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Plasma membrane-associated nucleoside diphosphate kinase (nm23) in the heart is regulated by β-adrenergic signaling

[1]Susanne Lutz, [1]Roman A. Mura, [1]Hans Joerg Hippe, [1]Christiane Tiefenbacher & *,[1]Feraydoon Niroomand

[1]Klinikum der Universität Heidelberg, Innere Medizin III, Bergheimer Str. 58, Heidelberg D-69115, Germany

1 A receptor-independent activation of heterotrimeric G proteins by plasma membrane-associated nucleoside diphosphate kinase (NDPK) has been demonstrated *in vivo*, and elevated levels of NDPK were found in purified sarcolemmal membranes of patients with end-stage heart failure.

2 Among 22 consecutive patients with chronic heart failure who underwent cardiac transplantation, those treated with a β-blocker ($n=8$) had a 65% lower NDPK content and activity in the cardiac sarcolemma, compared to patients with similar base line characteristics who had no β-blocker therapy ($n=14$).

3 The lower NDPK was associated with a reduced NDPK-dependent, G_i-mediated inhibition of adenylyl cyclase activity, as assessed by *in vitro* measurement of adenylyl cyclase activity in the presence of GDP or its kinase-resistant analog guanosine 5′-*O*-(2-thio)diphosphate (GDPβS).

4 We further tested whether treatment with a β-adrenergic agonist would induce an increase in sarcolemmal NDPK. Rats treated with isoproterenol developed myocardial hypertrophy, and NDPK in the sarcolemma rose by 60% during 14 days of treatment. The β-blocker propranolol prevented both effects. When hypertrophy was induced with thyroid hormone, NDPK did not increase.

5 In conclusion, chronic activation of β-adrenergic receptors increases the binding of NDPK to cardiac sarcolemma, where it may activate heterotrimeric G proteins.
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Abbreviations: βARK, β-adrenergic receptor kinase; ANOVA, one-way analysis of variance; GDPβS, guanosine 5′-*O*-(2-thio)diphosphate; β-HF and HF, patients treated with β-blockers and controls, respectively; NDPK, nucleoside diphosphate kinase; T3, 3,3′, 5-triiodo-L-thyronine

Introduction

Nucleoside diphosphate kinase (NDPK) catalyzes the transfer of terminal phosphate groups from 5′-triphosphate to 5′-diphosphate nucleotides. In the cell, the major reaction is a phosphate transfer from ATP to GDP to maintain levels of GTP. Only a small fraction of cellular NDPK binds to the plasma membrane, where it may serve the synthesis of GTP, required for the activation of G proteins (Otero, 1990; Kimura, 1993; Piacentini & Niroomand, 1996). We previously demonstrated a receptor-independent regulation of cardiac adenylyl cyclase activity by G proteins and NDPK in cardiac sarcolemmal membranes (Niroomand *et al*., 1997). More recently, we found a receptor-independent activation of the heterotrimeric adenylyl cyclase stimulating G protein G_s by NDPK (Hippe *et al*., 2003) in a cell line derived from neonatal rat heart myocytes. This *in vivo* activation is mediated by a intermediate phosphotransfer to the G protein β-subunit (Wieland *et al*., 1993; Cuello *et al*., 2003). Furthermore, we had found a three-fold increase of plasma membrane-associated NDPK in hearts from patients with severe congestive heart failure (Lutz *et al*., 2001). This increase was confined to the plasma membrane and was not observed in other cell compartments, where the vast majority of NDPK is located. The net effect of NDPK activity on adenylyl cyclase in the plasma membrane had a profound inhibition of activity that can be explained by the increased inhibitory G_i proteins under this condition (Feldman *et al*., 1988; Neumann *et al*., 1988). Beside the activation of heterotrimeric G proteins, NDPK has also been shown to phosphorylate and regulate other components of cellular signal transduction such as small G proteins (Zhu *et al*., 1999), MAP kinases (Moon *et al*., 2003), KSR (Hartsough *et al*., 2002), ICAP-1α (Fournier *et al*., 2002) and Tiam1 (Otsuki *et al*., 2001). Since congestive heart failure is associated with excessive catecholamine stimulation of the heart, we wondered if binding of NDPK to the plasma membrane is regulated through the activation of β-adrenergic receptors.

