Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes

Laura Pentassuglia, Philippe Heim, Sonia Lebboukh, Christian Morandi, Lifen Xu, and Marijke Brink

Department of Biomedicine, University of Basel and University Hospital Basel, Basel, Switzerland

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Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes. Am J Physiol Endocrinol Metab 310: E782–E794, 2016. First published March 15, 2016; doi:10.1152/ajpendo.00259.2015.—Nrg1β is critically involved in cardiac development and also maintains function of the adult heart. Studies conducted in animal models showed that it improves cardiac performance under a range of pathological conditions, which led to its introduction in clinical trials to treat heart failure. Recent work also implicated Nrg1β in the regenerative potential of neonatal and adult hearts. The molecular mechanisms whereby Nrg1β acts in cardiac cells are still poorly understood. In the present study, we analyzed the effects of Nrg1β on glucose uptake in neonatal rat ventricular myocytes and investigated to what extent mTOR/Akt signaling pathways are implicated. We show that Nrg1β enhances glucose uptake in cardiomyocytes as efficiently as IGF-I and insulin. Nrg1β causes phosphorylation of ErbB2 and ErbB4 and rapidly induces the phosphorylation of FAK (Tyr641), Akt (Thr308 and Ser473), and its effector AS160 (Thr642). Knockdown of ErbB2 or ErbB4 reduces Akt phosphorylation and blocks the glucose uptake. The Akt inhibitor VIII and the PI3K inhibitors LY-294002 and BCI-719 abolish Nrg1β-induced phosphorylation and glucose uptake. Finally, specific mTORC2 inactivation after knockdown of rictor blocks the Nrg1β-induced increases in Akt-p-Ser473 but does not modify AS160-p-Thr642 or the glucose uptake responses to Nrg1β. In conclusion, our study demonstrates that Nrg1β enhances glucose uptake in cardiomyocytes via ErbB2/ErbB4 heterodimers, PI3K/Akt, and Akt. Furthermore, although Nrg1β activates mTORC2, the resulting Akt-Ser473 phosphorylation is not essential for glucose uptake induction. These new insights into pathways whereby Nrg1β regulates glucose uptake in cardiomyocytes may contribute to the understanding of its regenerative capacity and protective function in heart failure.

phosphatidylinositol 3-kinase; metabolism; tyrosine kinase; ErbB; signaling; protein synthesis

Address for reprint requests and other correspondence: M. Brink, Dept. of Biomedicine, Cardiobiology, Univ. of Basel and University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland (e-mail: marijke.brink@unibas.ch).

neuronal isoform glial growth factor 2 (GGF2), are to be expected in the near future. Recent work also implicated Nrg1β in the regenerative potential of neonatal and adult hearts (3, 25, 53). Nevertheless, the molecular mechanisms whereby Nrg1β exerts these effects in cardiac cells are still poorly understood.

Upon stimulation of cardiomyocytes by Nrg1β, the ErbB receptors act via the Src/focal adhesion kinase (FAK), the extracellular-regulated kinase (Erk)1 and 2, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways, which have been linked to distinct functions (50). Cardiac developmental and postnatal growth as well as physiological or pathological adaptations of the adult heart are regulated by the serine/threonine kinase mammalian target of rapamycin (mTOR). mTOR modulates cellular processes such as protein synthesis and energy metabolism (10, 37) and has distinct functions depending on whether it is part of mTOR complex (mTORC)1 or mTORC2. In the developing and adult heart mTORC1 activity is associated with protein synthesis and physiological hypertrophy, and mTORC2 may modulate glucose uptake, as demonstrated previously in skeletal muscle (35, 40, 57). Increased glucose uptake is critical for the survival of cardiomyocytes during the acute phase of ischemic injury, when lipid metabolism will become not only insufficient for the energy demands of the heart but will also lead to a significant increase in oxidative stress (3, 46, 72). Nrg1β is cardioprotective during ischemia (38), but its effects on glucose uptake and the involvement of mTOR have not been investigated.

