UTP Transactivates Epidermal Growth Factor Receptors and Promotes Cardiomyocyte Hypertrophy Despite Inhibiting Transcription of the Hypertrophic Marker Gene, Atrial Natriuretic Peptide*

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In neonatal rat ventricular myocytes, activation of receptors that couple to the Go family of heterotrimeric G proteins causes hypertrophic growth, together with expression of “hypertrophic marker” genes, such as atrial natriuretic peptide (ANP) and myosin light chain 2 (MLC2). As reported previously for other Go-coupled receptors, stimulation of α1-adrenergic receptors with phenylephrine (50 μM) caused phosphorylation of epidermal growth factor (EGF) receptors as well as activation of ERK1/2, cellular growth, and ANP transcription. These responses depended on EGF receptor activation. In marked contrast, stimulation of Go-coupled purinergic receptors with UTP caused EGF receptor phosphorylation, ERK1/2 activation, and cellular growth but minimal increases in ANP transcription. UTP inhibited phenylephrine-dependent transcription from ANP and MLC2 promoters but not transcription from myoglobin promoters or from AP-1 elements. Myocardin is a muscle-specific transcription enhancer that activates transcription from ANP and MLC2 promoters but not myoglobin promoters or AP-1 elements. UTP inhibited ANP and MLC2 responses to overexpressed myocardin but did not inhibit responses to c-Jun, GATA4, or serum response factor, all of which are active in nonmuscle cells. Thus, UTP inhibits transcriptional responses to phenylephrine only at cardiac-specific promoters, and this may involve the muscle-specific transcription enhancer, myocardin. These studies show that EGF receptor activation is necessary but not sufficient for ANP and MLC2 responses to activation of Go-coupled receptors in ventricular myocytes, because inhibitory mechanisms can oppose such stimulation. ANP is a compensatory and protective factor in cardiac hypertrophy, and mechanisms that reduce its generation need to be defined.

Hearts respond to a range of pathological insults by increasing muscle mass, caused primarily by hypertrophic growth of the cardiomyocytes and by specific changes in the program of gene transcription. Hypertrophy in response to pressure overload involves heterotrimeric G proteins of the Goq class (1) and activation of Goq is sufficient to cause hypertrophy in vivo (2), as well as in cell models (3). In keeping with this, activation of Go-coupled receptors including α1-adrenergic receptors (α1-AR)1 ET₄ receptors or AT₁ receptors on neonatal rat ventricular myocytes (NRVM) causes increased growth of the cardiomyocytes, as well as increasing transcription from “hypertrophic” marker genes, such as ANP and MLC2 (3). Signaling pathways associated with increased protein synthesis, and hence cell size, are known to be partly independent of those causing re-expression of ANP and MLC2, but both can be activated by Goq (4). Thus, it might be expected that any receptor that activates Goq would initiate hypertrophic growth and marker gene expression in NRVM. In agreement with this, we have recently reported that activation of Goq-coupled purinergic receptors with UTP increases cardiomyocyte size and stimulates downstream MAP kinase activation, in an apparently similar manner to activation of α₁-AR with phenylephrine (PE) (5).

In addition to stimulating phospholipase Cβ (PLCβ) isoforms, Go-coupled receptors can induce transactivation of certain growth factor receptors (6). In NRVM, exogenous AT₁ receptors mediate phosphorylation of epidermal growth factor receptors (EGFR), and this appears to be critical for subsequent growth responses (7). Direct activation of Goq by means of an activating toxin (from Pasteurella multocida) has similar effects, implying Goq mediation (8). However, whether all Goq-coupled receptors transactivate EGFR and whether EGFR activation is critical for growth or gene transcription are still not certain. Possibly of more importance, it is not known whether EGFR activation is sufficient for any of these responses and whether downstream responses to EGFR phosphorylation vary depending on the nature of the stimulus.

In the current studies, we have compared responses to stimulation of purinergic receptors with UTP to stimulation of α1-AR with PE. Both factors caused phosphorylation of EGFR, and both activated ERK1/2 via mechanisms involving EGFR. 

