# Calcium Signaling, Calcineurin and NFAT3 Molecular Pathway in Reprogramming of Fetal Gene (BNP) Expression in Cardiac Hypertrophy and Inhibitors of Cardiac Hypertrophy-Review

**Kousalya.P[1,2*] and Doss VA[3]**

[1]*Research Scholar, Department of Biochemistry, PSG College of Arts & Science, Coimbatore-641014, Tamilnadu, India.*
[2]*Assistant Professor, PG and Research Department of Biochemistry, Bharathidasan College of Arts and Science, Erode-638116, Tamilnadu, India.*
[3]*Associate Professor, Department of Biochemistry, PSG College of Arts and Science, Coimbatore-641014, Taminadu, India.*
[1,2]kousiviswa@gmail.com,[3]victordoss64@gmail.com

***Corresponding Author:** Kousalya.P- Research Scholar, Department of Biochemistry, PSG College of Arts & Science, Coimbatore-641014, Tamilnadu, India.Email-Id: kousiviswa@gmail.com

***Abstract****: G-Protein Coupled Receptor (GPCR) plays a major role in cardiac hypertrophy. Isoproterenol is an agonist which binds to GPCR. This results in the activation of GS subunit. Activated Gs subunit stimulates cAMP mediated pathway. This activates the calcium signaling and elevates the calcium level. Increased calcium activates the phosphatase activity of calcineurin. Activated calcineurin dephosphorylates NFAT in cytoplasm. Dephosphorylated NFAT translocates to nucleus and binds to target region in DNA and activates re-expression of fetal genes in synergy with GATA-4 trascription factor. Reprogramming of fetal genes by NFAT-3 and GATA-4 results in cardiac hypertrophy. Calcineurin inhibitors and rephosphorylation of NFAT can prevent cardiac hypertrophy.*

**Key Words:** Calcium signaling, Cardiac hypertrophy, Isoproterenol, Calcineurin, NFAT, B-type natriuretic peptide (BNP).

## 1. Introduction

The heart consists of cardiac myocytes, non-myocytes and the extracellular matrix surrounding them. Cardiac myocytes are the muscle cells of the heart such as fibroblast, mast cells, endothelial cells and smooth muscle cells[1]. Cardiac hypertrophy is a response that is adaptive to pressure overload, volume stress and sarcomeric protein mutation. Many types of heart disease are characterized by hypertrophic development especially hypertension, ischemic disease, valvular disease and heart failure[2–4]. Cardiomyocyte hypertrophy is associated with a significant increase in size of the cell, elevated protein synthesis and expanded sarcomere organization at cellular level[5].

## 2. Physiological and Pathological Cardiac Hypertrophy

Cardiac hypertrophy is classified as physiological cardiac hypertrophy and pathological cardiac hypertrophy (Figure-1). Pathological cardiac hypertrophy is accompanied by disease like hypertension. It is the major risk factor for the failure of the heart[6]. Elevated interstitial fibrosis cardiac dysfunction and cell death are correlated with pathological hypertrophy[6]. Physiological cardiac hypertrophy is due to prolonged exercise. It is defined by normal morphology of heart. There is no fibrosis and apoptosis of cells. In physiological cardiac hypertrophy cardiac function is increased or normal[6].