In other contexts, ErbB receptor activity has been related to mTOR signaling. In breast cancer, pathological ErbB2 overexpression is associated with constitutive activation of Akt/mTOR and predicts tumor progression (52, 67, 79), and mTOR inhibitors improve the outcome of ErbB2-positive breast cancer (72). Whereas mTOR inhibition appears to be of therapeutic value in cancer, cardiomyocyte mTORC1 deficiency leads to cardiac dysfunction in mice (62, 77). The observation that recombinant human GGF2 causes phosphorylation of the mTORC1 target 70-kDa ribosomal S6 kinase (p70S6K) in cardiomyocytes (2) and that Nrg1β causes phosphorylation of Akt on the mTORC2 target site Ser473 (59, 76) led us to investigate whether and how mTORC1 and mTORC2 mediate one or more of the Nrg/ErbB-related cardioprotective activities.

Our study demonstrates in a model of rat neonatal cardiomyocytes that Nrg/ErbB signaling enhances glucose uptake and protein synthesis. The glucose uptake is mediated by PI3K/Akt/AS160. Nrg1β-induced mTORC1 activation plays a small role in the protein synthesis, whereas mTORC2 appears to not be implicated in the glucose uptake.
**MATERIALS AND METHODS**

**Growth factors and inhibitors.** Nrg1β was from R & D Systems, IGF-I was from Genentech, insulin, PP242, and wortmannin were from Sigma, Lapatinib and Dasatinib were from LC Laboratories, and PP2, PP3, LY-294002, Akt inhibitor VIII, U-0126, SB-203580, rapamycin, and PFS73228 were from Calbiochem. Bty-719, TGX-221, Cal101, and AS605240 were kind gifts from Matthias P. Wymann, University of Basel.

**Antibodies.** Antibodies against mTOR-p-Ser2448, mTOR-p-Ser2481, mTOR, Erk1/2-p-Thr202/Tyr204, Akt-p-Thr308, Akt-p-Ser473, Akt, p70S6K1-p-Thr389, p70S6K1, ULK1-p-Ser757, eukaryotic initiation factor 4E-binding protein-1 (4E-BP1)-p-Ser65, 4E-BP1, phosphorylated Akt substrate, AS160, and AS160-pThr422 were from Cell Signaling Technology. Antibodies against GAPDH, FAK, c-Src, ErbB2-p-Tyr1248, ErbB2, and ErbB4 as well as normal goat IgG were from Santa Cruz Biotechnology. Antibodies against FAK-p-Tyr925 and ErbB4-p-Tyr1248 were from Abcam. Antibodies against FAK-p-Tyr925 were from BD Biosciences. Antibodies against Src-p-Tyr415 were from ECM Biosciences, and antibodies to ULK1 were from Sigma.

**Primary neonatal cardiomyocyte isolation and transfection.** Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old rats and transfected with nontarget ErbB2 and ErbB4 siRNA (Dharmacon) at 1 μg/3 × 10^6 cells using cardiomyocyte AMAXA nucleofactor (Lonza), as published previously (26). Two days later, the cells were treated with inhibitors and growth factors after an overnight incubation in serum-free albumin, carnitine, taurine and taurophilic-medium enriched medium (ACCT). ACCT medium consisted of 2 g/l albumin, 2 mM L-carnitine, 5 mM creatine, and 5 mM taurine (all from Sigma) in DMEM (Gibco).

**Glucose uptake.** NRVMs were treated with inhibitors for 30 min, followed by 30 min in the presence of growth factors and another 30 min in the presence of deoxy-o-glucose, 2-[1,2-3H(N)] (Perkin-Elmer) and deoxy-d-glucose (1 μM/ml and 100 μM, respectively) in Krebs-Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 24 mM NaHCO_3, 10 mM HEPES, pH 7.4, and 0.1% BSA). The glucose uptake was stopped by three washes with ice-cold PBS and lysis in NaOH. Part of the lysate was mixed with scintillation liquid, and ^3H was measured with a β-counter. A micro BCA protein assay (Thermo Scientific) was performed with the remaining lysate to normalize the counts/min.

**Protein synthesis.** To analyze the pathways by which Nrg1β stimulates protein synthesis, NRVMs were incubated for 30 min with inhibitors, as indicated in RESULTS, and then stimulated in the presence of 1 μg/ml [3H]phenylalanine (Amersham Biosciences) for 24 h. Cells were then washed with ice-cold PBS, precipitated with 10% ice-cold trichloroacetic acid for 30 min, washed with glacial EtOH 95%, dried, and lysed in NaOH for 45 min. Part of the lysate was mixed with scintillation liquid for measurement of ^3H with a β-counter. A DNA assay with Hoechst (Invitrogen) was performed with the remaining lysate and used to normalize the counts/min.

