Phosphatidylinositol Metabolism in Hypertrophic Rat Heart

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The accumulation of inositol 1,4,5-trisphosphate (IP3) after hormonal stimulation has a physiological role, possibly by alteration of Ca2+ levels in cardiac myocyte. However, this accumulation has not been studied under pathophysiological conditions. In this report, we examine phosphatidylinositol metabolism during cellular response to norepinephrine in pressure-overloaded hypertrophic rat heart. After stimulation with norepinephrine, the accumulations of IP3 and diacylglyceride significantly increased in isolated myocytes from stroke-prone spontaneously hypertensive rat (SHRSP) heart, indicating phosphatidylinositol-specific phospholipase C activity increased in SHRSP heart cells. Protein kinase C activity was also enhanced in SHRSP, with a marked increase in particulate activity. We determined the intracellular calcium concentration and found it to be higher in SHRSP than in Wistar-Kyoto (WKY) rats at 30–40 weeks of age. Ca2+ influx was also elevated in SHRSP stimulated by norepinephrine. In SHRSP heart, cytosolic Ca2+ concentration may rise quickly in response to some stimuli, such as α1-adrenergic stimulation, which is shown to be one of the pathways that increases cytosolic Ca2+ levels in hypertrophied rat heart. These data suggest that a part of the phosphatidylinositol-turnover pathway, such as the phosphatidylinositol 4,5-bisphosphate-IP3-Ca2+ pathway or the diacylglycerol-protein kinase C pathway, may play an important role in the development of hypertrophy in SHRSP heart. (Circulation Research 1993;72:966–972)

KEY WORDS • phosphatidylinositol-specific phospholipase C • inositol 1,4,5-trisphosphate • inositol trisphosphate kinase • inositol trisphosphate phosphatase

Previous studies have raised the possibility that catecholamines may be one molecular signal that links increased circulatory demand to myocardial hypertrophy.1–3 It has recently been reported, using neonatal rat heart cell cultures, that norepinephrine (NE)-stimulated hypertrophy is mediated through α1-adrenergic receptors, and that α1-adrenergic stimulation by NE, phorbol esters, and serum induces an increase in the size of cardiac myocytes.4,5 Expression of proto-oncogene c-myc, which increases in growth factor–induced cell division, has also been studied in cardiac myocytes.6 NE has been shown to increase levels of c-myc–encoded mRNA 10-fold over control levels.5 This response was eliminated by α1-antagonists but was not affected by β-adrenergic antagonists.3 These earlier findings suggest that α1-adrenergic stimulation plays an important role in inducing neonatal cardiac myocyte hypertrophy. However, its role in adult cardiac myocyte hypertrophy is not yet clear.

The primary step in the function of many different hormones and neurotransmitters involves receptor-mediated stimulation of the breakdown of plasma membrane inositol phospholipids.6–8 This phosphatidylinositol (PI)-turnover pathway generates two second messengers, inositol 1,4,5-trisphosphate (IP3) and sn1,2-diacylglycerol (DAG).9,10 DAG stimulates membrane-bound, phospholipid-dependent, Ca2+-dependent protein kinase C (PKC),11 while IP3 releases Ca2+ from endoplasmic reticulum stores.12,13 This PI-turnover pathway may have an important role in inducing cardiac myocyte hypertrophy, even in adult rat myocytes. Recently, we showed that the PI-turnover pathway was more active in cardiomyopathic hamster heart cells than in control cells.14 Cardiomyopathic hamster heart cell develops hypertrophy without any pressure overload. Therefore, whether PI-turnover pathways increase in myocytes with a pressure overload remains to be shown. We reported earlier that basal phosphatidylinositol and polyphosphoinositide metabolism15 and IP3 kinase activity16 increased in spontaneously hypertensive rat heart without any stimulation. The purpose of the present experiment was to study the polyphosphoinositide metabolism in pressure-overloaded hypertrophic adult rat heart subjected to α1-adrenergic stimulation.