Methods

Patient selection, tissue source and purification of sarcolemmal membranes

Ventricular myocardium of failing human hearts was obtained after written consent from 22 consecutive patients who underwent cardiac transplantation due to end-stage heart

*Author for correspondence;
E-mail: Feraydoon_Niroomand@med.uni-heidelberg.de
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failure. After removal, all explanted hearts were placed immediately on ice, cut into $1 \times 1\,cm^2$ pieces and stored at $-80°C$ until use. Ventricular myocardium from 14 patients without β-blocker therapy was compared with that of eight patients who had been treated with a β-blocker during the last 6 months prior to transplantation. Sarcolemmal membranes were prepared by homogenization and differential sedimentation (Jones, 1988). Cytosolic fractions were prepared by centrifugation of supernatants from the homogenate at $100{,}000 \times g \times 60\,min$. Residual particulate fractions refer to all membranous components of the myocardial tissue, separated from the purified sarcolemmal membranes. Membranes and the cytosol were frozen in liquid nitrogen and stored prior to use at $-80°C$.

Animal studies

Male Wistar rats (190–220 g) were anesthetized by intraperitoneal injection of an 8% solution of chloralhydrate (0.8–1.4 ml) and a 10% solution ketamine (0.2 ml). Osmotic minipumps (Alzet ML 2, Palo Alto, CA, U.S.A.), containing the indicated substances were implanted subcutaneously. At the end of the indicated time intervals, rats were anesthetized with intraperitoneal pentobarbital (0.7 ml) and the hearts were excised, weighted and frozen in liquid nitrogen. Sarcolemmal membranes, cytosol and residual particulate fractions were prepared as described for the human hearts. In order to have sufficient tissue for membrane preparation, three rat hearts had to be pooled for one preparation. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Enzymatic activities

NDPK activity was determined using [^{3}H]GDP (1 μM; 10 Ci mmol^{-1}) as described previously with either ATP or ATPγS as substrate (Hippe *et al*., 2003). 5′ nucleotidase activity was assayed according to the method of Emmelot *et al*. (1964). Phosphate was determined according to Fiske & Subbarow (1925). Adenylyl cyclase activity was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP (Jakobs *et al*., 1976). The assay volume was 100 μl containing 0.1 mM ATP with $0.5–5 \times 10^6$ c.p.m. of [α-^{32}P] ATP (3000 Ci mmol^{-1}), 3 mM $MgCl_2$, 0.1 mM cAMP, 1 mM EDTA, 0.5 mM dithiothreitol and 75 mM triethanolamine, pH 7.6. The membranes (2.5–3.0 μg protein) were preincubated with alamethicin for 20 min at 4°C at a 1 : 1 ratio (w w^{-1}) to unmask latent adenylyl cyclase activity. This peptide ionophore increases the accessibility of substrates to the adenylyl cyclase in sealed sarcolemmal vesicles without affecting the functional coupling to receptors (Jones *et al*., 1980). The adenylyl cyclase reaction was started by the addition of membrane protein and continued for 10 min at 37°C.

β-*adrenergic receptor radioligand binding*

A measure of 3 μg of membrane were incubated with 300 pM [^{125}J]cyano pindolol (2200 Ci mmol^{-1}) in 250 μl of Tris-HCl (50 mM, pH 7.5) for 90 min at 30°C. Unspecific binding was determined in the presence of 10 μM propranolol. Incubation was terminated by vacuum filtration through glass-fiber filters (Millipore, Eschborn, Germany).

Western blots

Membrane-enriched and cytosolic fractions were suspended in SDS buffer for SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The separated proteins were transferred electrophoretically to nitrocellulose membranes and immunodetection was carried out using a specific antibody against human NDPK-B (nm23-H2, kind donation of Ioan Lascu, Bordeaux, France) or a commercial rat NDPK antibody (Santa Cruz Biotechnology, sc-343, Santa Cruz, CA, U.S.A.). Binding of the primary antibody was visualized using a second antibody and Lumilight plus (Roche, Mannheim, Germany). Chemiluminescence was quantified with a FluorS-MultImager (BioRad, Munich, Germany).

Data analysis

All the experiments were carried out in triplicate and were repeated at least three times. Values are given as means ± s.d. For statistical analysis, a two-tailed Student's *t*-test (comparison of two groups) or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (three or more groups) was performed for continuous variables and a Fisher's test was performed for nominal variables with the GraphPad PRISM 3 software.