**Protein extraction and Western blot analysis.** Total protein was extracted with RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 0.1% SDS, 5 mM EDTA, and 0.5% phosphatase inhibitor cocktail 2 and 3 (Sigma)), and 1% protease inhibitor cocktail (Sigma) was separated by SDS-PAGE and transferred to a PVDF membrane (Amersham-GE Healthcare). After incubation with antibodies, the signal was revealed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), CL-XPosure Film (Thermo Scientific), or the ChemiDoc MP System (Bio-Rad). Blots were quantified with Image Lab (Bio-Rad) and ImageJ (National Institutes of Health).

**Immunoprecipitation.** All procedures for immunoprecipitation were done at 4°C. Protein lysates (30 μg) were cleared with 25 μl of protein A-agarose (Amersham-GE Healthcare) and incubated over-night with 2 μg of antibody to AS160 or normal goat IgG. A 50% slurry of protein A-agarose (40 μl) was added for 4 h, and the beads were then washed five times with RIPA buffer and collected by centrifugation for 3 min at 3,000 rpm. The beads were resuspended in loading buffer and heated at 95°C. Supernatants were loaded on an 8% SDS-PAGE, and proteins transferred to PVDF, and phosphorylated Akt substrate and AS160 were detected as described above.

**Isolation of adult mouse ventricular myocytes.** Hearts were dissected from C57BL/6 mice, briefly washed in ice-cold Ca^2+−free perfusion buffer (135 mM NaCl, 4 mM KCl, 1 mM MgCl_2, 10 mM HEPES, 0.33 mM NaH_2PO_4, 10 mM glucose, 10 mM 2,3-butanedi-one-monoxime, and 5 mM taurine), and cannulated through the aorta for retrograde perfusion. After 5 min of acclimatization at 37°C, hearts were perfused for 7 min with digestion solution, consisting of 5,000 U collagenase (Worthington) and 5.24 U protease (Sigma) in Ca^2+−free perfusion buffer. The dissociated myocytes were passed through a 100-μm cell strainer and incubated with increasing concentrations of Ca^2+ (0, 0.06, 0.24, 0.6, and 1.2 mM) that were obtained by mixing appropriate amounts of transfer buffer A (perfusion buffer with 5 mg/ml of BSA) with transfer buffer B (137 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl_2, 10 mM HEPES, 1.2 mM CaCl_2, and 5 mM glucose). Cells were seeded on laminin-coated dishes and kept in ACCT medium overnight before treatment.

**Statistics.** All results are expressed as means ± SE. One-way ANOVA analysis was followed by Sidak’s post hoc testing using Prism 6 (GraphPad).

**RESULTS**

**Nrg1β induces phosphorylation of the mTORC1 targets 4E-BP1, S6K, and ULK and the mTORC2 target Akt-p-Ser473**. First, we analyzed the temporal pattern of activation of kinases known to be part of the mTOR and Akt signaling pathways using NRVMs. Figure 1A shows that at 5 min, Nrg1β treatment caused phosphorylation of both ErbB2 and ErbB4 at Tyr1248. At the same time, the phosphorylation of FAK and Akt was already strongly increased. The phosphorylated amounts of mTOR and the mTORC1 targets 4E-BP1, p70-S6K1, and ULK were increased later at 15 and 30 min. Lapatinib, a well known inhibitor of ErbB1 and ErbB2, blocked the phosphorylation of ErbB2 and ErbB4 as well as that of all downstream effectors (Fig. 1A). Moreover, a dose response experiment at 30 min confirmed specificity of the Akt response and established 10 ng/ml as optimal Nrg1β concentration for further experiments (Fig. 1B). Nrg1β also activated mTOR signaling in cardiomyocytes isolated from adult mouse hearts, in which increases in mTOR-pS2448 were similar to those obtained with IGF-I, and an upward bandshift was observed for 4E-BP1, indicating increased phosphorylation (Fig. 1C). Thus, we conclude that Nrg1β has immediate and specific stimulatory effects on mTOR-mediated signaling cascades in cardiomyocytes.