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1 The abbreviations used are: AR, adrenergic receptor; NRVM, neonatal rat ventricular myocyte(s); ANP, atrial natriuretic peptide; MLC2, myosin light chain 2; EGF, epidermal growth factor; EGFR, EGF receptor; PLC, phospholipase C; PE, phenylephrine; ERK, extra-cellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; 2xTRE, TRE2PRL−368; JNK, c-Jun N-terminal kinase; SRF, serum response factor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; InsP, [3H]inositol phosphate; DN, dominant negative; Mb, myoglobin; MKK, mitogen-activated kinase kinase.

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However, α1-AR activation caused increased transcription of ANP, whereas UTP was relatively ineffective, even though it increased cell growth. Recent studies have presented evidence that ANP and the related peptides BNP and CNP have beneficial effects on the hypertrophying cardiomyocytes by limiting cellular growth responses (9). Thus, the finding that purinergic receptors initiate cardiomyocyte growth without substantial increase in ANP transcription suggests the possibility of a more malignant type of hypertrophy (10). The current manuscript investigates the reason for this difference in ANP response to α1-AR agonists versus purinergic stimulation.

EXPERIMENTAL PROCEDURES

Culture of Neonatal Cardiomyocytes—NRVM cultures were prepared from 1–3-day-old Sprague-Dawley rat pups as previously described (11). The cells were preplated twice for 30 min each to remove nonmyocytes and left to attach for 18 h in DMEM, 10% fetal calf serum, 0.1 mM brotromoxyureidine, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate on uncoated dishes. The medium was then replaced with a defined serum-free medium consisting of DMEM, 10 μg/ml human insulin, 10 μg/ml bovine apotransferrin, 0.1 mM bromoethoxyureidine, 50 units/ml penicillin G, 50 μg/ml streptomycin sulfate, and 125 μg/ml fungizone. Bromoethoxurydine was omitted after 3 days.

Assessment of NRVM Hypertrophy—NRVM (400 cells/mm²) were treated with 100 μM UTP or 50 μM PE (plus 1 μM propranolol) with fresh additions of UTP every 8 h. After 48 h the cells were harvested into buffer containing 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, and 2 mM EGTA, pH 7.7, and the samples were assayed for protein (12) and DNA (15).

ERK1/2 Phosphorylation—NRVM plated on 9-cm dishes were washed twice with ice-cold phosphate-buffered saline and scraped in 100 μl of ice-cold lysis buffer at pH 7.7 containing 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM NaF, together with 5 μg/ml okadaic acid, 1 μM peptide A, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1% L-leucyl-o-phenylalanine, 0.5% sodium deoxycholate, and 1% Triton X-100. The lysates were cleared by centrifugation, and the protein content of the supernatants was measured (12). Proteins from whole cell lysates (150–300 μg of protein) were separated by SDS-PAGE (14) and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were stained with Ponceau S (Sigma), blocked for 1 h in 5% fat-free milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), diluted in 5% bovine serum albumin in TBS-T. Secondary antibodies and probed overnight at 4°C. Enhanced chemiluminescence detection was carried out according to the manufacturer’s instructions (Amersham Biosciences). The blots were subsequently stripped and reprobed with antibodies to total ERK1/2 (12/2000).

Transient Transfection and Reporter Gene Activity—The ANP-luciferase plasmid (pANP–638/L5) and the MLC2-luciferase plasmid (pMLC–125/85), the AP-1 luciferase plasmid (TRE2PRL–30) (2XCRE), and the myoglobin-lucerase construct have been described previously (15, 16). Plasmids encoding constitutively active JNK-1, c-Jun, and MKK7 were obtained from Dr. N. Dhanasekaran (Temple University School of Medicine, Philadelphia, PA). Plasmids expressing GATA4, SRF, and myocardin were provided by Prof. R. Harv ery (Victor Chang Cardiac Research Institute, Sydney, Australia) and have been described elsewhere (17). The Gαq, hemagglutinin plasmid was provided by Dr. R. Hannan (Baker Institute). Transfection experiments were performed in triplicate, using cardiomyocytes (400/500) on 35-mm wells 1 day after isolation. Transient transfection, using a total of 4.8 μg of DNA/well, was performed by the calcium phosphate method (11). Ctysollic fractions were harvested into lysis buffer containing 0.1 M KCl, 5 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, and 0.5% Triton X-100. Proteinase-free DNA (13). The activity was measured in 100 μM Tricine, 10 mM MgSO4, 2 mM EDTA, 2 mM ATP, 75 μM luciferin, pH 7.8, for 2 min using a Lumat LB9507 Luminometer.