Results

β-*blocker therapy reduces sarcolemmal NDPK in patients with chronic congestive heart failure*

In the 22 patients of this study with dilated cardiomyopathy and severe congestive heart failure (NYHA III–IV), who underwent cardiac transplantation, base line characteristics were similar for those treated with β-blockers (β-HF) and control (HF) patients (Table 1).

From the explanted hearts, sarcolemmal membranes, a cytosolic fraction and a residual particulate fraction were prepared. In the purified sarcolemmal membranes, 5′ nucleotidase activity and basal, G-protein-independent adenylyl cyclase activity (measured in the presence of manganese) and adenylyl cyclase activity with guanosine 5′-*O*-(2thio)diphosphate (GDPβS) were similar. A pronounced difference in sarcolemmal membranes from patients who had been treated with a β-blocker had a 60% lower activity and amount (Figure 1) of NDPK. The decreased NDPK activity in the β-HF group was confined to the sarcolemmal membrane fraction, and was not seen in the cytosol or the residual particulate fraction. We had observed the same change in distribution pattern, when we compared NDPK levels in failing *vs* nonfailing hearts, where NDPK in the sarcolemmal fraction from failing hearts was increased more than threefold, but the amount and activity of NDPK in other cellular compartments remained unchanged (Lutz *et al*., 2001). Hence, β-blocker therapy partially reverses the increased binding of NDPK to the plasma membrane in heart failure patients.

Table 1 Patient characteristics

	HF (n = *14*)	β-*HF* (n = *8*)	P-*value*
Age (years)	55 ± 12.2	50 ± 14.9	NS
Male (*n*)	12	7	NS
β-blocker			
Metoprolol (4) (mg day^{-1})	0	87.5 ± 25.0	
Carvedilol (4) (mg day^{-1})	0	21.9 ± 6.3	
ACE inhibitors (*n*)	12	7	NS
Digitalis (*n*)	12	6	NS
Diuretics (*n*)	12	8	NS
Peak VO_2 (ml kg^{-1} min^{-1})	12.3 ± 3.3	13.0 ± 3.2	NS
Heart rate	85.0 ± 7.8	82.1 ± 18.3	NS
Blood pressure			
Systolic	106 ± 15	114 ± 26	NS
Diastolic	75 ± 10	80 ± 23	NS
Ejection fraction (%)	18.7 ± 7.9	17.3 ± 8.3	NS
PC wedge	17.7 ± 8.4	18.6 ± 8.7	NS
Cardiac index (L (min m^2)$^{-1}$)	2.01 ± 0.48	2.11 ± 0.68	NS

Heart failure patients without (HF) and with *β*-blocker therapy (*β*-HF) showed similar base line characteristics. Peak VO_2: peak oxygen uptake during exercise; PC wedge: pulmonary capillary wedge pressure; NS: not significant. Pressure is given in mmHg.

