Amphetamine activates connexin43 gene expression in cultured neonatal rat cardiomyocytes through JNK and AP-1 pathway

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Abstract

\textbf{Objective:} Amphetamine has been known to induce cardiac dysrhythmia and sudden death. However, the molecular mechanism for the induction of dysrhythmia is not known. Connexin43 (Cx43) plays an important role for arrhythmogenesis. This study was undertaken to test the hypothesis that amphetamine could induce Cx43 expression in cardiac myocytes. \textbf{Methods:} Neonatal Wistar rat cardiac myocytes were cultured under the stimulation of amphetamine. Cx43 mRNA and protein expression were examined by Northern and Western blots, respectively. We used c-Jun N-terminal kinase (JNK) inhibitor, SP600125, and JNK1 dsRNAi to investigate the signal pathway of amphetamine-induced expression of Cx43. \textbf{Results:} The level of Cx43 protein significantly increased from 4 to 24 h after addition of amphetamine (10\textsuperscript{A}M). The Cx43 mRNA increased maximally to 4.2-fold at 6 h after addition of amphetamine and returned to the baseline level at 48 h. These increases of Cx43 protein at 24 h were completely attenuated ($P<0.001$) by SP600125 (20\textsuperscript{A}M) and JNK1 dsRNAi. Amphetamine increased and SP600125 decreased the phosphorylated c-Jun proteins. Gel-shifting assay showed that DNA-binding activity of AP-1 increased after addition of amphetamine and SP600125 and JNK1 dsRNAi abolished the binding activity induced by amphetamine. \textbf{Conclusions:} These findings indicate that amphetamine activates Cx43 gene expression in cultured rat neonatal cardiac myocytes. Amphetamine mediates the Cx43 gene expression, at least in part, through the JNK pathway. These findings from our study suggest that Cx43 plays a role for the molecular mechanism of amphetamine-induced cardiac dysrhythmias.

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1. Introduction

Amphetamine is one of the most widely abused among the illicit stimulants \cite{1}. Addiction to amphetamine poses enormous social and economic burdens. Serious and sometimes fatal cardiovascular toxicity associated with the use of this drug has been reported \cite{2–4}. The illicit use of amphetamines has been shown to lead to tachyarrhythmias and sudden death \cite{5,6}. Although the electrophysiologic mechanisms of amphetamine-induced arrhythmias have been shown to include trigger activity and automaticity \cite{7,8}, there is nothing in the literature about the molecular mechanism of arrhythmias-induced by amphetamine.

Gap junctions play an important role for arrhythmogenesis \cite{9,10}. The cardiac myocytes express multiple gap junction proteins (connexins) \cite{11–13}. Connexin43 (Cx43) is the physiological predominant connexin of myocardial cells \cite{14}. There is growing evidence suggesting that changes in pattern and velocity of conduction of myocardial electrical activity can affect cardiac rhythm and coordination of contraction \cite{15}. An abnormal coupling between cardiomyocytes through gap junctions is, therefore, increasingly considered an important factor in various pathophysiologic conditions including potentially life-threatening arrhythmias.
The effect of amphetamine on myocardial Cx43 expression remains unknown.

The present study was designed to test whether amphetamine could induce Cx43 expression in cardiac myocytes and to investigate the possible signal pathway that mediates the induction of Cx43 by amphetamine.