EGF Receptor Phosphorylation—NRVM plated on 9-cm dishes were washed with ice-cold saline and harvested by rocking in 800 μl of lysis buffer, followed by centrifugation to remove particulate matter. Equal quantities of protein (1.5–3 mg) were diluted to 2.5 ml with Dulbecco’s phosphate-buffered saline (JRH Biosciences), pH 7.0, and immunoprecipitated overnight with anti-EGFR antibody (12/2000) at 4°C. Immunocomplexes were harvested using protein A-Sepharose, separated by SDS-PAGE on 10% gels, and probed with antibodies to phosphorysine and EGFR. Similar protocols were used for ErbB2 and ErbB4 phosphorylation.

Quantification of ANP mRNA—Total RNA was extracted using acid guanidinium thiocyanate-phenol-chloroform (18). ANP mRNA was measured using RNase protection analysis, as described previously (19).

Measurement of PLC Activity—NRVM were labeled with [3H]inositol (5 μCi/ml), washed with unlabeled medium, and pretreated with 10 mM LiCl in DMEM for 10 min prior to the addition of agonists. [3H]Inositol phosphates (InsPs) were extracted with ice-cold 5% trichloroacetic acid, 2.5 mM EDTA, and 50 mM Tris-HCl, 100 mM NaCl, 2 mM EGTA, pH 7.7, and the samples were assayed for [3H]InsPs as described previously.

Treatment of Data—The differences between treatment groups were assessed by one-way analysis of variance with Tukey’s test for multiple comparisons and accepted as statistically significant at p < 0.05. Unless otherwise noted, the results shown are from representative experiments performed in triplicate, which were performed in independent NRVM preparations at least three times.

Materials—Fetal calf serum used during NRVM isolation was specially selected for low endotoxin and obtained from the Commonwealth Serum Laboratories (Parkville, Australia). DMEM, Heps, and other materials for the preparation of cell culture solutions and media were cell culture grade, obtained from Sigma, and dissolved in milliQ H2O. Other reagents were obtained from Sigma or BDH/A AnaR and were of analytical reagent grade. The antibodies were from the following sources: phospho-ERK1/2, ERK1/2, and ErbB2 were from Cell Signaling Technology; ErbB1 and phosphotyrosine (clone 4G10) were from Upstate Biotechnology, Inc.; and ErbB4 was from Santa Cruz.

RESULTS

UTP and PE Cause Growth of NRVM, but Only PE Substantially Increases Transcription of ANP—We have recently reported that UTP causes hypertrophic growth of NRVM via Gαq-coupled P2Y-purinergic receptors (5). In the current study we examined this hypertrophic response with a view to defining critical signaling pathways. NRVM were treated with the purinergic receptor agonist UTP (100 μM) or the α1-AR agonist PE (50 μM, plus 1 μM propranolol), for 48 h. Total cell lysates were prepared and assayed for DNA and protein. As shown in Fig. 1, UTP and PE caused similar increases in the protein/DNA ratio. Furthermore, the EGFR kinase inhibitor AG-1478 (5 μM) reduced responses to both agonists, implying involvement of transactivated EGFR.

We next compared the abilities of UTP and PE to induce ANP transcription, a well-established marker for cardiomyocyte hypertrophy. NRVM were treated with UTP (100 μM) or
PE (50 μM, plus 1 μM propranolol) for 48 h. RNA was extracted, and ANP mRNA was quantified by RNase protection. PE caused a substantial increase in ANP transcription, but in contrast, UTP caused only a small increase when added alone and inhibited the ANP mRNA responses to PE when the two agonists were added together (Fig. 2).