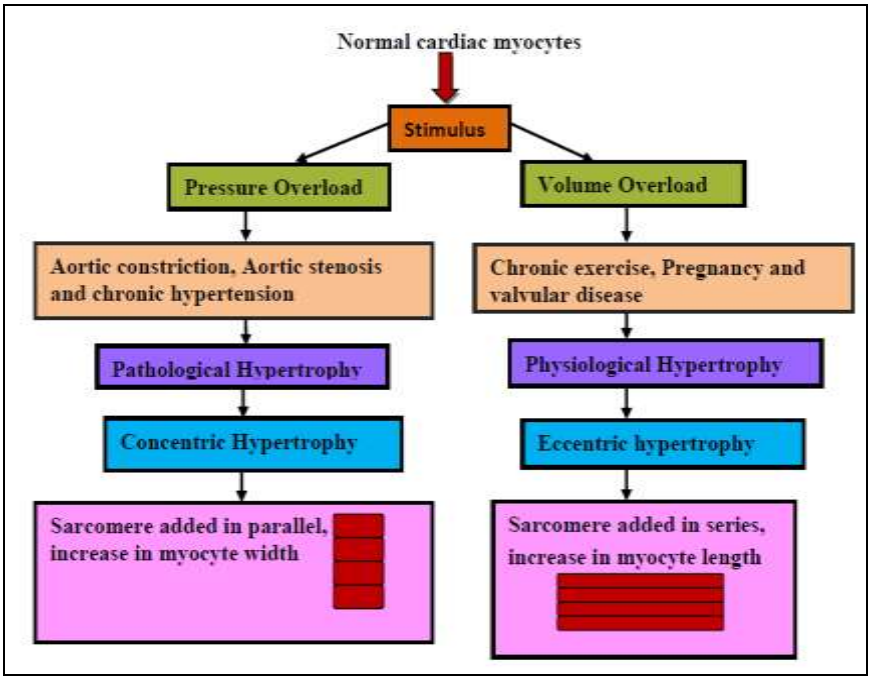


**Figure-1: Physiological and Pathological Cardiac Hypertrophy**

Physiological and pathological hypertrophy is categorized as concentric and eccentric hypertrophy. This classification is based on shape changes[5]. Prolonged left ventricular pressure overload due to pathological stimuli primarily induces thickening of wall and concentric hypertrophy. In concentric hypertrophy addition of sarcomere in parallel contributes to an increase in the width of myocyte cells[6]. Prolonged left ventricular volume overload due to physiological stimuli results in enlargement of chamber and an eccentric hypertrophy(4). In eccentric hypertrophy addition of sarcomere in series contributes to an increase in the length of myocyte cells and increased volume in the chamber[6].

## 3. Cardiac Energy Metabolism for Normal Heart and Hypertrophised Heart

The distortion of the metabolism of the cardiac energy substrate plays a crucial role in failure of the heart. In normal heart fatty acid is the primary metabolic pathway which gives highest energy. In pathological condition glucose is utilized as a primary substrate for metabolism to produce energy[7].

## 4. Calcium Signaling in Cardiac Hypertrophy

Calcium is an important second messenger[8]. Calcium plays an essential role in the regulation of heart contractility, fertilization, growth, differentiation and gene expression[9,10]. Hypertrophic cardiac development and cardiac failure are triggered by calcium [9]. Calcium level in the cells are elevated by the stimuli[10]. Calcium regulates the cellular activities through calmodulin, a calcium binding protein[11]. Increased expression of calmodulin cause cardiomyocyte hypertrophy[12]. Increased calcium via calmodulin stimulates the calcineurin activity[13].

## 5. Activation of G-Protein Coupled Receptors (GPCR) by Isoproterenol

G-protein coupled receptor is made up of a seven transmembrane α-helix structure. Adrenergic receptors are major class of G-protein coupled receptors (GPCR)[14,15]. There are three subtypes of β-Adrenergic receptors are present, they are β1-AR, β2-AR and β3-AR. Among these adrenergic receptors, β1 adrenergic receptor (80%) is the predominant type found in cardiac[14–16]. Stimulation or increased expression of Gαs coupled β1-AR by agonist cause cardiac hypertrophy[17–19].