Materials and Methods

Chemicals
d-[inositol-2-3H]-{1,4}-bisphosphate (IP3, 2–10 Ci/ mmol), d-[inositol-1-3H]-{1,4,5}-trisphosphate (17 Ci/
Experimental Protocol

Experiments were conducted on male stroke-prone spontaneously hypertensive rats (SHRSP) aged 5, 10, 20, 30, and 40 weeks and age-matched male Wistar-Kyoto (WKY) rats. Each age group comprised six animals. The left ventricle was excised from the rat heart, and blood was carefully washed out before weighing. Six experiments with cells from different hearts were used for each assay at each age. Each assay was performed in triplicate.

Cell Preparation

Cardiac myocytes of SHRSP and WKY rats were prepared in phosphate buffer (PB) according to a previously reported method,14,17 cultured in Ham F-10 medium with 10% fetal calf serum (FCS), and used within 2 hours for labeling of radioisotope. There were no differences in yield of myocytes between SHRSP and WKY rats. (We were able to prepare about 107 cells/heart, which were 5 weeks old.) The freshly prepared cells were maintained at 37°C in a humidified 5% CO2–95% air atmosphere.18 In this medium (0.3 mM CaCl2), more than 90% of cells were rod shaped and did not beat until the addition of NE and 1 mM CaCl2 (final concentration). The cells were lightly attached to the dishes. The viability of myocytes used for assay was determined by microscopic observation after resuspending the cells in 1.0 mM Ca2+. Myocytes were considered viable when they had a rod-shaped appearance and showed no evidence of blebs or granulations. Only cell suspensions with a viability greater than 90% were used for assay.

Cellular Response to NE

For determination of the cellular response of PKC activity to NE, myocytes (3×104) were prelabeled with 50 μCi [3H]myo-inositol for 24 hours in 10 ml PB with 0.3% FCS in 10-cm dishes. The cells were then washed with PB three times in 50-ml Falcon centrifuge tubes and subcultured (3×105 cells/dish) in 1 ml of PB containing 1 mM CaCl2 in 35-mm dishes for assays.14 The preliminary experiments revealed that the incorporation of [3H]myo-inositol into cardiac cells from WKY rats reached 3.5±0.6% at 16 hours of incubation and plateaued for 24–72 hours. Its maximal incorporation rates into SHRSP and WKY rats were 4.6±1.0% and 4.9±1.0%, per 10-cm dish, respectively. In these experiments, cells were labeled with [3H]myo-inositol for 24 hours at 37°C. The cell viability after labeling was about 90%. Cells were incubated with the indicated concentrations of NE, 5 mM 2,3-DPG,19 and 10 mM LiCl for the indicated periods in the presence of 1 μM metoprolol (CIBA-GEIGY, Osaka, Japan) to preclude the effect of β1-adrenergic receptor stimulation. The assay was terminated with 30 μl of 1 N HCl, and then lipids were extracted with ice-cold 3 ml chloroform/methanol (2:1 vol/vol).20 The incubation mixture was centrifuged at 2,500 rpm for 5 minutes at 4°C, and then the aqueous phase was applied to an AG1×8 column. Inositol monophosphate (IP1), IP2, IP3, and IP4 were separated by elution from AG1×8 columns in formate form (100–200 mesh) by a gradient of ammonium formate (0.2–1.2 M) plus 0.1 M formic acid.21,22 To compare the migration of these fractions from AG1×8, the aliquots (10,000 dpm each from IP1 and IP2) of samples from SHRSP heart cells treated with 1 μM NE were desalted by elution from Dowex chloride column, filtered, and separated by high-performance liquid chromatography (HPLC, Whatman Partisil 10 SAX anion-exchange column with a guard column) with a gradient of ammonium formate and phosphate.23 Figure 1 shows the recoveries in these fractions from AG1×8 column. The recoveries of IP3 and IP4 were 70±3% and 60±5%, respectively. Each fraction was clearly separated. These preliminary experiments revealed that AG1×8 column chromatography instead of HPLC was enough to separate polyphosphoinositides. This column chromatography system was used in this study. For determination of diacylglyceride release, cells were prelabeled for 24 hours with [3H]arachidonic acid (1 μCi/dish) as described above. The preliminary experiments revealed that the incorporation of [3H]arachidonic acid into cardiac cells from WKY rats reached 16.5±0.6% at 16 hours of incubation and plateaued for 24 to 72 hours. Its maximal incorporation rates into SHRSP and WKY rats were 22.6±1.0% and 22.5±1.0%, per 10-cm dish, respectively. Cardiac myocytes (3×105 cells per 35-mm dish) were incubated at 37°C for up to 15 minutes with NE in 1 ml of PB.
containing 1 mM CaCl₂. The released DAG and free arachidonic acid were extracted by the method of Folch et al.²⁰ The chloroform phase was pooled and evaporated under vacuum. The residues were applied to thin-layer chromatography, which was developed in diethyl ether-acetic acid (96:4 [vol/vol]) and then again in a solvent system containing petroleum ether/diethyl ether/acetic acid (90:10:1 [vol/vol/vol]).²⁴ The respective spots of monoglyceride, diglyceride, triglyceride, and arachidonic acid were scraped, counted with a scintillation spectrometer, and analyzed according to previous studies.²⁸

