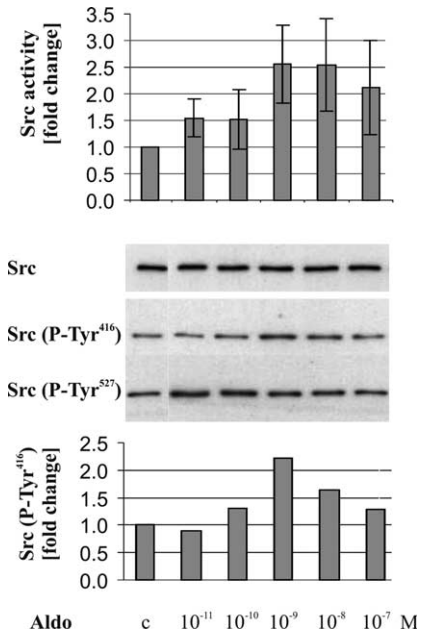

Fig. 3. Dose dependency of aldosterone-induced Src kinase activation. Src was immunoprecipitated, activity tests and Western blotting analyses were done. Representative Western blots using anti-Src, anti-Src (P-Tyr416) and anti-Src (P-Tyr527) antibodies are shown. The signals of the anti-Src (P-Tyr416) Western blot were densitometrically analyzed and are shown as fold change vs. control.

leads to Src activation as it is known for the androgen, estrogen and progesterone receptor. However, in Src immunoprecipitates of cells treated with aldosterone, no MR was detectable using Western blotting analysis and a MR specific antibody (not shown). This does not necessarily mean that MR is not involved. Therefore, we tried a second approach, namely treatment of cells with the MR antagonist spironolactone. Spironolactone prevented the aldosterone-induced Src kinase activation completely (Fig. 4).

For most rapid aldosterone effects described so far the involvement of the classical mineralocorticoid receptor could be ruled out, since spironolactone did not block these effects. Yet, in a few publications sensitivity of rapid aldosterone effects towards spironolactone is reported [16,17]. Apparently, there seem to be at least two different pathways for rapid aldosterone action as identified by the effect of sensitivity towards spironolactone. This is not uncommon if compared with other steroids; e.g., estrogens have been demonstrated to expose rapid actions which are mostly sensitive to the "classic" inhibitor ICI 182,780. Some reports on rapid actions fail to demonstrate that sensitivity, however [18].

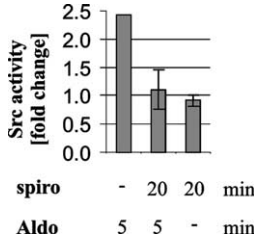

Fig. 4. Sensitivity of aldosterone (10^{-9} M)-induced Src kinase activation towards spironolactone (10^{-6} M).

3.5. Src kinase associates with HSP84 upon aldosterone treatment

In order to elucidate the mechanism by which aldosterone leads to Src kinase activation, we analyzed Src-associated proteins in Src immunoprecipitates by MALDI-MS. Among the identified proteins was HSP84, the mouse homolog of human heat shock protein 90β. HSP84 was identified with a Mowse score of 76 (significance limit = 62) and 15% sequence coverage. The mean error in mass accuracy was 34 ppm. The apparent molecular weight observed in SDS–PAGE corresponded to the calculated mass. The identity of the protein band was further confirmed using anti-HSP84 antibody.

Association between Src and HSP84 increased upon treatment of cells with aldosterone; no increase was seen when cells were preincubated with spironolactone, indicating the involvement of the mineralocorticoid receptor in aldosterone-induced HSP84–Src association (Fig. 5A).

Heat shock proteins are found in mineralocorticoid receptor complexes and are released upon aldosterone binding [19]. Since we see that more HSP84 bound to Src shortly after aldosterone administration, our results are compatible with a mechanism for rapid aldosterone action that includes aldosterone-induced release of HSP84 from the aldosterone–mineralocorticoid receptor complex and subsequent association of HSP84 with Src kinase. Such an involvement of HSP release in rapid aldosterone action was also suggested by Tumlin et al. [20] for non-genomic aldosterone-induced calcineurin activation. Similarly, Uhrenholt et al. [16] concluded that aldosterone rapidly inhibits vasoconstriction in renal afferent arterioles by a mechanism initiated by the mineralocorticoid receptor and involving HSP90.

To further corroborate our theory, we treated cells with rapamycin. Rapamycin stabilizes HSP–steroid receptor complexes, slowing the release of HSPs after steroid binding [21]. Consequently, in the presence of rapamycin we would not expect increased HSP84–Src association after administration of aldosterone. This is what we saw: in the presence of rapamycin, aldosterone did not lead to an increased HSP84–Src association. In addition, aldosterone-induced Src kinase activation was prevented by rapamycin (Fig. 5B).

Taking the results together, aldosterone seems to utilize a mechanism of Src activation different from the one of other steroid hormone receptors. In contrast to, e.g., androgen, estrogen and progesterone receptor, the mineralocorticoid receptor does obviously not directly bind to Src, but exerts its stimulatory effect likely via heat shock proteins which are released upon aldosterone binding. Both, strong positive effects of HSP90 on v-src activity [22] as well as inhibition [23], have been reported in in vitro studies, whereas other enzymes such as eNOS and protein kinase Akt/PKB are activated by interaction with HSP90 [24]. In our system, Src kinase activity is obviously stimulated by HSP association. Additional studies are necessary to further elucidate the mechanism of HSP-mediated Src activation by aldosterone and the physiological consequences. However, from our study here and studies from Krug et al. [5] and Gekle et al. [10], it seems very likely that in kidney cells Src kinase is one of the most upstream targets of rapid aldosterone signaling, which via a network of downstream targets finally leads to activation of Na^+/H^+ exchange.