2. Materials and methods

2.1. Cardiac myocyte culture

Cardiac myocytes were obtained from Wistar rats aged 3 days old by trypsinization, as described previously [18]. Briefly, hearts were minced and subjected to 15-min cycles of exposure to 0.125% trypsin (GIBCO, Grand Island, NY, USA) at 37 °C. The trypsin-digested cells were collected by centrifugation at 300 × g for 10 min. The cell pellet was resuspended in serum-containing medium and poured into a petri dish and incubated for 2.5 h with 5% CO2 at 37 °C to let the cells attach to the dish. The unattached cardiac myocytes in the medium were collected and plated at a cell density of 1.6 × 10⁶ cells/well on to six-well culture plates in Ham’s F-10 containing 10% horse serum and 10% fetal calf serum. After 2 days in culture, cells were transferred to serum-free medium (Ham’s F-12/DMEM; 1:1) and maintained for another 2 days. Myocytes culture thus obtained were >95% pure, as revealed by observation of their contractile characteristics under a light microscope and stained with anti-desmin antibody. To determine the roles of c-Jun N-terminal kinase (JNK), or p42/p44 MAP kinase in the expression of amphetamine-induced Cx43 expression, Cardiac myocytes were pretreated with SP600125 (20 μM, Calbiochem, San Diego, CA, USA), or PD98059 (50 μM) for 30 min, respectively, followed by addition of amphetamine. The amphetamine used in this study was the D-isomer (D-amphetamine sulfate purchased from Sigma), which is four times as potent as the l-isomer as a central nervous stimulant [7]. SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK. PD98059 is a specific and potent inhibitor of p42/p44 MAP kinase. The study conforms with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Western blot analysis

The myocytes were rinsed with PBS, scraped into PBS containing 10 μg/100 ml aprotinin and then centrifuged (300 × g) for 10 min at 4 °C. The pellet was resuspended, homogenized in a Reporter Lysis Buffer (Promega, Madison, WI, USA), and then centrifuged at 10,000 rpm for 20 min. Protein content of the supernatant was determined by the Bio-Rad Protein assay using bovine serum albumin as the standard. Equal amounts of protein extracts (15 μg) were loaded into 12.5% SDS-polyacrylamide gels. Proteins were electroblotted onto nitrocellulose. The membranes were blocked overnight in 5% skim milk in PBS and proteins of interest were revealed with specific antibodies as indicated at a dilution of 1:250 in PBS containing 0.1% Tween 20 (TPBS). After five washes in TPBS, the blots were incubated with horseradish peroxidase-conjugated goat antimouse IgG which was diluted 1:3,000 in TPBS. After being rinsed 6 times for 5 min each in TPBS, the blots were incubated for 1 min in ECL solution and exposed to X-ray film. The proteins were detected on membranes with an enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, England). Densitometric quantitation of signal intensity was then measured. Equal protein loading of the samples was further verified by staining with GAPDH-specific monoclonal antibody.

2.3. Northern blot analysis

Total RNA was prepared by solubilizing myocytes in Ultraspec™ RNA kit (Biotex Laboratory, Houston, TX, USA). Aliquots of 20 μg of total RNA were fractionated in formaldehyde-agarose gels, transferred to Hybond-N+ nylon membrane, and hybridized with [α³²P]dCTP-labeled cDNA probes, generated from rat Cx43 DNA. The membrane was prehybridized at 65 °C for 1 h, and hybridized with radioactively labeled probes at 65 °C for 3 h in Rapid-hyb buffer (Amersham). Post-hybridization wash was performed with a final stringency of 0.2 × standard saline

![Fig. 1. Amphetamine increased connexin 43 (Cx43) in cardiac myocytes.](image-url)
citrate containing 0.1% SDS at 65 °C. Quantitative analysis was performed with a PhosphorImager.

2.4. Immunohistochemistry

Cells were washed three times with PBS and fixed in 4% paraformaldehyde for 20 min, treated in 3% hydrogen peroxide/PBS for 25 min, blocked in 5% normal rabbit serum for 20 min, blocked with biotin/avidin for 15 min each, and incubated with primary antibodies for 2 h at room temperature as described [19]. Staining was performed with a peroxidase detection kit (Vector Laboratories, Burlingame, CA, USA). The cells were finally examined with light microscope.