Protein Kinase C

Myocytes used in assay of PKC were suspended at 8×10⁴ to 20×10⁴ cells/ml in Hanks’ balanced salt solution containing 1 mM MgCl₂, 1 mM CaCl₂, 15 mM glucose, and 0.5% bovine serum albumin in siliconized 25-ml Erlenmeyer flasks and gassed with 95% O₂–5% CO₂ at 37°C. After treatment with 10⁻⁶ M NE in 1.0 ml cell suspension in the presence of 1 μM metoprolol for 10 minutes, the assay solution was pelleted by centrifugation in microfuge for 3–5 seconds, the medium was removed, and cells were quickly suspended and homogenized with Teflon-glass homogenizer at 10⁴ cell/ml in a cold buffer containing 0.25 M sucrose, 20 mM HEPES, 5 mM EGTA, 5 mM dithiothreitol (DTT), 1 mg/ml fatty acid-free bovine serum albumin, and 50 μg/ml leupeptin (pH 7.5). The homogenate was centrifuged at 700g for 10 minutes, and the resultant supernatant was centrifuged at 9,000g for 20 minutes. The pellet obtained was washed twice with the same buffer. The supernatant was centrifuged at 105,000g for 60 minutes. The particulate fraction was resuspended in an equal volume of homogenizing buffer. Both cytosolic and particulate fractions were treated with 0.4% Triton X-100 for 1 hour on ice and then diluted with extraction buffer (1:3) immediately before assay. PKC activities in soluble and particulate fractions were assayed by measuring Ca²⁺- and phospholipid-dependent transfer of [³²P]ATP to histone H1. The reaction mixture contained 25 mM HEPES, 10 mM MgCl₂, 5 mM DTT, 20 μg histone, 40 μM [³²P]ATP (0.5 μCi), and 0.5 mM EGTA plus or minus 2.5 μg phosphatidyl-serine, 50 ng diolein, 0.75 mM CaCl₂, and 10 μl of sample in a total volume of 100 μl (pH 7.5). Phosphorylated histone H1 was isolated and measured as previously described.¹¹,²⁶ PKC activity was calculated as the increase in activity produced by lipids and excess Ca²⁺ above activity observed with EGTA alone.

Measurement of Intracellular Ca²⁺

Cytosolic Ca²⁺ concentrations ([Ca²⁺]c) were also measured using fura 2-AM (acetoxyethyl ester)-loaded myocytes. For loading with fura 2-AM, cell suspensions (1x10⁶/ml) were incubated with 5 μM of fura 2-AM for 30 minutes in PB containing 0.1% bovine serum albumin. Cells were then washed with Hanks’ balanced salt solution with 1.26 mM calcium and incubated in 0.7 ml of Hanks’ balanced salt solution (also containing 1.26 mM Ca²⁺). The ratio of excitation (340 nm) and emission (380 nm) wavelengths was monitored by fluorospectrometry.¹⁴ The [Ca²⁺]c was calibrated by exposing cells to 4–10 μM ionomycin followed by the addition of 6.6 mM EGTA.

