DOPAMINE D2 RECEPTOR STIMULATION INHIBITS ANGIOTENSIN II-INDUCED HYPERTROPHY IN CULTURED NEONATAL RAT VENTRICULAR MYOCYTES

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SUMMARY

1. Myocardial hypertrophy is a common pathological change that accompanies cardiovascular disease. Dopamine D2 receptors have been demonstrated in cardiovascular tissues. However, the pathophysiological involvement of D2 receptors in myocardial hypertrophy is unclear. Therefore, the effects of the D2 receptor agonist bromocriptine and the D2 receptor antagonist haloperidol on angiotensin (Ang) II- or endothelin (ET)-I-induced hypertrophy of cultured neonatal rat ventricular myocytes were investigated in the present study.

2. Protein content and protein synthesis, determined by examining [3H]-leucine uptake, were used as estimates of cardiomyocyte hypertrophy. The expression of D2 receptor protein in neonatal rat ventricular myocytes was determined using western blotting. Changes in [Ca2+]i, in cardiomyocytes were observed by laser scanning confocal microscopy.

3. Angiotensin II and ET-1, both at 10 nmol/L, induced myocyte hypertrophy, as demonstrated by increased protein content and synthesis, [Ca2+]i levels, protein kinase C (PKC) activity and phosphorylation of extracellular signal-regulated kinase, c-Jun N-terminal kinase and mitogen-activated protein kinase (MAPK) p38 (p38). Concomitant treatment of cells with 10 nmol/L AngII plus 10 μmol/L bromocriptine significantly inhibited cardiomyocyte hypertrophy, MAPK phosphorylation and PKC activity in the membrane, as well as [Ca2+]i signalling pathways, compared with the effects of AngII alone. In addition, 10 μmol/L bromocriptine significantly inhibited cardiomyocyte hypertrophy induced by 10 nmol/L ET-1. However, pretreatment with haloperidol (10 μmol/L) had no significant effects on cardiomyocyte hypertrophy induced by either AngII or ET-1.

4. In conclusion, D2 receptor stimulation inhibits AngII-induced hypertrophy of cultured neonatal rat ventricular myocytes via inhibition of MAPK, PKC and [Ca2+]i, signalling pathways.

Key words: cardiomyocytes, dopamine D2 receptor, hypertrophy, rat.

INTRODUCTION

Dopamine is a very important catecholamine neurotransmitter in the mammalian brain that has multiple roles in peripheral tissues. Its effects are exerted via stimulation of dopamine D1–D5 receptors. Binding of dopamine to D1 and D5 receptors stimulates adenyl cyclase and phospholipase C (PLC), as well as activating calcium channels. Conversely, stimulation of D2, D3 and D4 receptors inhibits adenyl cyclase and calcium channels and activates the opening of single K+ channels, resulting in an increase in K+ conductance and associated membrane hyperpolarization.

Myocardial hypertrophy, which is an adaptive response to various mechanical changes and humoral stimuli, eventually leads to heart failure. Because cardiomyocytes rapidly lose their ability to divide under basal conditions both in vivo and in culture, their growth response to various stimuli primarily involves the hypertrophy of individual cells. Many studies have demonstrated that angiotensin (Ang) II is a potent growth promoter of cardiomyocytes by stimulating different signal transduction pathways, including the activity of Gq, which facilitates activation of the PLC/protein kinase C (PKC) pathway. Activated mitogen-activated protein kinase (MAPK) may stimulate various transcription factors and induce increased gene expression and protein synthesis.

In a previous study, we detected D2 receptor mRNA and protein expression in normal rat cardiac tissues and reported, for the first time, that expression decreased in an animal model of cardiac hypertrophy. Furthermore, we showed that the D2 receptor is also present in cultured neonatal rat ventricular myocytes. Thus, the aim of the present study was to determine the effects of D2 receptor activation/inhibition on the hypertrophic response of cardiomyocytes to AngII and the mechanisms involved.

METHODS

Cells and treatment

Neonatal rat ventricular myocytes were isolated from 2-day-old Wistar rats by enzymatic digestion with 0.25% trypsin, as described previously. The culture medium was changed to serum-free medium for 24 h before treatment. Dishes from each culture preparation were randomly assigned to one of the following experimental groups, each comprising eight dishes:
Cardiomyocyte purity and cell viability

Cardiomyocyte purity was monitored using an antibody to cardiac sarcomeric α-actin (Boster Biological Technology, Wuhan, China) according to the manufacturer’s instructions. Cell viability was analysed by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) assay, performed in triplicate. Cells and dye crystals were solubilized with 200 μL dimethylsulphoxide and absorbance was measured at 490 nm, using a model ELX-800 microplate assay reader (One Lambda, Canoga Park, CA, USA).