Fig. 2. Time course of Cx43 expression induced by amphetamine at 10 μM. (A) Representative Western blot for Cx43 and Cx40 in neonatal cardiac myocytes incubation with amphetamine for various periods of time. (B) Quantitative analysis of Cx43 protein levels. The values from amphetamine-treated cells have been normalized to values in control cells (n = 3 per group). *P < 0.01 vs. control, **P < 0.05 vs. control. (C) Representative Western blot for Cx43 in adult cardiac myocytes incubation with amphetamine for various periods of time. Similar results were observed in another 2 independent experiments. (D) Representative Western blot for Cx43 in neonatal cardiac myocytes for various periods of time after incubation with three times of amphetamine in 6 h.
2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear protein concentrations from cultured myocytes were determined by BioRad protein assay. Consensus and control oligonucleotides (Santa Cruz Biotechnology, CA, USA) were labeled by polynucleotides kinase incorporation of $[^{32}P]$-dATP. Oligonucleotide sequences included the activating protein 1 (AP-1) consensus 5'-CGCTTGATGACTTCAGCCGGAA-3'. The AP-1 mutant oligonucleotide sequences were 5'-CGCTTGATGACTTGGCCGGAA-3'. After the oligonucleotide was radiolabeled, the nuclear extracts (4 μg of protein in 2 μl of nuclear extract) were mixed with 20 pmol of the appropriate $[^{32}P]$-dATP-labeled consensus or mutant oligonucleotide in a total volume of 20 μl for 30 min at room temperature. The samples were then resolved on a 4% polyacrylamide gel. Gels were dried and imaged by autoradiography. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labeled sequences.

2.6. RNA interference

Neonatal cardiac myocytes were transfected with 800 ng JNK1 annealed dsRNAi oligonucleotide: sense, 5'-CGUG-GAUUUAUGGUCUGUGdTdT-3' and antisense, 5'-CACAGACCAUAACCUCCACCdTdT-3' (Dharmacon, Lafayette, CO, USA). For negative control, green fluorescent protein (GFP) dsRNAi was used: sense, 5'-P.GGCUACGUC-CAGGAGCGACC-3' and antisense, 5'-P.UGCGCUC-CUGGACGUAGCCUU-3' (Dharmacon). After incubation at 37 °C for 24 h, cells were treated with amphetamine as indicated for 24 h, and subjected to analysis of Western blot, immunohistochemistry, EMSA, and Dye transfer assay.

2.7. Dye transfer assay

Gap junctional communication in cardiac myocyte cultures was assayed by microinjection of 5% Lucifer yellow CH dye in 0.1 mol/l LiCl solution. Cells were visualized by using an inverted phase-contrast/epifluorescence microscope. The dye-labeled cells directly adjacent to the microinjected dye-loaded cells were considered to represent cell–cell coupling properties. The total number of fluorescent cells per injection was counted 5 min after injection and photographed. For each treatment condition, 10 cells were microinjected in each of 4 dishes.

2.8. Statistical analysis

The data were expressed as mean ± S.E.M. Statistical significance was performed with analysis of variance fol-

![Fig. 3. Effect of amphetamine on Cx43 mRNA expression in cardiac myocytes. (A) Representative Northern blot for Cx43 mRNA in cardiac myocytes treated with amphetamine (10 μM) for various periods of time. (B) Quantitative analysis of Cx43 Northern blots. The values from amphetamine-treated cells have been normalized to matched GAPDH measurement and then expressed as a ration of normalized values to mRNA in control cells (n=3–4 per group). *P<0.01 vs. control. **P<0.05 vs. control.](image-url)
followed by post-hoc analysis. A value of \( P < 0.05 \) was considered to denote statistical significance.

3. Results

3.1. Amphetamine enhances connexin 43 protein and mRNA expression in cardiac myocytes

To investigate the optimal effect of amphetamine on Cx43 protein expression in cultured myocytes, different doses of amphetamines from 1 to 100 \( \mu \text{M} \) were used. As shown in Fig. 1, all the three different doses of amphetamine increased Cx43 protein expression after 24 h of incubation and 10 \( \mu \text{M} \) of amphetamine had the optimal effect. Therefore, the dose of 10 \( \mu \text{M} \) amphetamine was used in the subsequent studies. Cx43 protein expression was enhanced by 2 h after incubation with amphetamine, and increased 5.9-fold (to its maximum) by 24 h (Fig. 2). The protein expression was less enhanced after incubation with amphetamine by 48 h. In addition to induction of Cx43, amphetamine also induced Cx40 expression in neonatal cardiomyocytes (Fig. 2A). However, the expression of Cx40 is weaker than that of Cx43. To test whether there is a similar effect of amphetamine on Cx43 expression of adult cells, we cultured adult cardiomyocytes from Wistar rats weighing around 100g. As