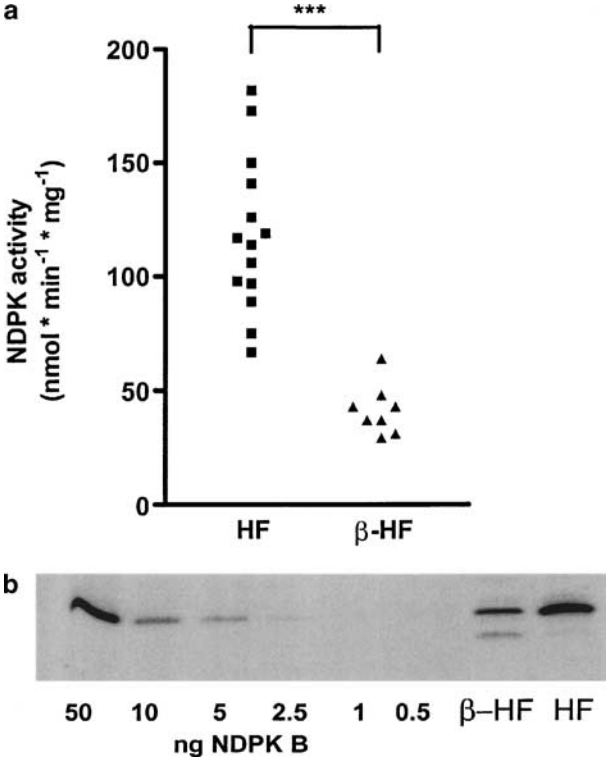


Figure 1 Lower sarcolemmal NDPK in patients treated with a *β*-blocker. (a) NDPK activity was determined in purified sarcolemmal membranes from the hearts of 14 patients without (HF) and of eight patients with *β*-blocker (*β*-HF) therapy. (b) Representative Western blot with recombinant human NDPK B (nm23-H2; 0.5–50 ng) as standard. The antibody is specific for the NDPK B isoform. Both activity and content of NDPK were reduced in treated patients (***$P<0.0001$, as determined by a two-tailed unpaired *t*-test).

Reduction of sarcolemmal NDPK leads to a reduced inhibition of NDPK-dependent adenylyl cyclase activity

We proceeded to examine the influence of altered sarcolemmal NDPK on adenylyl cyclase activity. This may be achieved by measuring adenylyl cyclase activity in the presence of ATP and GDP, and by comparison to the activity with GDP*β*S and ATP. GDP*β* shares the same properties with GDP, regarding its binding to and inactivation of G proteins. However, GDP*β*S is a very poor substrate for the NDPK in most systems and in the membranes studied here (data not shown), and thus may be used in comparison with GDP to discriminate NDPK-mediated effects. While basal adenylyl cyclase activities and activities in the presence of GDP*β*S were similar in both groups, at maximal effective concentrations of GDP (0.1 mM GDP), NDPK-dependent adenylyl cylase activity in patients with *β*-blockers was inhibited only by 15%, compared to a 45% inhibition in patients without *β*-blockers (Figure 2).

Isoproterenol and thyroid hormone induce myocardial hypertrophy and have opposite effects on β*-adrenergic receptor density*

As *β*-blocker therapy apparently attenuated the binding of NDPK to the plasma membrane in patients with heart failure, we wondered whether chronic treatment with a *β*-receptor agonist could induce this binding. In rats, treated with the implantation of osmotic minipumps, the specific *β*-adrenergic receptor agonist isoproterenol (2.4 mg kg^{-1} day^{-1}), induced an increase in the ratio of heart weight to body weight by 41% after 14 days of treatment. Propranolol (10 mg kg^{-1} day^{-1}), used to antagonize *β*-adrenergic receptor activation by isoproternol, prevented the hypertrophic response to isopro-

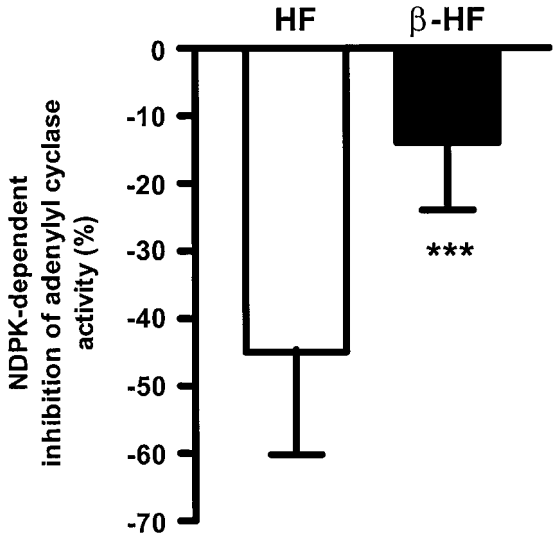


Figure 2 Influence of NDPK on cAMP synthesis. Adenylyl cyclase activity was determined in sarcolemmal membranes from failing human hearts. NDPK-dependent adenylyl cyclase activity was determined as the difference of activity in the presence of GDP*β*S (poor substrate for NDPK) and of GDP. NDPK-mediated inhibition of adenylyl cyclase was lower ***($P=0.001$, as determined by a two-tailed unpaired *t*-test) in sarcolemma from heart failure patients with *β*-blocker therapy (*β*-HF, $n=8$), compared to the untreated group (HF, $n=14$).