Measurement of Ca²⁺ Influx

Cells were prepared in 35-mm Petri dishes as described above. For determination of Ca²⁺ influx, cells were washed with Hanks’ balanced salt solution with 1.26 mM Ca²⁺ and incubated in 0.7 ml of Hanks’ balanced salt solution (containing 1.26 mM Ca²⁺) in the presence of NE (1 μM), and 1 μCi of ⁴⁰Ca²⁺ with or without metoprolol (1 μM) for 1 minute at 37°C. Thereafter, cells were scraped from dishes, filtered through a 0.45-μm Millipore filter, washed three times with 4 ml of Hanks’ balanced salt solution, and counted in 10 ml of Aquasol.²⁷

Other Methods

Protein was determined by the method of Lowry et al.²⁸ with bovine serum albumin as the standard.

Statistical Analysis

Six assays in triplicate were analyzed in all experiments. Results are expressed as mean±SEM. Statistical significance was estimated using the previously described method (ANOVA), taking p<0.05 as the limit of significance.²⁹

Results

Left Ventricular Weight

At 5 weeks old, the left ventricular weight (mg/100 g body wt) of SHRSP was not different from WKY rats. The ventricular weight was found to increase with age. This weight increase was greater in SHRSP aged 10–40 weeks than in age-matched WKY rats (Figure 2).

Cellular Responses by NE

We determined the effects of NE on IP₃ and DAG release from [³H]myoinositol prelabeled myocytes. When myocardial cells isolated from 10-week-old SHRSP and WKY rats were incubated with the indicated concentrations of NE with 1 μM of metoprolol, IP₃ rapidly decomposed and IP₃ was released from the myocardial cells (10⁻⁸ to 10⁻⁶ M) (Figure 3). In SHRSP, this IP₃ release was higher than in WKY rats for all concentrations of NE used, Figure 4 shows the time course of IP₃ release induced by 10⁻⁶ M NE. A detectable IP₃ release occurred within 15 seconds and continued for 90 seconds. The release of IP₃ was also observed.

![Figure 2](image-url)
FIGURE 3. Graph showing the effects of norepinephrine (NE) on inositol 1,4,5-trisphosphate (IP3) release in cells of stroke-prone spontaneously hypertensive rats (■) and Wistar-Kyoto (○) rats aged 10 weeks determined as described in "Materials and Methods" in the presence of 1 μM metoprolol. **p<0.001 compared with Wistar-Kyoto rats.

in WKY rats during the 15–90-second interval but was smaller than that observed in SHRSP. The time course of IP3 is slightly longer compared with other cells. A distinct possibility exists that nonbeating cardiac myocytes proceed through different metabolic pathways. This point merits investigation. To determine the effect of NE on α1-adrenergic receptor–mediated myocardial cell phospholipase C activity, we also determined DAG release from the myocytes. The release of DAG from myocytes increased markedly during stimulation with NE in the presence of 1 μM metoprolol (Figure 5). This release was higher in SHRSP than in WKY rats for all concentrations of NE used.

As shown in Figure 6, the effect of age on IP3 was determined. The release of IP3 in isolated NE-stimulated cells was significantly higher in SHRSP than in WKY rats. The increase in IP3 release in SHRSP aged 10–40 weeks was significant (p<0.001) compared with SHRSP aged 5 weeks. This increase was also significant in WKY rats (p<0.05, aged 10 weeks; p<0.001, aged 10–40 weeks) compared with WKY rats aged 5 weeks.

It is well known that DAG stimulates PKC activity. In hypertrophic cells from SHRSP, DAG release was higher in SHRSP than in WKY rats, as described above. It is possible that DAG stimulates PKC in SHRSP. We also determined PKC activity after treatment with NE and found it to be higher in SHRSP heart cells aged 5–40 weeks than in WKY rats (Figure 7). The PKC activity in the particulate was especially enhanced in SHRSP (Figure 7A). The largest rate increase for PKC activity occurred at 5–10 weeks of age. This increase is similar to that found in left ventricular weight at the same ages (Figure 2). The PKC activity in the cytosolic fraction was observed and found to increase with age. However, the PKC activity was lower than that found in the particulate fraction (Figure 7B).