**PE and UTP Phosphorylate EGF Receptors**—Sustained intense activation of Gq in NRVM, either by stimulating overexpressed AT1 receptors (7) or by treatment with an activating toxin (recombinant P. multocida toxin, rPMT) (8), causes phosphorylation of EGF receptors (ErbB1, EGFR) via a transactivation process and initiates downstream hypertrophic signaling pathways. Transactivation of EGFR by endogenously expressed receptors is less well documented. AG-1478 had inhibited growth responses to both UTP and PE, and we next measured EGFR phosphorylation in response to activation of endogenously expressed purinergic receptors with UTP or α1-AR with PE.

NRVM were treated with UTP (100 μM) or PE (50 μM, plus 1 μM propranolol) for 10 min. The lysates were prepared, immunoprecipitated with anti-EGFR antibody, subjected to SDS-PAGE, transferred, and probed with anti-phosphotyrosine antibodies. PE and UTP both caused transactivation of EGFR (ErbB1), as shown by increased tyrosine phosphorylation (Fig. 3). Neither UTP nor PE caused detectable phosphorylation of ErbB2 or ErbB4, even though these responded to neuregulin (NRG2) (10 nM). Thus, purinergic receptors and α1-AR transactivate ErbB family members in an apparently similar manner.

**UTP and PE Activate ERK1/2 via EGFR Transactivation**—We next examined whether downstream signaling from these two receptor classes required EGFR transactivation. NRVM were pretreated with AG-1478 (5 μM) for 10 min and then UTP (100 μM) or PE (50 μM, plus 1 μM propranolol) was added for a further 10 min. The cells were lysed, and the extracts were subjected to SDS-PAGE, transferred, and probed with antibodies to phosphorylated ERK1/2. UTP and PE both caused activation of ERK1/2, and responses to both agonists were substantially inhibited by AG-1478 (Fig. 4). We have previously reported that neither of these agonists caused detectable activation of JNKs or p38 MAPKs in these cells under the conditions of our experiments (5).

**PE Increases Transcription of ANP via Gαq and EGFR Transactivation**—Given the failure of UTP to induce a robust ANP transcriptional response, even though UTP couples to Gq and transactivates EGFR, we next examined the ANP response to PE and its dependence on Gq and EGFR. To do this, we transfected NRVM with an ANP-luciferase construct, together with other plasmids expressing Gαq or a dominant negative EGFR (EGFR-DN) and measured luciferase activity as an index of ANP gene transcription. PE (50 μM, plus 1 μM propranolol) was added, and the cells were harvested after a further 20 h. Overexpression of Gαq increased ANP transcription and markedly enhanced responses to PE. PE responses were inhibited by overexpression of EGFR-DN or by the addition of 5 μM AG-1478 (Fig. 5). Neither the EGFR-DN nor AG-1478 inhibited ANP responses to overexpression of MKK7, the immediate activator of JNK (Fig. 5, inset), confirming that the inhibition by EGFR-DN and AG-1478 was not nonspecific nor a consequence of cytotoxicity. The experiments show that the ANP response to PE involves Gq and EGFR, even though UTP, which also activates Gq and EGFR, is relatively ineffective in increasing ANP transcription and inhibits responses to PE. Similar results were obtained when MLC2 transcription was measured using an MLC-luciferase construct (data not shown).
UTP Inhibits ANP and MLC Transcription when Activated by PE or Gαq, but Not Early Signaling Responses—We next investigated the UTP inhibition of PE-activated transcription from ANP and MLC2 promoters. NRVM were transfected with ANP-luciferase or MLC2-luciferase constructs as indicated, PE (50 μM, plus 1 μM propranolol) or AG-1478 (5 μM) was added, and luciferase activity was measured after 20 h. The values shown are the luciferase activity relative to protein (means ± S.E., n = 3). **, p < 0.01 relative to no PE. ††, p < 0.01 relative to vector control. The inset shows effects of AG-1478 and MKK7-DN on responses to overexpressed constitutively active MKK7. The experiment was performed three times with similar results.