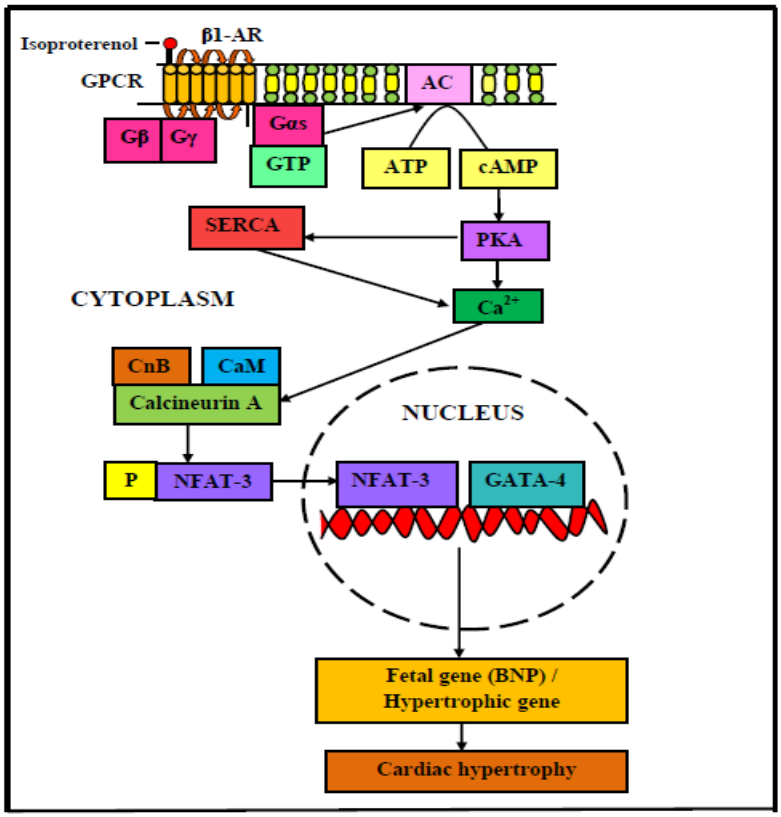


**Figure-2: Molecular mechanism of cardiac hypertrophy.** NFAT-3-Nuclear Factor of Activated T cell, PKA-Protein Kinase A, CnB-CalcineurinB, AC-adenylyl cyclase, CaM-Cadmodulin, GATA-4-transcription factor, SERCA- Sarco-Enoplasmic Reticulum $Ca^{2+}$ ATPase and B-type natriuretic peptide (BNP).

Isoproterenol is an agonist for β1-AR. Binding of isoproterenol to Gs coupled β1-AR induce cardiac hypertrophy through Gαs-cAMP mediated signaling pathway[17,20–22]. Binding of isoproterenol to Gαs coupled β1-AR stimulates the adenylyl cyclase (AC). Activated adenylyl cycle stimulate the formation of second messenger cAMP from ATP. This cAMP activates the protein kinase A (PKA). Activated PKA phosphorylares membrane ion channels and increase the Ca2+ cycling. Increased calcium stimulates the calcineurin activity through calmodulin. Activated calcineurin dephosphorylates the NFAT in cytosol (Figure-2). Dephosphorylated NFAT enters the nucleus and activates transcription factors and results in cardiac hypertrophy[17,21,23–25].

**6. Activation of Calcineurin and NFAT Molecular Pathway by Calcium Signalling**

Calcineurin, a serine threonine phosphatase which is also recognized as protein phosphatase 2B or PP2B which is foundin cytoplasm (Figure-3). Calcineurin is an enzyme complex which is regulated by calcium. Calcineurin is a heterodimer consists of two subunits calcineurin A (CnA)- 60 kDa catalytic subunit and calcineurin B (CnB)-19 kDa regulatory subunit[26–28]. Calcineurin A (CnA) has a catalytic domain, a domain for binding of calcineurin B (CnB), a domain for calmodulin(CaM) binding and an auto inhibitory domain(AID)[27,29,30]. Calcineurin A is encoded by three distinct genes in mammals named as α, β and γ. Calcineurin Aγ is expressed in testis. Calcineurin Aα and calcineurin Aβ is expressed in heart (26).Calcineurin B(CnB) contains four conserved Ca2+ binding EF-hand motifs[27,29,30]. Calcineurin B is encoded by two distinct gene- calcineurin B1 and calcineurin B2[31,32].