![Fig. 4. JNK inhibitors inhibited Cx43 protein expression induced by amphetamine. (A) Representative Western blot for Cx43 protein levels in cardiac myocytes treated with amphetamine for 24 h in the absence or presence of inhibitors. (B) Quantitative analysis of Cx43 Western blots. The values from amphetamine-treated myocytes have been normalized to values in control cells (n=3 per group).](image-url)

shown in Fig. 2C, the induced Cx43 expression pattern of adult cells by amphetamine at 10 μM was similar to that of neonatal cells. We also added three times of amphetamine (10 μM) in 6 h and observed the change of Cx43 protein expression. As shown in Fig. 2D, the change of Cx43 in three times of adding amphetamine was similar to that of one time stimulation with amphetamine. The Northern blots showed that Cx43 messages increased significantly after 6 and 24 h of incubation with amphetamine (Fig. 3). The Cx43 mRNA then returned to its baseline level. No increase in release of LDH was observed following 10 μM for 24 h and trypan blue staining also did not show any significant cell damage under these conditions (data not shown). These data indicated that amphetamine at 10 μM did not induce serious injury on cardiac myocyte.

3.2. Amphetamine-induced Cx43 protein expression in cardiac myocytes is mediated by JNK pathway

To investigate the possible signal pathway that mediates the amphetamine-induced Cx43 in cardiac myocytes, the cardiac myocytes were incubated with amphetamines for 24 h in the absence or presence of inhibitors. As shown in Fig. 4, the amphetamine-induced increases of Cx43 proteins were completely blocked ($P < 0.001$) after the addition of SP600125 (20 μM) 30 min before the addition of amphetamine. The addition of PD98059 (50 μM) did not significantly block the amphetamine-induced Cx43 protein expression. To test the specific effect of JNK pathway mediating the expression of Cx43, JNK1 dsRNAi was transfected to neonatal cardiomyocytes before addition of

Fig. 5. Representative microscopic images of the Cx43 immunoreactive signal (arrow) in control cells (A) and amphetamine-treated cardiac myocytes for 24 h in the absence (B) or presence (C) of JNK inhibitor, SP600125, and after addition of JNK1 dsRNAi (D).
amphetamine. As shown in Fig. 4A, JNK1 dsRNAi also completely blocked the Cx43 expression induced by amphetamine ($P < 0.001$). The GFP dsRNAi did not affect the Cx43 expression induced by amphetamine. The DMSO alone as a vehicle did not affect the Cx43 proteins induced by amphetamine. Another inhibitor of JNK, JNK inhibitor I (Calbiochem), also reduced expression of Cx43 induced by amphetamine. Amphetamine also increased immunoreactive signals of Cx43 in cardiac myocytes. The anti-Cx43 antibody produces punctate labeling along the appositional membrane between cells. Amphetamine had increased Cx43 immunoreactive signal by 24 h when compared to control cells without addition of amphetamine (Fig. 5). This immunoreactive signal decreased after addition of SP600125. JNK1 dsRNAi also decreased the immunoreactive signal induced by amphetamine. These findings

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**Fig. 6. Effect of amphetamine on phosphorylated JNK and c-Jun protein.** (A) Representative Western blots for phosphorylated JNK and c-Jun protein levels in cardiac myocytes treated with amphetamine (10 µM) for various periods of time and in the presence of SP600125. Quantitative analysis of phosphorylated JNK (B) and c-Jun (C) Western blots. ($n = 3 – 4$ per group). *$P < 0.01$ vs. control. **$P < 0.001$ vs. control.
implicated that JNK pathways mediated the induction of Cx43 proteins by amphetamine in cardiac myocytes.