In Fig. 1D, the effects of Nrg1β are compared with those of IGF-I to evaluate the potency of either growth factor to activate mTOR and its effectors over a longer period of time. At 30 min, IGF-I and Nrg1β similarly increased mTOR-p-Ser2448, p70-S6K1-p-Thr389, Akt-p-Ser473, and 4E-BP1 phosphorylation. For Nrg1β, the p70-S6K1-p-Thr389 and Akt-p-Ser473 signals decreased more rapidly than for IGF-I. Thus, at 3 h the Nrg1β-treated samples already displayed a much lesser increase compared with controls than the IGF-I-treated samples, for which the increase remained very pronounced for at least 6 h. On the other hand, the phosphorylation of 4E-BP1 persisted for up to 24 h for both growth factors. PP242, an mTOR inhibitor that blocks mTORC1 as well as mTORC2, abolished all of the Nrg1β-induced increases in phosphorylation (Fig.
1D). As expected, rapamycin decreased phosphorylation of p70-S6K1 and 4E-BP1 in line with its function as mTORC1 inhibitor in short-term experiments. Interestingly, rapamycin increased IGF-I-induced phosphorylation of the mTORC2 target site Ser473 in Akt, consistent with earlier studies (44, 68, 69, 74), whereas it did not modify the Nrg1β-induced phosphorylation of Akt at Ser473. We conclude that Nrg1β/ErbB activates mTORC1-4E-BP1 in a similar manner as IGF-I, whereas it activates p70-S6K1 and mTORC2-Akt more transiently than IGF-I.

Nrg1β stimulates protein synthesis. Given the well-established function of mTORC1, we tested whether Nrg1β stimulates protein synthesis in cardiomyocytes via mTORC1. Incubation with Nrg1β for 24 h increased phenylalanine incorporation in a Lapatinib-sensitive manner (Fig. 2A), and rapamycin and PP242 both diminished this effect to a similar

Fig. 1. Neuregulin-1β (Nrg1β) activates mammalian target of rapamycin (mTOR) complex (mTORC)1 and mTORC2 in ventricular myocytes. A: after overnight incubation in serum-free albumin, carnitine, creatine, and taurine-enriched medium, neonatal rat ventricular myocytes (NRVMs) were treated with Nrg1β (10 ng/ml) for 5, 15, and 30 min. Cells were pretreated with Lapatinib (Lap; 10 μM) or vehicle (DMSO) for 30 min. B: dose response for Nrg1β in NRVMs at 30 min. C: adult mouse ventricular myocytes were treated with Nrg1β (10 ng/ml) or IGF-I (20 ng/ml) and lysed for analysis by Western blotting at the time points indicated. D: NRVMs were treated with Nrg1β (10 ng/ml) or IGF-I (20 ng/ml) for ≤24 h in the absence or presence of rapamycin (Rap; 20 ng/ml) or PP242 (20 μM), and lysates were analyzed as in A. D: total proteins (20 μg) were analyzed by Western blotting to test for total and phosphorylated proteins as indicated. FAK, focal adhesion kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; Ctl, control; S6K, p70S6K1.

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extent (Fig. 2B), which suggests that mTORC1 contributes to the Nrg1β-induced protein synthesis. However, since a strong increase persisted after PP242 or Rap inhibition, mTOR-independent alternate pathways are also important in the Nrg1β-induced phenylalanine uptake. Consistent with previous studies, the Erk1/2 inhibitor U-0126 partially inhibited phenylalanine incorporation (Fig. 2C). Moreover, the Akt inhibitor VIII abolished the protein synthesis response, in line with the established role of Akt as upstream activator of mTORC1. We conclude that Akt and Erk1/2, as well as mTORC1 via sustained phosphorylation of 4E-BP1 (Fig. 1D), are implicated in the enhanced global protein synthesis after Nrg1β treatment of neonatal cardiomyocytes.