UTP Does Not Inhibit Responses from an NFAT-sensitive Myoglobin Promoter or from AP-1 Response Elements—PE activates promoters other than ANP and MLC2 in cardiomyocytes, and we next examined whether UTP inhibited all of these responses. Similar experiments to those described above were performed using myoglobin (Mb)-luciferase or 2xTRE-luciferase constructs responsive to NFAT or AP-1 elements, respectively. NRVM were transfected with Mb-luciferase or 2xTRE-luciferase, subsequently treated with 50 μM PE (plus 1 μM propranolol) or with 100 μM UTP and luciferase activity was measured after 20 h. Both PE and UTP caused increased transcription from Mb-luciferase and 2xTRE-luciferase reporter constructs, as shown in Fig. 8. Thus, the inhibitory effect of UTP is restricted to cardiac-specific promoters, even though NFAT and AP-1 elements are activated by hypertrophic stimuli such as PE (20, 21).

UTP Selectively Inhibits ANP and MLC Responses to Myocardin—The data had shown that UTP selectively inhibited ANP and MLC2 responses to PE but not events close to the

Fig. 5. PE-activated ANP transcription is mediated by Gαq and EGFR. NRVM were transfected with plasmids expressing ANP-luciferase together with plasmids expressing Goq, dominant negative EGFR (EGFR-DN), or constitutively active MKK7, as indicated. PE (50 μM, plus 1 μM propranolol) and/or AG-1478 (5 μM) was added, and luciferase activity was measured after 20 h. Open bars, no additions. Black bars, PE. The values shown are the luciferase activity relative to protein (means ± S.E., n = 3). **, p < 0.01 relative to no PE. ††, p < 0.01 relative to vector control. The inset shows effects of AG-1478 and EGFR-DN on responses to overexpressed constitutively active MKK7. The experiment was performed three times with similar results.
receptors. This pointed to a UTP inhibited step beyond immediate signaling responses. The failure of UTP to inhibit PE responses at myoglobin promoters or AP-1 elements suggested selective inhibition of cardiac-specific promoters. Myocardin is a newly described transcription enhancer that confers muscle specificity on the ubiquitous transcription factor, SRF (17). We next investigated whether UTP inhibited ANP and MLC2 responses to overexpression of myocardin. NRVM were transfected with ANP- or MLC2-luciferase constructs together with plasmids expressing myocardin. UTP (100 μM) was added, and luciferase activity was measured after 20 h. Myocardin caused substantial activation of ANP transcription at all concentrations tested, with very high activation observed at the highest concentration of added DNA, as reported previously (17). UTP inhibited ANP transcription at all of these myocardin doses (Fig. 9A). For comparison, the experiments were also performed with overexpressed GATA4, c-Jun + JNK, and SRF, transcription factors not restricted to muscle-specific genes. All of the transcription factors increased ANP transcription, but in contrast to myocardin, the responses were not inhibited by UTP (Fig. 9B). Responses to myocardin were synergistic with the PE response, pointing to a role for myocardin in the ANP response to PE (Fig. 9C).

Similar experiments were performed using MLC-luciferase. As with ANP-luciferase, GATA4, c-Jun + JNK, SRF, and myocardin all activated MLC2 transcription, but only the response to myocardin was inhibited by UTP. As described previously (17), the maximal MLC2 response to myocardin was lower than the ANP response (Figs. 9A and 10A). Myocardin substantially increased PE-induced MLC2 transcription (Fig. 10B).

Myocardin has been reported to activate muscle-specific promoters exclusively (17). In the current study we confirmed that myocardin did not activate Mb-luciferase or 2xTRE-luciferase constructs. NRVM were transfected with Mb-luciferase or 2xTRE-luciferase, 50 μM PE together with 1 μM propranolol was added, and luciferase activity measured after 20 h. Over-

![Fig. 8. UTP does not inhibit transcription from myoglobin- or AP-1-luciferase constructs.](image)

![Fig. 9. UTP selectively inhibits myocardin-induced ANP transcription.](image)

![Fig. 10. UTP selectively inhibits MLC2 transcriptional responses to myocardin.](image)
The experiments were performed three times with similar results.