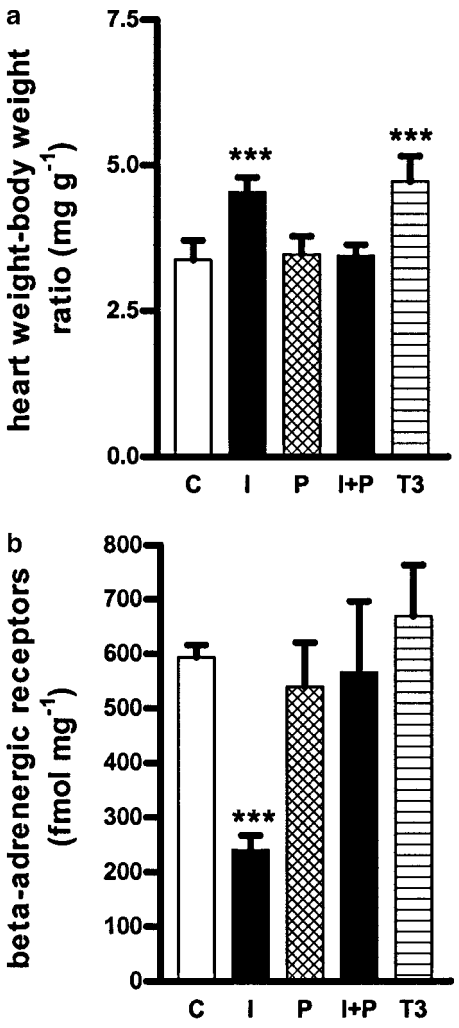


Figure 3 Myocardial hypertrophy and β-adrenergic receptors after 2 weeks of treatment with isoproterenol or thyroid hormone. (a) Effect of treatment in rats on myocardial hypertrophy and (b) density of β-adrenergic receptors in sarcolemmal membranes. The treatments were: saline (C, $n=21/7$), isoproterenol (I, $n=21/7$), propranolol (P, $n=9/3$), isoproterenol and propranolol combined (I + P, $n=9/3$), and triiodo-thyronine (T3, $n=18/6$). For n, the first value indicates number of treated animals and the second, number of sarcolemmal membrane preparations, where three hearts were pooled for each preparation. Both isoproterenol and T3 significantly increased the heart weight–body weight ratio. Treatment with isoproterenol resulted in a significant downregulation of β-adrenergic receptors. $^{***}P<0.001$ according to ANOVA followed by Dunett's multiple comparison test.

terenol completely (Ostman-Smith, 1995). 3,3′,5-triiodo-L-thyronine (T3; $0.5\,mg\,kg^{-1}\,day^{-1}$) induced a similar hypertrophic response as isoproterenol that is independent of β-adrenergic receptor activation, and thereby served as an additional control for the evaluation of β-adrenergic effects on NDPK (Figure 3a).

From these hearts, plasma membranes, cytosols and a residual particulate fraction were prepared. For each preparation, three hearts were pooled to obtain the minimal amount of tissue required for membrane purification. 5′nucleotidase, used as a plasma membrane marker, was not significantly different among the treated groups (data not shown). As expected, isoproterenol induced a significant downregulation of β-adrenergic receptors by more than 50% in the sarcolemmal fraction. Again, this effect was prevented completely by concomitant application of propranolol. T3 induced a small increase in the density of β-adrenergic receptors that did not reach statistical significance (Figure 3b).

Isoproterenol and thyroid hormone have opposing effects on sarcolemmal NDPK

Treatment with isoproterenol led to a progressive myocardial hypertrophy during 14 days (Figure 4a). It had no effect on NDPK activity in the cytosol or residual particulate fraction at any time (data not shown). In plasma membranes, however, after 7 days a 22% increase in NDPK activity was observed that reached 60% after 14 days of treatment with isoproterenol when compared to the saline-treated groups (Figure 4b).

To investigate the mechanism underlying the binding of NDPK to the plasma membrane during chronic treatment with isoproterenol, the β-adrenergic receptor antagonist propranolol was coadministered. Propranolol completely prevented the upregulation of NDPK in the plasma membrane with isoproterenol (Figure 4c and d), indicating the involvement of β-adrenergic receptors. However, a remaining issue was that upregulation of NDPK could be an epiphenomenon of myocardial hypertrophy rather than being related to β-adrenergic receptor stimulation.

Therefore, rats were treated for 14 days with T3. This treatment led to a similar hypertrophic response as that seen with the β-receptor agonist (Figure 3a) However, T3 did not induce an upregulation of NDPK, rather a small but significant downregulation of NDPK activity in the plasma membrane was observed (Figure 4c and d).