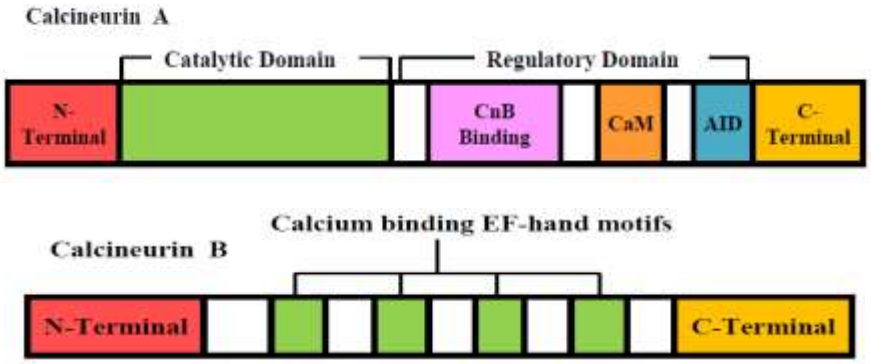


**Figure-3: Calcineurin A and Calcineurin B**

In the unactivted cells phosphatase activity of calcineurin is inhibited by auto inhibitory domain by interacting with catalytic cleft[33,34]. In the activated cell, elevated calcium concentration stimulates the calmodulin which replaces the auto inhibitory domain in the catalytic domain. This results in binding of CnB to CnA and activation of calcineurin[33,34]. The activated calcineurin dephosphorylates NFAT present in the cytoplasm and dephosphorylated NFAT translocates to the nucleus[33–36]. NFAT is the substrate for calcineurin[33,37].

Nuclear factor of activated T cells (NFAT) is a transcription factor which is involved in differentiation of adipocyte, expression of cytokine gene and cardiac hypertrophy[38–40]. Five genes (NFATc1, NFATc2, NFATc3, NFATc4 and NFATc5) encoding NFAT protein were identified and named as NFATc1 (NFATc/NFAT2), NFATc2 (NFATp/NFAT1), NFATc3 (NFAT4/NFATx), NFATc4 (NFAT3) and NFAT5 (Ton EBP)[41–45].

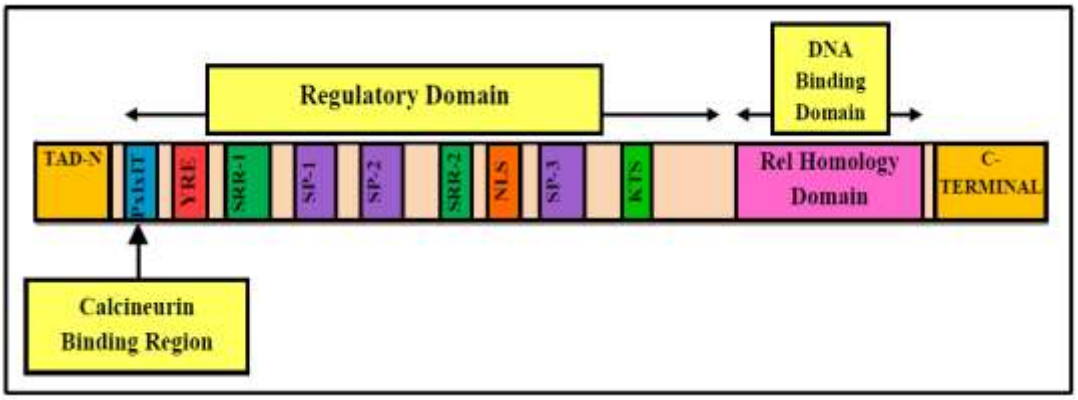


**Figure-4: Calcineurin binding region and DNA binding region in NFAT**

NFAT structure contains regulatory NFAT homology domain at NH2 terminal which is conserved and DNA binding domain of rel homology family of transcription factor at COOH terminal[42,46,47]. Regulatory domain contains 300 amino acids and in a single exon this regulatory domain is encoded[48]. Trans activation domain (TAD) is present at NH2 terminal and regulatory domain contains calcineurin binding region, serine rich motifs and nuclear localization sequence (NLS)[30]. The regulatory NFAT homology domain at NH2 terminal contains PXIXIT motifs, serine rich region (SRR) and serine-proline (SP) motifs[41,42]. These sequences were observed in all members of NFAT. Calcineurin activates NFATc1, NFATc2, NFATc3 and NFATc4. NFAT-3 is expressed in heart and other NFAT are expressed in T cells and skeletal muscle[40]. Nuclear translocation of NFAT-3 depends on the activation by calcineurin[49]. NFAT-3 activity is regulated by Ca2+ and calcineurin (Figure-4).