Intracellular Ca2+ may stimulate the activities of phospholipase C, PKC, and protein synthesis. Intracellular Ca2+ concentration and Ca2+ influx were also

FIGURE 4. Graph showing the effects of norepinephrine on inositol 1,4,5-trisphosphate (IP3) release. Time course of IP3 release in cells of stroke-prone spontaneously hypertensive rats (■) and Wistar-Kyoto (○) rats aged 10 weeks were determined as described in "Materials and Methods" by the stimulation of norepinephrine (1 μM) in the presence of 1 μM metoprolol. **p<0.001 compared with Wistar-Kyoto rats.

FIGURE 5. Graph showing the effect of norepinephrine (NE) on diacylglyceride (DAG) release in cells of stroke-prone spontaneously hypertensive rats (■) and Wistar-Kyoto (○) rats aged 10 weeks determined as described in "Materials and Methods" in the presence of 1 μM metoprolol. **p<0.001.

FIGURE 6. Graph showing the effect of age on inositol 1,4,5-trisphosphate (IP3) release. IP3 release in cells of stroke-prone spontaneously hypertensive rats (■) and Wistar-Kyoto (○) rats aged 5 to 40 weeks were determined as described in "Materials and Methods" by the stimulation of norepinephrine (1 μM) in the presence of 1 μM metoprolol. *p<0.05 and **p<0.001 compared with Wistar-Kyoto rats.
determined to study whether a relation exists between intracellular Ca\(^{2+}\) and myocardial cell hypertrophy. The basal intracellular Ca\(^{2+}\) concentration without NE stimulation was higher in older SHRSP than in age-matched WKY rats. There were no significant differences in intracellular Ca\(^{2+}\) levels between SHRSP and WKY rats aged 5–20 weeks. However, SHRSP aged 30–40 weeks had significantly higher Ca\(^{2+}\) levels than those in age-matched WKY rats (Figure 8). The influx of Ca\(^{2+}\), stimulated by 1 \(\mu\)M NE, was also determined and found to be higher in SHRSP aged 5–40 weeks than in age-matched WKY rats (Figure 9).

**Discussion**

In the present experiments performed using isolated cardiac cells, we demonstrated that phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) breakdown was followed by the release of IP\(_3\) and DAG. This release was more pronounced in SHRSP than in WKY rats.

In many tissues, the physiological second messenger mediating the intracellular response to \(\alpha\)-adrenergic stimulation is 1,4,5-IP\(_3\). The metabolism of 1,4,5-IP\(_3\) has been studied extensively in many types of tissues. The results of these studies indicate that dephosphorylation to 1,4-IP\(_2\) and phosphorylation to 1,3,4,5-IP\(_4\) are the immediate metabolic routes.\(^{30-35}\) The 1,4-IP\(_3\) is further dephosphorylated to 4-IP while 1,3,4,5-IP\(_4\) is dephosphorylated to form another isomer of inositol trisphosphate, 1,3,4-IP\(_3\).\(^{23}\) The 1,4,5-IP\(_3\) mobilizes intracellular Ca\(^{2+}\) and as such produces physiological alterations in the characteristics of specific hormonal stimulation. In human cardiac tissue, the \(\alpha\)-adrenergic receptor-mediated increase in IP\(_3\) has been previously reported.\(^{36}\) It was also reported that \(\alpha\)-adrenergic stimulation caused IP\(_3\) to accumulate in adult canine myocytes.\(^{37}\) These reports suggest that the accumulations of IP\(_3\) and IP\(_4\) after hormonal stimulation play a physiological role, possibly by altering Ca\(^{2+}\) levels in cardiac tissues. However, studies concerning the accumulation of inositol polyphosphate under pathophysiological conditions are few. We recently reported that IP\(_3\) release increased in cardiomyopathic hamster heart cells\(^{14}\) and IP\(_3\) kinase markedly increased in SHRSP heart.\(^{15}\) The present experiment investigated IP\(_3\) breakdown, and IP\(_3\) release was shown to increase in SHRSP hearts.