Measurement of beating rate, myocyte diameter, protein content and protein synthesis

The beating rate of myocardial cells was recorded with a JVC (Kanagawa-ku, Japan) GZ-MG505AC digital recorder. Ten fields were chosen at random for every group and 10 cells were evaluated in each field. The number of beats over a 60 s period was counted manually.

Total cell protein of 5 × 10⁵ cells was measured using a modification of the method of Lowry et al. and bovine serum albumin as a standard. The total DNA content of each plate was quantified using ultraviolet spectrophotometry (DU-65 Spectrophotometer; Beckman, Woodland Hills, CA, USA). Each experiment was repeated 12 times.

[p[H]-Leucine uptake was used as an index of protein synthesis, as described previously. To correct for any minor differences in cell number between treatment groups, protein synthesis and protein content were analysed by [p[H]-leucine uptake/DNA content.

Preparation of PKC reagents and PKC activity assay

Cells were scraped into cool protein lysate (Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, China) containing 1% phenylmethylsulphonyl fluoride (Amresco, Solon, OH, USA). Samples were centrifuged at 10 303 g for 15 min at 4°C and the supernatant, representing the cytosolic fraction, was collected. Pellets were suspended in protein lysate containing 0.1% Triton X-100 (Amresco). After homogenization, samples were kept at 4°C for 1 h, agitated every 20 min for 15 s and then centrifuged at 10 303 g for 20 min at 4°C. Protein kinase C activity from cytosolic and membrane fractions was determined according to the method provided with the Protein Kinase C Assay System (Promega, Madison, WI, USA). The incorporation of [γ-32P]-ATP (111 GBq/mmol, 0.37 MBq/μL; Beijing Furei Biotechnology, Beijing, China) was determined by liquid scintillation and the activity of PKC was determined by subtracting the activity of the enzyme in the absence of phospholipids (control buffer) from that of the enzyme in the presence of phospholipids (activation buffer). In the present study, PKC activity was calculated as pmol phosphate radioactivity transferred/min per mg protein.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting

Samples (50 μg protein) from different experimental groups were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) by electroblotting (100 V for 1.5 h). Membranes were then blocked at 37°C for 1 h in 5% (w/v) skimmed milk powder in TBS (Tris 10 mmol/L, NaCl 150 mmol/L, pH 8.0; Beijing Chemical Reagents Factory, Beijing, China) and incubated with mouse anti-D2 receptor (1: 200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-extracellular signal-regulated kinase (ERK; 1: 2000 dilution; Promega), anti-p-c-Jun N-terminal kinase (JNK; 1: 1000 dilution; Promega), anti-p-p38 (1: 1000 dilution; Neomarker, Fremont, CA, USA) or anti-actin (1: 200 dilution; Boster) antibodies overnight at 4°C. Alkaline phosphatase-conjugated goat anti-mouse IgG (1: 2500 dilution; Promega) antibodies were added and membranes were incubated at 37°C for 1 h. Immunodetection was performed using a Bio2000 Imaging Analysis System (Chengdu Taimeng Sci-Tec, Chengdu, China). β-Actin was used as an internal control for semiquantitative assays.

Measurement of intracellular Ca²⁺

Free intracellular calcium concentrations ([Ca²⁺]) in myocardial cells was determined using the Fluo-3/AM (Dojindo Laboratories, Kumamoto, Japan) probe as follows. After treatment with AngII, bromocriptine or haloperidol alone, cells were incubated with 5 μmol/L Fluo-3/AM for 40 min at 37°C under a 95 : 5 air : CO₂ atmosphere, washed three times with phosphate-buffered saline (PBS) and further incubated for 20 min in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Invitrogen, Carlsbad, CA, USA) in the presence of 10 mmol/L AngII. Dynamic changes in [Ca²⁺] in myocardial cells were measured after stimulation with AngII for 30 min by measuring Flu-3 fluorescence (excitation at 488 nm and emission at 525 nm). Fluorescence intensity was measured to determine changes in [Ca²⁺]. The fluorescence intensity was observed in eight randomly chosen cells using a laser scanning confocal microscope (TCS SP2; Leica, Mannheim, Germany) to calculate average fluorescence intensity for all cells.