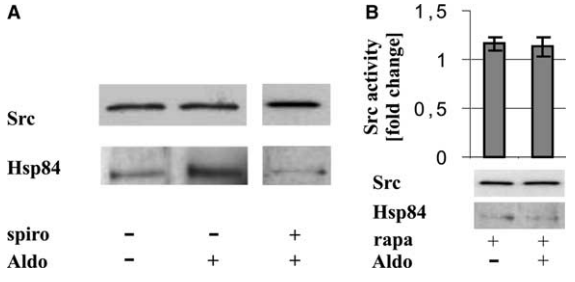

Fig. 5. A) HSP84–Src association after aldosterone (10^{-9} M, 5 min) and spironolactone (10^{-6} M, 20 min preincubation) treatment of M-1 cells. Anti-src immunoprecipitates were analyzed by Western blotting using anti-src and anti-HSP84 antibodies. (B) Effect of rapamycin (12 μM) on HSP84–Src association and Src activation in the absence and presence of aldosterone (10^{-9} M, 5 min).

References

[1] Boldyreff, B. and Wehling, M. (2003) J. Steroid Biochem. Mol. Biol. 85, 375–381.
[2] Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M.V., Ametrano, D., Zannini, M.S., Abbondanza, C. and Auricchio, F. (2000) EMBO J. 19, 5406–5417.
[3] Boonyaratanakornkit, V., Scott, M.P., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T. and Edwards, D.P. (2001) Mol. Cell 8, 269–280.
[4] Buitrago, C., Vazquez, G., De Boland, A.R. and Boland, R.L. (2000) J. Cell. Biochem. 79, 274–281.
[5] Krug, A.W., Schuster, C., Grassner, B., Freudinger, R., Mildenberger, S., Troppmair, J. and Gekle, M. (2002) J. Biol. Chem. 277, 45892–45897.
[6] Stoos, B.A., Narray-Fejes-Toth, A., Carretero, O.A., Ido, S. and Fejes-Toth, G. (1991) Kidney Int. 39, 1168–1175.
[7] Boldyreff, B. and Wehling, M. (2003) Nephrol. Dial. Transplant. 18, 1693–1695.
[8] Harvey, B.J. and Higgins, M. (2000) Kidney Int. 57, 1395–1403.
[9] Gekle, M., Golenhofen, N., Oberleithner, H. and Silbernagl, S. (1996) Proc. Natl. Acad. Sci. USA 93, 10500–10504.
[10] Gekle, M., Freudinger, R., Mildenberger, S. and Siblenagl, S. (2002) Am. J. Physiol. Renal Physiol. 282, F669–F679.
[11] Ookawara, S., Tabei, K., Furuya, H. and Asano, Y. (1999) Miner. Electrolyte Metab. 25, 191–198.
[12] Migliaccio, A., Di Domenico, M., Castoria, G., De Falco, A., Bontempo, P., Nola, E. and Auricchio, F. (1996) EMBO J. 15, 1292–1300.
[13] Urbach, V. and Harvey, B.J. (2001) J. Physiol. 573, 267–275.
[14] Vetter, W., Vetter, H. and Siegenthaler, W. (1993) Acta Endocrinol. 74, 558–567.
[15] Superti-Furga, G. (1995) FEBS Lett. 369, 62–66.
[16] Uhrenholt, T.R., Schjerning, J., Hansen, P.B., Norregaard, R., Jensen, B.L., Lyster, G.L. and Skott, O. (2003) Circ. Res. 93, 1258–1266.
[17] Liu, S.L., Schmuck, S., Chorazczewski, J.Z., Gros, R. and Feldman, R.D. (2003) Circulation 108, 2400–2406.
[18] Wade, C.B., Robinson, S., Shapiro, R.A. and Dorsa, D.M. (2001) Endocrinology 142, 2336–2342.
[19] Bruner, K.L., Derfoul, A., Robertson, N.M., Guerriero, G., Fernandes-Alnemri, T., Alnemri, E.S. and Litwack, G. (1997) Recept. Signal Transduct. 7, 85–98.
[20] Tumlin, J.A., Lea, J.P., Swanson, C.E., Smith, C.L., Edge, S.S. and Someren, J.S. (1997) J. Clin. Invest. 99, 1217–1223.
[21] Renoir, J.M., Le Behan, S., Mercier-Bodard, C., Gold, A., Arjomandi, M., Radanyi, C. and Baulieu, E.E. (1994) J. Steroid Biochem. Molec. Biol. 48, 101–110.
[22] Yang, X. and Lindquist, S. (1993) Proc. Natl. Acad. Sci. USA 90, 7074–7078.
[23] Hutchison, K.A., Brott, B.K., De Leon, J.H., Perdew, G.H., Jove, R. and Pratt, W.B. (1992) J. Biol. Chem. 267, 2902–2908.
[24] Balligand, J.L. (2002) Circ. Res. 90, 838–841.