**Nrg1β enhances glucose uptake.** Given our observation that the effect of PP242 on protein synthesis was only modest, we next aimed at identifying other functions of mTOR in cardiomyocytes. Very little is known about the cardiac function of mTORC2, and therefore, we decided to focus on the function of the Nrg1β-induced phosphorylation of Akt at Ser473, which in Fig. 1D was shown to be rapamycin resistant and PP242 sensitive and, therefore, most likely mTORC2 dependent. Akt is implicated in the translocation of glucose transporters to the plasma membrane and thereby contributes to insulin-induced glucose uptake after feeding (34). As mTORC2 has been reported to regulate glucose metabolism in some tissues (35, 40), we hypothesized that mTORC2-mediated phosphorylation of Akt contributes to Nrg1β-induced glucose uptake in our cardiomyocyte model. We tested this hypothesis while using IGF-I and insulin as a reference. The three stimuli induced similar increases in glucose uptake, and combinations of Nrg1β with either IGF-I or insulin did not yield any further increase (Fig. 3A), indicating that Nrg1β depends at least in part on the same signaling molecules as IGF-I and insulin. Lapatinib abolished the effect of Nrg1β on glucose uptake (Fig. 3B), implicating ErbB2 and/or ErbB4 in the response. Figure 3C shows that rapamycin did not have any effect at all, which excludes a role for mTORC1. On the other hand, PP242 preincubation reduced the Nrg1β-induced glucose uptake compared with that of DMSO-preincubated cells (Fig. 3C). In fact, PP242 reduced the Nrg1β-induced glucose uptake from 1.77- to 1.56-fold compared with the corresponding unstimulated DMSO- and PP242-preincubated controls, respectively, and therefore, the inhibition was 27% of the total increase. In additional independent experiments, PP242 reduced Nrg1β-, IGF-I-, and insulin-induced glucose uptake from 1.88-, 1.64-, and 1.90- to 1.52-, 1.56-, and 1.52-fold, respectively, vs. the corresponding unstimulated controls. This represents an inhibition of 41, 13, and 42%, respectively, for the three growth factors. Together, our data suggest that common pathways are used by the three growth factors to link receptor activation to glucose uptake and that mTORC2 may mediate part of the insulin- and Nrg1β-induced responses. Additional nonpharmacological approaches are required to prove this further (see below).

**The Nrg1β-induced glucose uptake is mediated by Akt and AS160.** To further characterize the PP242-sensitive signaling branch of the Nrg1β-induced glucose uptake, we assessed which kinases, in addition to those already shown in Fig. 1, were inhibited by PP242 (Fig. 4). At 30 min, Nrg1β enhanced Akt phosphorylation at Thr308 and Ser473, although the effect was somewhat weaker than that observed for IGF-I. PP242 abolished both increases completely for Nrg1β and partially for IGF-I (Fig. 4A). Together with the generally accepted view that Akt-p-Ser473 is a direct target of mTORC2 and our finding that rapamycin does not reduce Akt-p-Ser473 (Fig. 1D), this complete inhibition by PP242 supports that mTORC2 is implicated in this Nrg1β-induced Akt phosphorylation in cardiomyocytes. In Fig. 1A, we showed that Nrg1β increases Akt-p-Ser473 already at 5 min, before mTOR phosphorylation is detectable, and therefore, we tested whether Nrg1β has a rapid, mTOR-independent phase of Akt activation. Figure 4B shows that PP242 inhibited the Akt-Ser473 phosphorylation at 5 min after Nrg1β stimulation, which indicates that mTORC2 was active and required for this early phosphorylation of Akt. Notably, Akt-p-Thr308 was somewhat reduced but not abolished in the PP242-pretreated cells, consistent with the notion that PDK1-mediated Akt-Thr308 phosphorylation is possible in the absence of mTORC2-mediated Akt-Ser473 phosphorylation. These reduced Akt-p-Thr308 amounts may explain the partially reduced glucose uptake response to Nrg1β after PP242 pretreatment. Figure 4C shows that the Akt inhibitor VIII abolished the Nrg1β-induced gluc-
Glucose uptake, which indeed supports that Akt is a main actor in this effect.