In isolated SHRSP cells, Ca\(^{2+}\) influx was higher than in WKY rats aged 5–40 weeks. On the other hand, there were no significant differences in basal intracellular Ca\(^{2+}\) concentration between SHRSP and WKY rats aged 5–20 weeks. It is not clear whether 1,3,4,5-IP\(_4\) affected Ca\(^{2+}\) influx. Furthermore, \(\alpha\)-adrenergic stimulation may regulate intracellular Ca\(^{2+}\) concentration through some other mechanisms. For example, phosphatidic acid, a metabolite of phospholipids, increases Ca\(^{2+}\) influx as calcium ionophore.\(^{38,39}\) \(\alpha\)-Adrenergic stimulation activates Ca\(^{2+}\) channels,\(^{40,41}\) yet suppresses the Ca\(^{2+}\) pump.\(^{42}\) PKC regulates Ca\(^{2+}\) concentration by mediating Na\(^{+}\)-Ca\(^{2+}\) exchange.\(^{43,44}\) These routes for Ca\(^{2+}\) entry into a cell should be studied in hypertrophic heart.

**Figure 7.** Graphs showing the effect of age on PKC activity. PKC activity in cells of stroke-prone spontaneously hypertensive rats (●) and Wistar-Kyoto (○) rats aged 5–40 weeks were determined as described in “Materials and Methods” by the stimulation of norepinephrine (1 \(\mu\)M) in the presence of 1 \(\mu\)M of metoprolol. Panel A: Particulate fraction; Panel B: cytosol fraction. **p<0.001 compared with Wistar-Kyoto rats; *p<0.05 compared with Wistar-Kyoto rats.

**Figure 8.** Graph showing the effect of age on basal cytosolic free Ca\(^{2+}\) concentration. The intracellular free Ca\(^{2+}\) concentration using fura-2 AM–loaded cardiac myocytes was determined as described in “Materials and Methods.” ●, Stroke-prone spontaneously hypertensive rats; ○, Wistar-Kyoto rats. **p<0.001 compared with Wistar-Kyoto rats.

**Figure 9.** Graph showing the effect of age on Ca\(^{2+}\) influx. Ca\(^{2+}\) influx in stroke-prone spontaneously hypertensive rats (●) and Wistar-Kyoto (○) rats aged 5–40 weeks was determined as described in “Materials and Methods” by the stimulation of norepinephrine (1 \(\mu\)M) in the presence of 1 \(\mu\)M of metoprolol. **p<0.001 compared with Wistar-Kyoto rats.
cells. It has also been reported that PKC inhibits Ca\(^{2+}\) influx in aortic smooth muscle cells.\(^{45}\) In our cells, activated PKC could suppress Ca\(^{2+}\) influx. However, 1,3,4,5-IP\(_3\) and 1,4,5-IP\(_3\) may interact to release Ca\(^{2+}\) from the sarcoplasmic reticulum.\(^{46}\) Therefore, the intracellular Ca\(^{2+}\) concentration in SHRSP could be increased by NE in older ages. These results suggest that the intracellular calcium handling is disturbed in older ages in SHRSP. On the other hand, it appears to be well preserved in the early stages of hypertrophic heart in SHRSP. These results were also supported by a previous study that reported calcium uptake into sarcoplasmic reticulum to be preserved in hypertrophic cardiomyopathy hamsters compared with dilated cardiomyopathy hamsters.\(^{44}\)

According to a previous study, the stimulation of  \(\alpha_1\)-adrenergic receptor causes PI breakdown in heart cells,\(^{47-50}\) and the number of  \(\alpha_1\)-adrenergic receptors is greater in spontaneously hypertensive rats and hypertrophic hearts.\(^{49}\) In our experiments, NE enhanced IP, and DAG release in SHRSP hearts. The stimulatory effect of NE in SHRSP myocyte may be caused not only by the increased number of  \(\alpha_1\)-adrenergic receptors on the cellular membrane but also by the increased basal activity of this enzyme. Our study may have indicated one of the pathways that increases the cytosolic Ca\(^{2+}\) level in genetically hypertrophic rat hearts. Further experiments using experimental hypertension are under consideration.

The present findings suggest that the enhanced PIP\(_2\)→IP\(_3\)→Ca\(^{2+}\) or DAG-PKC pathway may increase protein synthesis in the SHRSP heart and contribute to the development of pressure-overloaded cardiac hypertrophy.

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