As shown in Fig. 6, phosphorylated JNK and c-Jun proteins were induced by amphetamine and the increase in phosphorylated JNK and c-Jun proteins were completely inhibited after addition of SP600125 30 min before addition of amphetamine. The pattern of increases in phosphorylated JNK and c-Jun proteins was similar to that of Cx43 proteins after addition of amphetamine. Amphetamine also increased the phosphorylated JNK and c-Jun proteins in adult cardiac myocytes (data not shown). To exclude the cytotoxicity of SP600125, MTT assay was performed. The absorbency at 570 nm demonstrated no difference among control cells and cells treated with SP600125 at different concentrations for up to 24 h (data not shown). These findings indicated that there was no cytotoxicity of SP600125 on cardiac myocytes.

3.3. Amphetamine increases AP-1 binding activity

After incubation of amphetamine with cardiac myocytes for 2–6 h, the DNA–protein binding activity of AP-1 (Fig. 7) increased significantly as compared to control cells without incubation with amphetamine. An excess of unlabelled AP-1 oligonucleotide competed with the probe for binding AP-1 protein, whereas an oligonucleotide containing a 3-bp substitution in the AP-1 binding site did not compete for binding. Addition of SP600125 30 min before use of amphetamine abolished the DNA–protein binding activity induced by amphetamine. JNK1 dsRNAi, similar to SP600125, also abolished the DNA–protein binding activity induced by amphetamine.

3.4. Amphetamine enhances Cx43 communication

To determine the functional effect of amphetamine on intercellular communication, a dye transfer assay was used to measure the level of coupling between adjacent cardiac myocytes in culture. As shown in Fig. 8, dye transfer was detected in an average of six neighboring cells in amphetamine-treated cardiomyocytes. In control cells, minimal dye transfer to neighboring cells was observed. The increased cell communication between cardiomyocytes induced by amphetamine was blocked by SP600125 and JNK1 dsRNAi.

4. Discussion

In this study, we demonstrated that amphetamine enhanced not only Cx43 mRNA and protein synthesis but also intercellular coupling in cultured cardiac myocytes. Cx43 mRNA and protein were upregulated in a time-dependent manner by amphetamine. Amphetamine induced Cx43 protein expression both in neonatal and adult cardiac myocytes. Amphetamine blood concentrations of 40 µM have been reported in some fatal cases with amphetamine abuse [20]. Plasma amphetamine concentrations of 1 µM from...
authentic toxicology cases have also been reported by a sensitive and reliable assay [21]. In this study, all three different doses of amphetamines from 1 to 100 μM increased Cx43 protein expression. Therefore, the effective concentration of amphetamine used in our in vitro studies is within the range that might be relevant to account for pharmacological actions of the drug. Cx43 is essential for cardiac electrical stability and altered gap junction expression is arrhythmogenic in vivo [22]. Reduction in the abundance of Cx43 protein through targeted deletion of a Cx43 allele directly leads to slowed ventricular conduction [23]. Recently, Nikolski et al. [24] demonstrated that Cx43 is the substrate of atrioventricular nodal reentrant tachycardia. These studies suggest that Cx43 is the possible molecule that could play a role in conduction heterogeneity. Simpson [25] reported that amphetamine increased blood

Fig. 8. Dye-transfer experiments for measuring cell–cell coupling in control cardiomyocytes (A), amphetamine-treated cardiomyocytes (B), addition of SP600125 before amphetamine treatment (C), and using silencing interference RNA for JNK1 before amphetamine treatment (D). Arrow indicates the dye injection site.
pressure and heart rate in rat. The illicit use of amphetamines may also lead to tachyarrhythmias and sudden death [5,6]. Hypertension increased Cx43 in aortic smooth muscle cells, but not in cardiac muscle [26]. Taken together with our study, the increased Cx43 in cardiac myocytes may contribute to the dysrhythmia induced by amphetamine.