Myocardin is a newly described transcription enhancer that associates with SRF, which, on its own, is functional in nonmuscle cells. Many of the other transcription factors known to bind the ANP promoter, including GATA4 and c-Jun, also activate promoters that are not muscle-specific (24, 25). In the current studies, we found that UTP treatment caused a selective inhibition of myocardin-induced ANP and MLC2 transcription. Responses to c-Jun, GATA4, or SRF were not inhibited. This myocardin selectivity potentially explains the selective inhibition of PE-induced transcription from ANP and MLC2 but not from myoglobin or AP-1 elements. The mechanism by which myocardin activity is regulated is not currently known, and a role in PE-mediated responses remains to be established. However, there are a number of factors that argue in favor of myocardin as the target of UTP inhibition of PE-induced ANP and MLC2 transcription. First, UTP treatment depressed transcriptional responses to myocardin. Second, PE responses were inhibited only at myocardin-sensitive promoters. Third, the PE response was markedly enhanced by overexpressed myocardin. Taken together these findings suggest that PE activation of ANP and MLC2 promoters involves myocardin, among other factors, and that UTP treatment reduces PE responses by interfering with myocardin functioning at these promoters. However, it must be stressed that direct evidence is lacking and that it is also possible that there are two separate and independent inhibitory actions of UTP one targeting myocardin and the other some other factor acting beyond immediate signaling responses to PE. It is also likely that other muscle-specific factors are involved in the responses described.

Cardiomyocytes express a number of different purinergic receptors of which the P2Y2 and P2Y4 receptors might be expected to respond robustly to UTP (26). In acute experiments, responses to UTP were mimicked by ATP (5). Thus, ATP enhanced PLC activity and caused EGFR phosphorylation and ERK1/2 activation, but because ATP is cytotoxic to cardiomyocytes with prolonged exposure (5), it is impossible to evaluate effects on transcriptional responses. Similarly, we found suramin, an antagonist at P2Y1, P2Y2, and P2Y4 receptors (26), to be toxic with chronic treatment. Therefore, it has not been possible to define the receptors responsible for the inhibitory action of UTP on ANP and MLC2 transcription.

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Pathological insults including chronically heightened blood pressure, myocardial infarction, valve disease, and some congenital defects lead to hypertrophic growth of cardiomyocytes (27, 28). In most cases this is associated with increased expression and release of ANP from the ventricular myocytes. Increased plasma ANP would be expected to increase sodium excretion and to cause dilatation of the vasculature, thereby reducing afterload on the heart (29, 30). In addition, a recent study has suggested direct beneficial effects of ANP on the cardiomyocytes themselves. Cardiac targeted knockout of ANP receptor type C lead to a worsened prognosis following pressure overload in vivo (9). Thus, a hypertrophic response that induces cellular growth without increased ANP expression might be expected to be particularly detrimental to the heart (10). It is not obvious how UTP would cause sustained myocardial responses in vivo, unless there is substantial local release from the heart under some circumstances. ATP, but not UTP, is released from cardiac sympathetic nerves during stimulation (31) and might cause chronic activation of purinergic receptors under some conditions. It is possible that the cytotoxic effect of ATP, observed in cell culture, is due to degradation products accumulating in the culture medium and that in vivo repeated release and clearance of ATP would allow for sustained growth responses, but this remains to be established. In any case, we have described an inhibition of ANP transcriptional responses by UTP, possibly involving the newly described transcription factor, myocardin. Further studies are needed to establish exactly how myocardin responses are regulated, whether myocardin...
din is involved in α₁-AR-mediated responses, and how inhibition is induced by chronic exposure to UTP.