IGF/insulin-induced Akt activation is known to cause the translocation of glucose transporters to the sarcolemma via its downstream mediator AS160 (56), but whether or not Nrg1β acts via this mechanism in cardiomyocytes is not known. Immunoprecipitation experiments demonstrated that Nrg1β raised the phosphorylation of Akt substrate AS160 in a manner similar to IGF-I (Fig. 4D). Moreover, an antibody to AS160-p-Thr642 confirmed its phosphorylation after Nrg1β stimulation (Fig. 4E), which is consistent with the known function of this specific phosphorylation site being indicative of enhanced glucose transporter type 4 (GLUT4) translocation to the sarcolemma. Together, these data support that Nrg1β induces glucose uptake in NRVMs via Akt and AS160 and suggest that mTORC2-mediated Akt-Ser473 phosphorylation may contribute to this response.

Nrg1β-induced mTORC2-mediated Akt phosphorylation at Ser473 is not required for the glucose uptake response. To further investigate the role of mTORC2, we knocked down its specific and essential component rictor using siRNA technologies. Figure 4F shows that reduced rictor protein levels were associated with lower Akt-p-Ser473, confirming that mTORC2 activity was efficiently reduced. However, this was not associated with lower AS160-p-Thr642 (Fig. 4F), and consistently, glucose uptake responses to Nrg1β were not impaired (Fig. 4G). Together with our observations that PP242 only modestly and Akt inhibitor VIII completely inhibited the glucose uptake, these data indicate that mTORC2-mediated Akt phosphorylation is not essential for this response to Nrg1β. Thus, whereas our data demonstrate that Nrg1β activates mTORC2 and stimulates glucose uptake, the mTORC2-mediated Akt-p-Ser473 phosphorylation appears not to modulate this glucose uptake.

Upstream of Akt, PI3K is implicated in the Nrg1β-induced glucose uptake. We next aimed at identifying other upstream signaling molecules that mediate the Nrg1β-induced Akt activation and glucose uptake. The class 1 PI3K inhibitor LY-294002 (LY) abolished the Nrg1β-induced glucose uptake (Fig. 5A), whereas effects of the p38 inhibitor SB203580 (SB) and the Erk1/2 inhibitor U-0126 did not reach significance (Fig. 5B). Consistently, LY blocked whereas SB only had small effects on Akt phosphorylation (Fig. 5E). Others have demonstrated that SB decreases glucose uptake independently of p38 MAPK inhibition (1). Therefore, PI3K is most likely the main actor upstream of Akt in the Nrg1β-induced glucose uptake. LY has been reported to also inhibit other kinases, including mTORC1, at concentrations similar to those that inhibit PI3K (13). Additional experiments with the isoform-specific PI3K inhibitor Bafilomycin A1 (Bafil) revealed that Nrg1β activates glucose uptake via PI3Kα (Fig. 5C), and this was associated with lower Akt (Thr308 and Ser473) and Akt substrate (160 kDa) phosphorylation (Fig. 5D), which suggests that PI3Kα is activating both PDK1 and mTORC2. Consistently, inhibition of PI3Kβ, -δ, and -γ with TGX-221, Cal101, and AS605240, respectively, had no effect (data not shown).