JNK and MAP kinase have been reported to be important intracellular signaling pathways that regulate Cx43 [27,28]. In this study, we demonstrated the complete inhibition of Cx43 by SP600125, a potent inhibitor of JNK [29] and partial inhibition of Cx43 by PD98059, a potent p42/p44 MAP kinase inhibitor. We also demonstrated that the JNK1 dsRNAi completely inhibited the Cx43 expression induced by amphetamine. Double-stranded RNAi can regulate gene expression at a translational level through interactions with its target messenger RNA [30]. JNK1 dsRNAi has been demonstrated to successfully block the JNK pathway [31]. The activation of JNK activity in cardiac myocytes after addition of amphetamine is correlated with the upregulation of Cx43. These data implicated that JNK pathway, but not the p42/p44 MAP kinase pathway, is the major pathway involved in the induction of Cx43 by amphetamine. Our findings differ from those of Petrich et al. [28] who have demonstrated that activation of JNK mediates the down-regulation of Cx43 in cardiac myocytes under the stimulation of anisomycin (a protein synthesis inhibitor) and sorbitol (an osmotic stressor). Petrich et al. [28] used transgenic heart-specific overexpression of a JNK activator to show the detrimental effects on Cx43 expression. There are two possible reasons to explain the contradictory findings between these two studies: First, it is likely that different stress may lead to activation or inhibition of Cx43 through the JNK signal pathway in cardiac myocytes and, second, different model of experiments (in vitro vs. in vivo) may result in different findings.

Ap-1 is a family of dimeric transcription factors comprised of either c-Jun homodimers or heterodimers of certain Jun/Fos family members. AP-1, a well-characterized downstream target of JNK, has been demonstrated to be needed for maximal Cx43 promoter activity in human myometrial smooth muscle [32]. Activation of AP-1 is highly regulated. Not only must it be a dimer, but it must also be trans-activated, which means the components of AP-1 are phosphorylated. In this study, we demonstrated that amphetamine stimulation of AP-1 DNA binding required at least phosphorylation of the c-Jun protein since JNK inhibitor and JNK1 dsRNAi abolished the AP-1 binding activity. C-Jun protein is phosphorylated by JNK. The increased phosphorylation of c-Jun by amphetamine indicated that JNK was activated. Therefore the phosphorylated c-Jun decreased after the activity of JNK was inhibited by the addition of JNK inhibitor, SP600125. These data implicated that amphetamine modulated activity of the AP-1 transcription factor via a JNK pathway. The JNK pathway is activated by amphetamine and is intimately linked with the regulation of AP-1 transcription activity. Activated JNK is capable of binding the NH2-terminal activation domain of c-Jun, activating AP-1 by phosphorylating its component c-Jun. AP-1 can then translocate into the nucleus to promote transcription of downstream genes. The protein levels of Cx43 increased markedly (3- to 4-fold) within 4 h after addition of amphetamine. This is before any detectable change in mRNA levels. The role for transcription factors (such as Jun/AP-1) mediating the Cx43 expression is therefore crucial in late times, but not in early times. The Cx43 protein stability may be enhanced by amphetamine in the early times.

Alterations in conduction are critical in the pathogenesis of sudden cardiac death due to reentrant ventricular arrhythmia [33]. Gap junctions are essential in maintaining normal cardiac conduction properties. Amphetamine-like psychostimulants are abused worldwide, and addition to these drugs poses enormous social and economic burdens. In this study, we demonstrated for the first time that amphetamine induced Cx43 through JNK and AP-1 pathway. Signal transduction pathways activated by amphetamine may cause remodeling of conduction pathway and lead to the development of anatomic substrate of arrhythmia. Activation of Cx43 may possibly be one of the molecular mechanisms for cardiac arrhythmia and sudden death induced by illicit use of amphetamine. However, the direct relationship of Cx43 activation induced by amphetamine and its clinical cardiac arrhythmia needs further study to ascertain.

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