Discussion

β-adrenergic receptor-mediated binding of NDPK to the plasma membrane

The major finding of this study is that chronic stimulation of β-adrenergic receptors increases binding of NDPK to the plasma membrane and that β-blockers are effective in preventing this mechanism in patients with chronic congestive heart failure. The mechanisms that regulate intracellular distribution of NDPK are not known. According to our current findings, the chronic activation of β-adrenergic receptors affects the content of NDPK only in the plasma membrane. Since total cellular NDPK content did not change in patients treated with a β-blocker or in rats treated with isoproterenol, an increased expression as mechanism is unlikely. NDPK in the plasma membrane accounts for a small fraction of total cellular NDPK. Therefore, a translocation of NDPK to the plasma membrane from the cytosol would not be accompanied by a detectable decrease in the latter. β-adrenergic receptor-mediated increase of plasma membrane-bound NDPK was seen only after several days of treatment. Apparently, the increase in sarcolemmal NDPK paralleled the progression of myocardial hypertrophy. However, when myocardial hypertrophy was induced by high doses of thyroid hormone, NDPK in the sarcolemma was even decreased. Thus, myocardial hypertrophy is not inevitably associated with an increased translocation of NDPK to the plasma membrane. It has to be assumed that

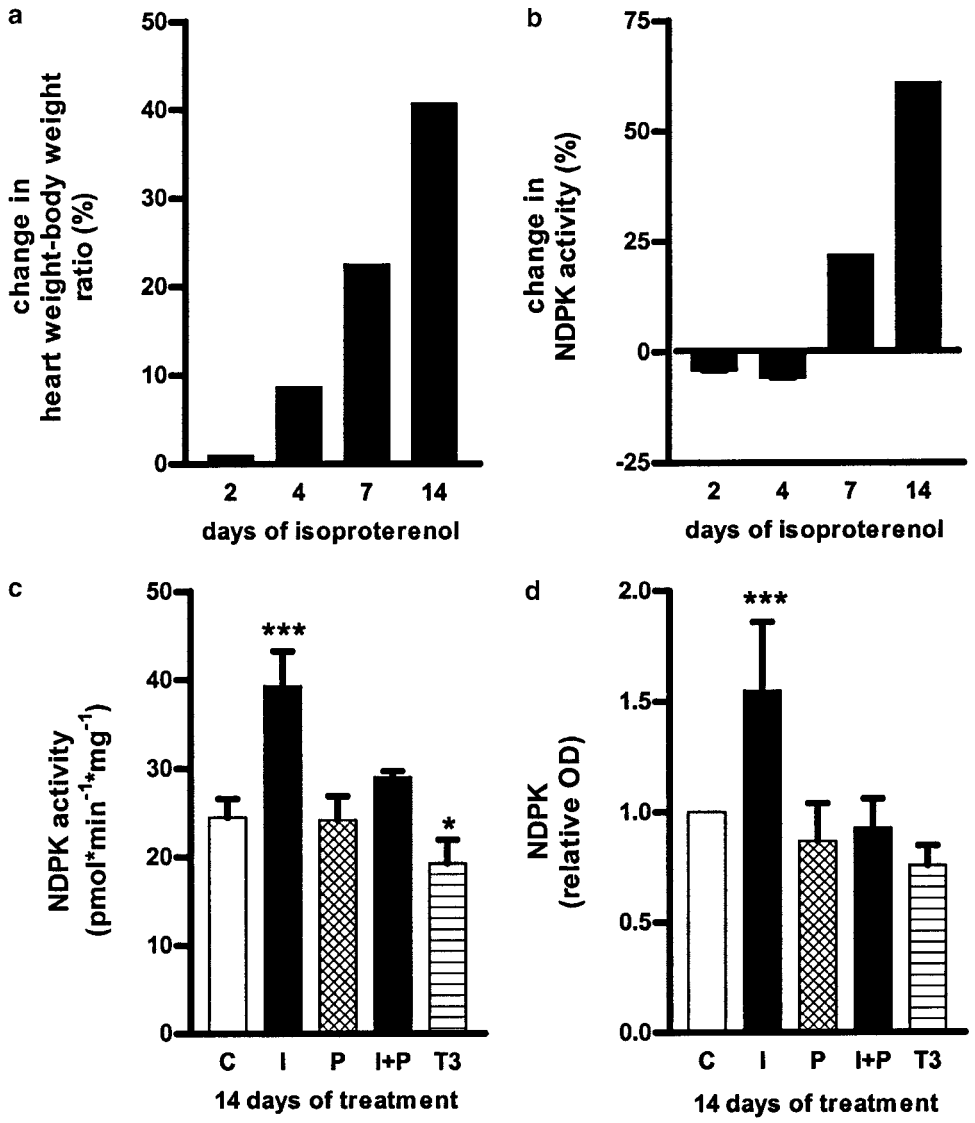
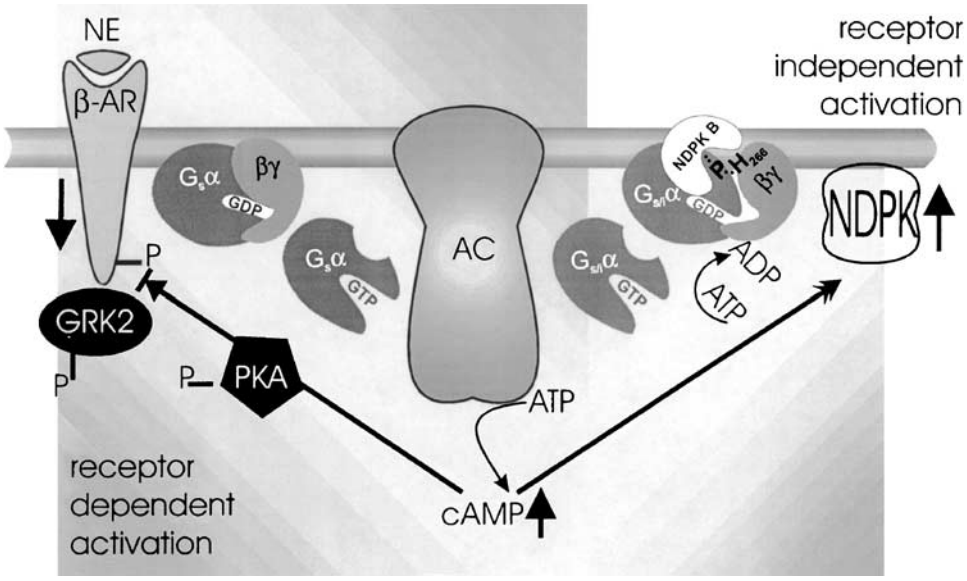