Effect of Src family kinase inhibitors on the Nrg1β-induced glucose uptake. How does Nrg1β-induced ErbB2/ErbB4 phosphorylation lead to PI3K activation? Given our observation that the Nrg1β-induced Akt phosphorylation was paralleled over time by increased FAK-p-Tyr861 (Fig. 1A), we tested whether Src family kinases are implicated, because FAK is one of their direct targets (7, 61). PP2, a compound that inhibits Src family protein kinases such as c-Src, reduced the Nrg1β-induced glucose uptake (Fig. 6A) as well as phosphorylation of FAK at Tyr996 and Akt at Thr308 and Ser473 (Figs. 5E and 5F), whereas it had no effect on p70-S6K1-p-Thr389 (Fig. 5E). None of the effects was observed with PP3, a negative control for PP2. Recent studies reported that PP2 may inhibit other kinases with similar affinities and that it is less Src selective than the Src family kinase inhibitor dasatinib (Das) (4). Therefore, we also tested Das and found that it efficiently reduced FAK-p-Tyr996, Akt at both tested
sites (Figs. 5E and 6E), and the 160-kDa Akt substrate (not shown). A dose response experiment demonstrated that strong inhibition was obtained already at 100 ng/ml and was complete at 1 μg/ml (data not shown). Notably, Das potently reduced the Nrg1β- but not the IGF-I-induced glucose uptake (Fig. 6B), indicating that Das sensitivity is a unique feature of Nrg1β-induced ErbB signaling. Similarly, only Nrg1β led to increased phosphorylation of FAK at Tyr861 (Fig. 6C). In an attempt to further identify the implicated kinase, we analyzed over time the phosphorylation of Src at Tyr215 and Tyr416, the latter site being indicative of c-Src activation. Src-p-Tyr215 increased at 30 min, which is much later than FAK-p-Tyr861, Akt, and Akt substrate phosphorylation, excluding a role of this site in the rapid activation of Akt (Fig. 6D). Src-p-Tyr416 was already high under basal conditions and did not increase any further after Nrg1β stimulation. Das but not PP2 inhibited phosphorylation of this site (Fig. 6E), whereas both inhibitors negatively affected Nrg1β-induced Akt activation and glucose uptake (Fig. 6, A and B), which altogether excludes a causative role of c-Src. In conclusion, our findings indicate that one or more members of the Src kinase family (but not c-Src) are involved in integrin activation involves, as a first step, autophosphorylation of FAK at Tyr861 (Fig. 6A). In an attempt to further identify the implicated kinase, we analyzed over time the phosphorylation of Src at Tyr215 (Fig. 6C), indicating that Das sensitivity is a unique feature of Nrg1β-induced ErbB signaling. Similarly, only Nrg1β led to increased phosphorylation of FAK at Tyr861 (Fig. 6C). In an attempt to further identify the implicated kinase, we analyzed over time the phosphorylation of FAK at Tyr861 (Fig. 6C) and Tyr416, the latter site being indicative of c-Src activation. Src-p-Tyr215 increased at 30 min, which is much later than FAK-p-Tyr861, Akt, and Akt substrate phosphorylation, excluding a role of this site in the rapid activation of Akt (Fig. 6D). Src-p-Tyr416 was already high under basal conditions and did not increase any further after Nrg1β stimulation. Das but not PP2 inhibited phosphorylation of this site (Fig. 6E), whereas both inhibitors negatively affected Nrg1β-induced Akt activation and glucose uptake (Fig. 6, A and B), which altogether excludes a causative role of c-Src. In conclusion, our findings indicate that one or more members of the Src kinase family (but not c-Src) are involved in the phosphorylation of FAK at Tyr861 and in the glucose uptake response to Nrg1β, although a causal relationship between FAK-p861 and glucose uptake and the precise mechanism involved remain to be established.

Nrg1β/ErbB-induced glucose uptake does not depend on integrin/FAK397. Src family kinases have been implicated in integrin signaling, and recent data suggest that integrin activation may promote glucose uptake (29). Ligand-induced integrin activation involves, as a first step, autophosphorylation of FAK at Tyr397, which induces a conformational change and exposes a binding motif for SH2-domain-
containing Src-family kinases. After binding, these kinases further phosphorylate FAK at multiple sites and thereby activate it (60). To investigate whether the Nrg1β/H9252-induced glucose uptake requires signaling via integrin/FAK-p-Tyr397, we used the potent FAK inhibitor PF-573228 (PF). Figure 7A shows that Nrg1β/H9252 increased FAK-p-Tyr861 but did not change FAK-p-Tyr397 compared with untreated controls. Consistent with an earlier report (33), PF had no effect on the Nrg1β/H9252-stimulated FAK-p-Tyr861, but it effectively reduced FAK-p-Tyr397, Src-p-Tyr416, and phosphorylation of the integrin-FAK target paxillin. Nevertheless, PF neither inhibited Nrg1β-induced mTOR and Akt signaling, as phosphorylation of mTOR and its targets Akt (Ser473) and p70S6K remained high (Fig. 7A), nor did it affect the glucose uptake response (Fig. 7B), from which we conclude that these effects are integrin/FAK-p-Tyr397/c-Src-independent. The data also indicate the presence of high basal integrin/FAK/Src activity in our model.