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REFERENCES
1. Esposito, G., Rapacciuolo, A., Naga Prasad, S. V., Takaoka, H., Thomas, S. A., Koch, W. J., and Rockman, H. A. (2001) Cardiovasc. Res. 50, 65–74
2. Dorn, G. W., and Brown, J. H. (1999) Trends Cardiovasc. Med. 9, 26–34
3. Adams, J. W., and Brown, J. H. (2001) Oncogene 20, 1626–1634
4. Boluyt, M. O., Zheng, J. S., Younes, A., Long, X. L., O’Neill, L., Silverman, H., Lakatta, E. G., and Crow, M. T. (1997) Circ. Res. 81, 176–186
5. Pham, T., Morris, J., Arthur, J., Post, G., Brown, J., and Woodcock, E. (2003) J. Mol. Cell Cardiol. 35, 287–292
6. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) EMBO J. 16, 7032–7044
7. Thomas, W. G., Brandenburger, Y., Autelitano, D. J., Pham, T., Qian, H. W., and Hannan, R. D. (2002) Circ. Res. 90, 135–142
8. Subri, A., Wilson, B. A., and Steinberg, S. F. (2002) Circ. Res. 90, 850–857
9. Holtwick, R., van Eickels, M., Skryabin, B. V., Baba, H. A., Babikat, A., Begrow, F., Schneider, M. D., Garbers, D. L., and Kuhn, M. (2003) J. Clin. Invest. 111, 1399–1407
10. Molkentin, J. D. (2003) J. Clin. Invest. 111, 1275–1277
11. Woodcock, E. A., Wang, B. H., Arthur, J. F., Lennard, A., Matkovich, S. J., Du, X.-J., Brown, J. H., and Hannan, R. D. (2002) J. Biol. Chem., 22734–22742
12. Peterson, G. L. (1979) Anal. Biochem. 100, 201–220
13. Burton, K. (1956) Biochem. J. 62, 315–323
14. Cannon-Carlson, S., and Tang, J. (1997) Anal. Biochem. 246, 146–148
15. Shubeta, H. E., Martinson, E. A., Vanhelen, M., Chien, K. R., and Brown, J. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1305–1309
16. Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Ross, O., Michael, L. F., Puigserver, P., Isotani, E., Olson, E. N., Lewell, B. B., Bassel-Duby, R., and Spiegelman, B. M. (2002) Nature 418, 797–801
17. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Cell 105, 851–862
18. Chomczynsky, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
19. Harrison, S. N., Autelitano, D. J., Wang, B. H., Milano, C., Du, X. J., and Woodcock, E. A. (1998) Circ. Res. 83, 1232–1240
20. Pu, W. T., Ma, Q., and Izumo, S. (2003) Circ. Res. 92, 725–731
21. Omura, T., Yoshiyama, M., Yoshida, K., Nakanura, Y., Kim, S., Iwao, H., Takeuchi, K., and Yosihikawa, J. (2002) Hypertension 39, 81–86
22. Adams, J. W., Sakata, Y., Davis, M. G., Sah, V. P., Wang, Y., Liggett, S. B., Chien, K. R., Brown, J. H., and II, G. W. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10140–10145
23. Arthur, J. F., Matkovich, S. J., Mitchell, C. J., Biden, T. J., and Woodcock, E. A. (2001) J. Biol. Chem. 276, 37341–37346
24. Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228
25. Chang, L. F., and Karin, M. (2001) Nature 410, 57–60
26. Vassort, G. (2001) Phys. Rev. E, 817–826
27. Schaub, M. C., Hehi, M. A., Harder, B. A., and Eppenberger, H. M. (1997) J. Mol. Med. 75, 911–920
28. Vassort, G. (2001) Phys. Rev. E, 817–826
29. Calderone, A. (2003) Heart Fail Rev. 8, 55–70
30. Costello-Boerrigter, L. C., Boerrigter, G., and Burnett, J. C., Jr. (2003) Mol. Clin. North Am. 87, 475–491
31. Sesti, C., Koyama, M., Broekman, M. J., Marcus, A. J., and Levi, R. (2003) J. Pharmacol. Exp. Ther. 306, 238–244