In normal resting cells NFAT proteins are phosphorylated and present in cytoplasm. Regulatory domain contains conserved serine rich motifs (SRR-1,SRR-2,SPxx repeats and KTS) which contains phosphorylated serine[46,50]. Three SPxx motifs (serine proline repeat motifs) is present in NFAT, they are designated as SP-1, SP-2 and SP-3 with sequence [SPxx]SPxxSPxxSPxxxxx[D/E][D/E][50,51]. Conserved YRE region and nuclear localization sequence (NLS) is present in NFAT[50,51].

Calcineurin activates NFAT-3 by dephosphorylation and this dephophorylated NFAT-3 translocate to nucleus and binds to target sequence. In nucleus NFAT-3 in synergy with GATA-4 transcription factors cause reprogramming of fetal genes[49,52]. Calcineurin phosphatase recognize conserved region which is present in regulatory domain at NH2 terminal of NFAT and dephosphorylates the three serine rich motifs in NFAT[49,53,54]. Dephosphorylated NFAT translocates to nucleus and binds to the target sequence in DNA[55,56]. Low concentration of activated calcineurin can dephosphorylate serine residues in SRR-1 region. SRR-1 region is present near to PxIxIT, which is the calcineurin docking region[50,51,56]. Calcineurin docking site (PxIxIT) sequence in NFAT1 and NFAT2 is SPRIEIT, NFAT3 sequence is CPSIQIT and NFAT4 sequence is CPSIRT. In all NFAT proteins PxIxIT motif are polar[51].

In nucleus NFAT-3 binds to target region and activates the transcription factors and hypertrophic genes expression which results cardiac hypertrophy[57]. Inducible phosphorylation site which is located in TAD (Trans Activation Domain) plays an major role in the transcription activity[50]. In cardiac hypertrophy fetal gene reprogramming takes place.

Brain natriuretic peptide (BNP), atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) are expressed in hypertrophised heart[58,59].

**7. Reprogramming and Expression of Fetal Genes by NFAT-3 and GATA-4 Transcription Factors**

In adult myocardium, hypertrophic stimulus cause reprogramming and re-expression of fetal genes[40,60] . Fetal genes like atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and α-skeletal actin (α-SKA) are upregulated in cardiac hypertrophy. Adult cardiac genes like α-MHC, α-cardiac actin, sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) and phospholamban are down regulated during cardiac hypertrophy[40,60].

At carboxyl terminal end NFAT3 contains rel homology domain (RHD) that mediates the DNA binding as monomer or dimer at consensus DNA binding sequence 5'-GGAAAAT-3'[40,61]. NFAT3 trans activate BNP in synergy with GATA4. The BNP promoter is a direct transcriptional target for the transcription factors NFAT-3 and GATA-4. In the presence of GATA-4, NFAT-3, and calcineurin, the BNP promoter was activated by over 100-fold[40]. In BNP promoter binding site for NFAT3 was located at -927 site[40,59,62,63]. The expression of the (BNP) gene is rapidly elevated in the infracted heart, and plasma BNP levels indicate the degree of left ventricular dysfunction and hypertrophy[64,65].

GATA transcription factors (GATA-1-6) are a class of double zinc finger transcription factors (CysX2-CysX17-CysX2-Cys) that selectively bind to the (A/T)GATA(A/G) consensus DNA binding sequence[61]. GATA-4, 5, 6 is expressed in heart. GATA-4 interacts with transcription factors like MEF-2, NFAT, Nkx-2.5, SRF, dHAND, and YY1 with C-terminal zinc finger domain[61]. The N-terminal domain of GATA-4 activity is controlled by numerous signaling pathways as a result of hypertrophic stimulation and is implicated in cardiac hypertrophy development[61].