Figure 4 NDPK in sarcolemmal membranes from rat hearts after treatment with isoproterenol or thyroid hormone. (a) Time-dependent increase of myocardial hypertrophy and (b) plasma membrane-associated NDPK activity in isoproterenol-treated rats, compared with saline-treated animals. (c) NDPK activity and (d) content (relative quantity, determined in Western blots) in plasma membranes from rat hearts after 14 days of treatment. The treatments were: saline (C, $n=21/7$), isoproterenol (I, $n=21/7$), propranolol (P, $n=9/3$), isoproterenol and propranolol combined (I + P, $n=9/3$) and triiodo-thyronine (T3, $n=18/6$). For n, the first value indicates number of treated animals and the second, number of plasma membrane preparations, where three hearts were pooled for each preparation. $^{***}P<0.0001$, $^{*}P<0.05$, according to ANOVA followed by Dunett's multiple comparison test.

sustained β-adrenergic receptor stimulation leads to increased expression of a distinct plasma membrane or carrier protein responsible for the translocation of NDPK.

Consequences of increased plasma membrane-associated NDPK

After several findings in the 1980s had suggested a role for NDPK in G protein activation (Kimura, 1993; Piacentini & Niroomand, 1996), serious concerns have been raised against this view (Otero, 2000). These concerns, however, only address the direct phosphotransfer from the NDPK to the GDP bound at the α-subunit of G proteins. Structural considerations regarding the localization of the guanine nucleotide-binding site on the G protein and the catalytic domain of the NDPK would tend to rule out such a direct interaction. Previous studies have demonstrated an activation of G proteins (Wieland *et al*., 1992) and regulation of adenylyl cyclase activity (Wieland *et al*., 1993) by phosphorylated G protein β subunits. We have recently demonstrated an activation of the adenylyl cyclase stimulating G_s protein by NDPK *in vivo* that was associated with an increase in Gβ phosphorylation (Hippe *et al*., 2003). Furthermore, we could show a tight association of NDPK with Gβ and phosphorylation of Gβ by NDPK (Cuello *et al*., 2003). It can be assumed that other heterotrimeric G proteins will be activated in the same manner; however, as yet this remains to be demonstrated. The increase in plasma membrane-bound NDPK may be viewed as a feedback mechanism that compensates in part for the downregulation of G-protein-coupled receptors (Scheme 1). It has been shown that even in the absence of receptor agonists, empty receptors with constitutive activity can activate G proteins, an effect that is inhibited by some receptor



Scheme 1 Chronic β-adrenergic stimulation causes desensitization of β-adrenergic receptors by protein kinase A (PKA)-mediated phosphorylation of the receptor and G-protein-coupled receptor kinase GRK2. In addition, according to the present study, NDPK is translocated to the plasma membrane, where it can activate heterotrimeric G proteins. The net effect of NDPK on cAMP synthesis will depend on the relative activation of inhibitory and stimulatory G proteins.

antagonists (Bond *et al*., 1995). With the downregulation and uncoupling of receptors, this tonic activation would be lost, and an upregulation of NDPK may compensate for this loss.

Possible implications

Despite the enormous impact, only little information is available how β-blockers act on cardiac signal transduction to improve cardiac function, symptoms and prognosis in patients with heart failure (Anonymous, 1999a, b). Clearly, an upregulation of β-adrenergic receptors as seen after chronic treatment with metoprolol (Heilbrunn *et al*., 1989; Witte *et al*., 1998) cannot be the only mechanism for the improved cardiac function and prognosis in these patients, because treatment with carvedilol is associated with these beneficial effects (Metra *et al*., 2000) even though the substance does not increase β-receptors (Yoshikawa *et al*., 1996). Moreover, carvedilol reduces plasma noradrenaline concentration, whereas another β-blocker, atenolol, has no such effect (Herman *et al*., 2003). The question then arises of why substances that reduce norepinephrine release like carvedilol (Gilbert *et al*., 1996; Herman *et al*., 2003), downregulate (metoprolol) and block the β-adrenergic receptor, increase systolic function of the heart (Squire & Barnett, 2000). Clearly, the immediate effect of β-blockers is a reduction in cardiac inotropy and the increase in ejection fraction is seen only after several weeks of treatment (Metra *et al*., 2000). This suggests that complex cellular changes have to precede, and that rapid changes within the classical β-adrenergic signal transduction pathway, like receptor or G protein density (Bristow *et al*., 1982; Feldman *et al*., 1988; Neumann *et al*., 1988; Brown & Harding, 1992; Sigmund *et al*., 1996), activity of β-adrenergic receptor kinase (Ungerer *et al*., 1993), phospholamban phosphorylation (Minamisawa *et al*., 1999) or phosphorylation of the ryanodine receptor (Yamamoto *et al*., 1999; Marx *et al*., 2000; Marks *et al*., 2002) alone cannot explain the positive inotropic effect of chronic β-blocker therapy. Sensitization of other G_s-coupled receptors like histamine (Sanders *et al*., 1996), 5-HT4 (Sanders *et al*., 1995) and β2 adrenergic receptors (Hall *et al*., 1990) has been demonstrated in patients with chronic β-blocker therapy; however, their particular contribution to improved cardiac function remains to be proven. In this regard, it is worth noting that carvedilol blocks both β1 and β2 adrenergic receptors and, as outlined previously, is at least as effective as selective β1 antagonists. The β receptor-mediated translocation of NDPK to the plasma membrane is a slow process that required weeks in our animal model. As outlined in the introduction, plasma membrane-associated NDPK exerts complex effects on various signal transduction pathways such as heterotrimeric G proteins, small G proteins and mitogen-activated protein kinases. Whether these effects are important in the setting of heart failure remains elusive. However, an increased activation of G_i proteins by NDPK may contribute to the impaired β-adrenergic signal transduction in chronic heart failure, and the reduction of sarcolemmal NDPK by treatment with β-blockers could improve cardiac function through a reduction of receptor-independent G_i activation.

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