Statistical analysis

Data are presented as the mean±SEM and were analysed using SPSS v. 11.5 software (SPSS, Chicago, IL, USA), with the number of observations indicated. Statistical significance was tested by post hoc analysis following one-way repeated-measures ANOVA. Significance was set at P < 0.05.

RESULTS

Cardiomyocyte purity, viability and diameter

The percentage of α-actin-positive cells was >95% of the total number of cells (Fig. 1). In preliminary experiments performed to determine the optimum concentration of all drugs, viability decreased after treatment of cells with 100 and 1000 nmol/L AngII or ET-1, but no adverse effects were observed after treatment of cells with 10 μmol/L bromocriptine or haloperidol (Fig. 2a). At 10 nmol/L, AngII and ET-1 both increased cell diameter and there were no obvious differences in the hypertrophy induced by 10, 100 and
1000 nmol/L AngII or ET-1. At this concentration (10 nmol/L), neither AngII nor ET-1 had any significant toxic effects, so this concentration was used in subsequent experiments. Bromocriptine and haloperidol alone (both at 1, 10, 100 and 1000 μmol/L) had no effect on cardiomyocyte dimater (Fig. 2b). Therefore, in all subsequent experiments, 10 μmol/L bromocriptine and haloperidol was used.

**D2 receptor protein expression in neonatal rat ventricular myocytes**

Expression of D2 receptor protein in neonatal rat ventricular myocytes was detected with western blotting. Protein levels of the D2 receptor were significantly lower in AngII- and ET-1-treated groups compared with the control group (Fig. 3a,b).

**Hypertrophy of neonatal rat ventricular myocytes**

Cardiomyocytes were pretreated for 10 h with bromocriptine or haloperidol, then treated with AngII or Et-1; the beating rate was measured 72 h later. The beating rate of AngII-treated cardiomyocytes was significantly higher than that of the control group. In contrast, bromocriptine, haloperidol and ET-1 alone had no effect on the beating rate of cardiomyocytes. Pretreatment of cells with bromocriptine prior to AngII decreased the beating rate of myocardial cells (Fig. 4a).

At 10 nmol/L, treatment of cardiomyocytes with AngII and ET-1 alone increased cell diameter compared with the control group. Pretreatment with 10 μmol/L bromocriptine reduced the increase in cell diameter induced by both 10 nmol/L AngII and ET-1 (Fig. 4b).

Protein content and synthesis were determined for each well (containing 5 × 10^5 cells). Compared with the control group, the protein content and synthesis of ventricular myocytes were significantly higher following treatment with AngII and ET-1 (both at 10 nmol/L). The D2 receptor agonist bromocriptine (10 μmol/L) significantly decreased the AngII- or ET-1-induced increase in [3H]-leucine incorporation and, thus, protein content. In contrast, the D2 receptor specific antagonist haloperidol had no significant effect on the AngII- or ET-1-induced increases in protein content and synthesis (Fig. 4c,d).

**Phosphorylation of ERK1/2, JNK and p38**

There was no difference in non-phosphorylated ERK between the different treatment groups. There was very little phosphorylation of ERK, JNK and p38 MAPK in normal neonatal ventricular myocytes. When 10 nmol/L AngII was added to the medium, there was a marked increase in phosphorylation of ERK1/2, JNK and p38 MAPK. However, in the bromocriptine-pretreated group, the AngII-induced phosphorylation of ERK and JNK was decreased significantly (P < 0.01; Fig. 5).

**Measurement of [Ca²⁺] and PKC activity**

From fluorescence images taken by a laser scanning confocal microscope, we found that AngII markedly increased [Ca²⁺], and that bromocriptine significantly inhibited this increase. However, the D2 receptor antagonist haloperidol had no effect on AngII-induced increase in [Ca²⁺], (Fig. 6).

Treatment of cardiomyocytes with AngII increased PKC activity in the membrane and cytosolic fractions compared with the control group. There was no difference in PKC activity in the cytosolic fraction between the AngII alone, AngII + bromocriptine and AngII + haloperidol groups. However, PKC activity in the membrane fraction decreased in the bromocriptine-pretreated AngII-treated group (Fig. 7).
we demonstrated that stimulation of dopamine D2 receptors inhibited the hypertrophic response. This inhibition was associated with activation of MAPK and [Ca\(^{2+}\)], signalling pathways.