Atrial natriuretic peptide, B-type natriuretic peptide and C-type natriuretic peptide are the three types of mammalian natriuretic peptides. Both ANP and BNP are expressed in heart [66]. In adult human heart BNP consists of 32 amino acids and synthesized by ventricles of heart. BNP possess diuretic, natriuretic and vasodilator properties[64,65]. BNP is made up of three exons separated by two introns that are encoded by a single gene (NPPB). In atrial tissue, BNP-32 is 60% prominent, while in ventricular tissue, proBNP-108 is 60% prominent [66,67]. After birth, the genes for ANF and BNP are downregulated in the ventricles[58].Their levels rise significantly in the adult mammalian heart during hypertrophy and heart failure. BNP gene expression can be used to assess the progression of hypertrophy[58]. In the process of myocyte hypertrophy and cardiac fibrosis, ANP and BNP plays a major role[68].

Atrial natriuretic factor and brain natriuretic peptide are encoded by the genes Nppa and Nppb, which are located next to each other in the vertebrate genome. Both genes are expressed in distinct patterns in the embryonic and fetal stages. Expression of Nppa and Nppb in ventricular myocardium is reported in certain cardiovascular failure[69].The ventricles are the major source of BNP expression in the embryonic mouse heart, which peaks at midgestation (E12.5)[70]. In human foetuses aged 12–17 weeks, no BNP mRNA nor BNP peptide were found[71,72]. The level of BNP mRNA in the mouse atrium and ventricle increased after birth, but did not reach the mid-gestation level[70].

ANF and BNP level in plasma is elevated after volume overload[73,74]. After atrial pacing, ANF and BNP were rapidly secreted, with a peak around 20–30 minutes later[75]. In ventricular stimulation ANP and BNP is secreted within 1-5 minutes. These results clearly demonstrate that myocytes respond quickly to stress[76]. With the development of hypertrophy and fibrosis, the level of BNP mRNA is increased[77]. BNP mRNA was elevated in the

ventricles of the individuals with chronic volume overload induced by a regurgitant cardiac valve lesion, although ANF mRNA levels were comparable to the control group. BNP gene expression can be used to track the course of hypertrophy[78].

BNP and NT-proBNP were elevated in the individuals with congenital heart defects like systemic right ventricle and univentricular heart[79]. In ventricular cardiomyocytes mechanical stress induces the expression of ANF, BNP and skeletal alpha-actin mRNA[80]. ANP and BNP expression were dramatically increased after isoproterenol stimulation. In rats with isoproterenol-induced cardiac hypertrophy, DCAE (*Dendrobium candidum* aqueous extract)dramatically reduced plasma levels of ANP and BNP[81]. Isoproterenol induced myocardial infarction in rats increases the BNP mRNA in the ventricles after 18 hours[73].

**8. Inhibitors of Calcineurin and NFAT Pathways in Cardiac Hypertrophy**

cGMP-mediated signalling of nitric oxide (NO) and cGMP-dependent protein kinase type I (PKG I) act as a negative regulator of cardiomyocyte hypertrophy. NO-cGMP-PKGI inhibits calcineurin-NFAT mediated hypertrophy by inhibiting the transcriptional activity of NFAT, BNP expression and cell enlargement[82]. Cardiomyocyte hypertrophy is suppressed by telmisartan, a type of angiotensin II receptor inhibitor . Telmisartan inhibits the nuclear translocation of NFAT, expression of ANP and BNP and cardiomyocyte apoptosis[83].

Vitexin, a flavone glucoside derived from the leaf of Crataeguspinnatifida Bunge, inhibits Ca2+ mediated calcineurin-NFATc3 and CaMKII signalling pathways, resulting in prevention of cardiac hypertrophy[84]. In cardiomyocyte hypertrophy, SIRT6, a NAD+-dependent class III histone deacetylase, has been shown to block NFATc4 dephosphorylation and nuclear translocation[85]. Endogenous calcineurin inhibitors include calcineurin-binding protein 1 (CABIN1) and Down's syndrome critical region 1 (DSCR1)[86]. NFAT kinases, including protein kinase A (PKA)[87] as glycogen-synthase kinase 3 (GSK3)[88], casein kinase 1 (CK1)[89],dual-specificity tyrosine-phosphorylation regulated kinase1/2(DYRK1 and DYRK2), extra-cellular signal related kinase (ERK) , p38, c-JUN kinase (JNK) and CK2(formerly casein kinase II)[90] rephosphorylate NFAT and inactivate NFAT proteins[86].

The activity of calcineurin is inhibited by calcineurin inhibitor (CAIN) or calcineurin-binding (CABIN) proteins[91]. Calcineurin interaction with the substrate is blocked by AKAP79 (A-kinase anchoring protein 79) a scaffold protein[92]. Calcineurin homologous protein (CHP) inhibits the phosphatase activity of calcineurin[93]. Calcineurin signaling is inhibited by modulatory calcineurin interacting proteins (MCIP), MCIP is also known as calcipressin/Dscr1/Rcn1)[94].

Gossypol, a polyphenolic aldehyde found in cotton plants, inhibits the phosphatase activity of calcineurin [95]. Phosphatase activity of calcineurin is inhibited by Lie120, a thiazole derivative[96]. In cell lysates, PD 144795, a benzothiophene derivative, inhibits enzymatic activity of Calcineurin[97].The enzymatic activity of calcineurin is inhibited by dibefurin, a fungus-derived phenolic compound [98] . Dipyridamole, a drug used in the treatment of strokes, prevents the interaction of calcineurin with NFAT[99].

NFATc dephosphorylation and nuclear translocation are inhibited by NCI3, a pyrazolopyrimidine derivative[100]. Microcystin LR, Dibefurin, PD 144795, Endothal Derivatives, Cantharidin, and Metal-Ligating Phosphonates are among the natural and synthetic chemicals that have been found to be strong inhibitors of calcineurin[27]. Punicalagin, a compound extracted from the fruit of the Punica granatum plant, inhibits nuclear translocation and DNA binding of NFAT[101]. Imperatorin, a furanocumarin isolated from *Oppopanax chironium*(L.), inhibits NFAT binding to DNA binding[102]. Trifluoperazine

binds to calmodulin, which prevents its interaction with calcineurin[103]. The Roc-1, 2 and 3 Rocaglamide derivatives block NFATc1 translocation into the nucleus, which is caused by activation[104]. Barbiturates that suppress phosphatase activity of calcineurin include thiopental, pentobarbital, thiamylal, and secobarbital[105].

## 9. Conclusion

G-Protein coupled receptor activation by isoproterenol increases the calcium level. Elevated calcium activates phosphatase activity of calcineurin. Active form of calcineurin dephosphorylates NFAT-3 in cytoplasm and this result in NFAT-3 nuclear translocation. In nucleus NFAT-3 in synergy with GATA-4 transcription factor reprograms the expression of fetal genes and hypertrophic genes, this result in cardiac hypertrophy. Calcineurin inhibitors like CsA, FK506, Cabin 1/Cain, AKAP79, CHP, MCIP/calcipressin inhibits the activity of calcineurin. Glycogen synthase kinase-3β, protein kinase A, MAP kinase, casein kinase 1, c-Jun N-terminal kinase are involved in rephosphorylation of NFAT and nuclear export[90]. Dibefurin, norcantharidin, gossypol, thiopental and tarcolimus blocks the enzymatic activity of calcineurin. Trifluoroperazine prevents binding of CaM to calcineurin. Dipyridamole prevents binding of calcineurin to NFAT. BTP1 prevents NFAT dephosphorylation. BTP2 reduces calcium influx into cytoplasm[86,106]. In future phosphorylation and dephosphorylation sites in NFAT3 can be studied in detail, some of the natural plant based bioactive compounds targeting to block the calcineurin and NFAT activity can be identified to prevent cardiac hypertrophy.

## Conflicts of interest statement

The authors declare no conflicts of interest.

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