Both D1 and D2 receptors have been identified in cardiac muscle. In a previous study, we detected the expression of D1 and D2 receptor mRNA and protein in normal rat cardiac tissues; interestingly, expression of D2 receptor mRNA and protein decreased in an animal model of cardiac hypertrophy induced by experimental aortic coarctation. In vivo, receptor downregulation may be related to many humoral and neural factors. Cultured neonatal cardiac myocytes have been used extensively as an experimental model in which to investigate the mechanisms of myocyte hypertrophy, avoiding interference from humoral and neural factors. In this system, adrenoceptor stimulation, AngII, endothelin and peptide growth factors cause myocyte hypertrophy without hyperplasia. Some studies have shown that AngII plays an important role in the initiation of proto-oncogene expression and growth in myocardial cells. In addition, angiotensin-converting enzyme inhibitors have been shown to prevent the development of cardiac remodelling after myocardial injury.

In the present study, we observed D2 receptor mRNA and protein expression in neonatal rat ventricular myocytes in vitro and found that expression decreased following treatment of cells with AngII. In order to determine whether decreases in D2 receptor mRNA and protein expression are unique to AngII, we examined D2 receptor expression ventricular myocytes after treatment with ET-1 and observed similar results. Therefore, we speculate that the decrease in the expression of D2 receptors is related to the reduction in the relative density of D2 receptors (as a proportion of the increased cell surface area resulting from hypertrophy).

Dopaminergic ligands easily discriminate between the different dopamine receptor subtypes. In the present study, we chose bromocriptine and haloperidol as specific D2 receptor agonists and antagonists, respectively. The results showed that bromocriptine significantly inhibited hypertrophy of ventricular myocytes, with a decrease in protein content, cellular protein synthesis and cell diameter. These findings are consistent with recent observations reported by Mejia-Rodriguez et al. In patients with end-stage renal disease treated with continuous ambulatory peritoneal dialysis, there was a 24.4% decrease in left ventricular mass index after treatment with bromocriptine compared with the control group. The authors concluded that bromocriptine inhibits noradrenaline release, antagonizes aldosterone and downregulates angiotensin AT1 receptors, which may lead to regression of left ventricular hypertrophy.

The results of the present study indicate that D2 receptor activation in vitro can also stimulate other pathways to inhibit myocardial hypertrophy, in addition to humoral and neural factors. Masson et al. have reported that CHF-1024, a D2 receptor agonist, blunts cardiac fibrosis in pressure overload and has no effect on cardiac mass. One possible explanation for this is that CHF-1024 is not able to reduce levels of AngII, a major hypertrophic factor. Hussain et al. have proposed that bromocriptine (1 mmol/L) alone stimulates Na\(^+/\)K\(^{-}\)-ATPase activity. They have also suggested that pre-activation of D2, D3 and D4 receptors by bromocriptine prior to AngII treatment abolishes AngII-mediated stimulation of Na\(^+/\)K\(^{-}\)-ATPase activity and inhibition of cAMP accumulation.

In vivo, bromocriptine induces tachycardia. The results of the present study show that the beating rate of isolated myocytes in vitro was not changed after treatment with bromocriptine, suggesting

**DISCUSSION**

Cardiac hypertrophy is an independent risk factor for the development of ischaemia, arrhythmia and sudden death. Evidence from in vivo studies suggests that both dopamine and its receptors are implicated in cardiac hypertrophy. The D2 receptor agonist bromocriptine induces regression of left ventricular hypertrophy in peritoneal dialysis patients. However, the mechanisms involved after D2 receptor activation were not determined in any of these previous studies.

In the present study, using an experimental model of AngII- or ET-1-mediated hypertrophy of neonatal rat cardiac myocytes, we demonstrated that stimulation of dopamine D2 receptors inhibited the hypertrophic response. This inhibition was associated with activation of MAPK and [Ca\(^{2+}\)], signalling pathways.

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Fig. 5 Western blot amplification of phosphorylated (a,b) extracellular signal-regulated kinase (ERK) 1/2, (c) c-Jun N-terminal kinase (JNK) and (d) p38 protein in neonatal ventricular myocytes. Levels of ERK1/2, JNK and p38 phosphorylation protein were quantified by densitometric analysis. The results are representative of six experiments. Data are the mean ± SEM. *P < 0.05 compared with the control group. AngII, 10 nmol/L angiotensin II; ET-1, 10 nmol/L endothelin-1; Bro, 10 μmol/L bromocriptine; Hal, 10 μmol/L haloperidol.

Fig. 6 Continuous record of [Ca^{2+}]_i in neonatal ventricular myocytes. (a) Dynamic changes in [Ca^{2+}]_i in myocardial cells were measured 1 h after stimulation with 10 nmol/L angiotensin (AngII). (b) Fluorescence intensity changes in [Ca^{2+}]_i were recorded continuously with a laser scanning confocal microscope in the different treatment groups. Angiotensin II increased the intracellular concentration of calcium. Bro, 10 μmol/L bromocriptine; Hal, 10 μmol/L haloperidol.

Fig. 7 Protein kinase C (PKC) activity in the (a) membrane and (b) cytosolic fraction of neonatal ventricular myocytes. Data are the mean ± SEM. *P < 0.05. AngII, 10 nmol/L angiotensin II; Bro, 10 μmol/L bromocriptine; Hal, 10 μmol/L haloperidol.
that bromocriptine increases the beating rate of cardiomyocytes in vitro by changing cardiac vagal or sympathetic tone.

Ganguly et al. have found evidence of a relationship between D1 receptors and hypertrophy. They have shown that SCH 23390, a D1 receptor antagonist, partially represses cardiac hypertrophic changes after aortic constriction. D1 and D2 receptors have different pharmacological characteristics in the PKC pathway: D1 receptor-stimulated adenyl cyclase strongly stimulates cAMP accumulation, whereas activation of D2 receptors inhibits adenyl cyclase. The fact that blockade of D1 receptors and stimulation of D2 receptors inhibit hypertrophy suggests that suppression of the PKC signalling pathway may cause inhibition of cardiac hypertrophy in vitro and in vivo. In addition, via a Gαi-protein, the D2 receptor participates in the activation of potassium conductance, resulting in inhibition of voltage-gated calcium currents in melanotrophs and stimulation of phospholipase D activity. In the present study, we found that the D2 receptor agonist alone had no effect on protein content, cellular protein synthesis or cell diameter. These results indicate that activation of D2 receptors interferes with AngII-mediated hypertrophic signalling.

Accumulating evidence from in vitro and in vivo studies suggests that AngII participates in the development of hypertrophy by activating many signalling pathways and that PKC, MAPK and phosphatidase–calcium pathways may be involved. In an attempt to understand the mechanism through which D2 receptor activation exerts its antihypertrophic effects, we investigated the role of the aforementioned AngII-related signalling pathways in our culture system. The results showed that AngII stimulation significantly increased [Ca2+]i. In contrast, 10 μmol/L bromocriptine significantly decreased [Ca2+]i, which was accompanied by inhibition of myoccardial hypertrophy. The increase in [Ca2+]i, in ventricular myocytes is connected directly to cell hypertrophy and calcium modulation by the D2 receptor may play a role in the inhibition of hypertrophy. Angiotensin II induced a marked increase in the phosphorylation of ERK and JNK. This was significantly reduced by 80% and 30%, respectively, after treatment with D2 receptor agonist bromocriptine.

Probably the most important finding of the present study is that D2 receptor activation can induce changes in other messenger molecules (e.g. a decrease in cAMP or nitric oxide), which results in the inhibition of phosphorylation of MAPK and an increase in [Ca2+]i. In the present study, the D2 receptor antagonist haloperidol did not promote the hypertrophic response or the changes in MAPK and [Ca2+]i. This infers that the D2 receptor was inactive in normal cells and was activated by the agonist, thus showed no significant changes following treatment with the D2 receptor antagonist haloperidol. The activity of p38 MAPK did not differ significantly between the groups, likely because p38 MAPK is involved mainly in cell apoptosis. We investigated changes in PKC activity in cytosolic and membrane fractions of ventricular myocytes and the results indicate that PKC was translocated to the cell membrane in AngII-treated cells. After treatment with bromocriptine, the translocation of PKC to the cell membrane was significantly reduced by 30%. Activated PKC inhibits L-type Ca2+ channels and decreases Ca2+ influx. We propose that bromocriptine inhibited activation of PKC and attenuated the deleterious increase in [Ca2+]i, that occurred during AngII-mediated hypertrophy of neonatal rat cardiac myocytes in vitro.

It is important to note that the present study dealt specifically with the effects of a D2 receptor agonist on neonatal myocardial cells. Further studies are needed to determine whether our findings are relevant to pressure overload in other species, as well as in humans with chronic cardiac hypertrophy.

In summary, D2 receptor stimulation partly inhibited AngII-induced hypertrophy in cultured neonatal rat ventricular myocytes via inhibition of MAPK, PKC and [Ca2+]i, signalling pathways.

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