Specific downregulation of ErbB2 and ErbB4 with siRNA. Lapatinib blocked the glucose uptake and all Nrg1β/H9252-induced signaling, but as it reduced phosphorylation of both ErbB2 and ErbB4 it did not distinguish between the two receptors (Fig. 1). To define the specific role of ErbB2 and ErbB4 in the Nrg1β-induced pathways that enhance glucose uptake, we used pools of siRNA specific for ErbB2 and ErbB4. Both targeted proteins were effectively downregulated, whereas GAPDH and vinculin were not altered (Fig. 8). Knockdown of ErbB4 resulted in reduced Nrg1β/H9252-stimulated levels of FAK-p-Tyr861, Akt-p-Ser473, Akt-p-Thr308, mTOR-p-Ser2448, ULK-p-Ser757, and p70-S6K1-p-Thr389. ErbB2 knockdown, on the other hand, only reduced Akt-p-Ser473 and Akt-p-Thr308 (Fig. 8). Both the ErbB2 and ErbB4 siRNA knockdown abolished the Nrg1β-induced glucose uptake, showing that both ErbB2 and ErbB4 are needed for this response. The signaling data suggest that ErbB4 is implicated in the Nrg1β-induced activation of mTORC1 as well as mTORC2. ErbB2 is implicated in mTORC2 but does not seem to be required for mTORC1 activation by Nrg1β. However, as our knockdown approach was more efficient for ErbB4 than for ErbB2, communoprecipitation and com-

**Fig. 5.** Upstream of Akt, PI3Kα is implicated in the Nrg1β-induced glucose uptake. A–D: NRVMs were pretreated with the PI3K inhibitor LY (10 μM), the p38 inhibitor SB-203580 (SB; 10 μM), and the PI3Kα inhibitor Byl-719 (1 μM; D) for 30 min. NRVMs were then stimulated with vehicle (Ctl) or Nrg1β (10 ng/ml) for 30 min, and glucose uptake was assessed as in Figs. 3 and 4. E: inhibitors were used and cells stimulated as in A–D and Fig. 6. Protein extracts were analyzed by Western blotting as in Fig. 1. *P < 0.05, **P < 0.01, and ****P < 0.0001 (vs. Ctl); $$$$$P < 0.0001, inhibitor vs. DMSO.
complete knockout approaches are required to further investigate receptor involvement. Our data are in line with the notion that ErbB4 is the main receptor for Nrg1β, whereas ErbB2 has no extracellular binding pocket for Nrg1β but transduces specific signals when it is part of a heterodimer complex with one of the other ErbB isoforms.

**DISCUSSION**

Under stress conditions, cardiac microvascular endothelial cells release Nrg1β, which in a paracrine manner activates the receptor dimers ErbB2/ErbB4 and ErbB4/ErbB4, both of which are expressed in cardiomyocytes (11, 78). Upon stimulation, the ErbB receptors may act via the Erk1/2, PI3K/Akt, and Src/FAK pathways, each of which has been linked to distinct protective functions. For example, Nrg1β diminishes doxorubicin-induced sarcomere disarray in cardiomyocytes via Erk (48, 51, 59), whereas PI3K/Akt activation is responsible for the protection against basal or anthracycline-induced apoptosis (16, 22, 78) in part by reducing oxidative stress and improving mitochondrial function, calcium handling, and contractility (22, 23, 66). Independently of PI3K/Akt and Erk1/2, Nrg1β influences focal adhesion formation via Src/FAK (36).

Consistent with previous studies (2, 11, 78), we show here that Nrg1β increases global protein synthesis in neonatal cardiomyocytes. Whereas early studies implicated ErbB2 and Erk1/2 in the protein synthesis response to GGF2, here we extend the mechanistic insights by demonstrating how Nrg1β activates mTORC1 over time and by providing data that suggest its involvement in protein synthesis. However, it should be mentioned here that a large part of the Nrg1β-induced protein synthesis was not inhibitable by the mTOR inhibitor PP242, indicating pathway redundancy. Nevertheless, we show that Nrg1β increases mTOR-p-Ser2448 (a site that indicates mTORC1 activity) and the phosphorylated levels of two direct mTORC1 targets that regulate protein synthesis, namely p70-S6K and 4E-BP1. Nrg1β-induced increase in 4E-BP1 phosphorylation lasted as long as that induced by IGF-I, which may explain the similar potency of the two factors to enhance protein synthesis. In contrast to IGF-I, Nrg1β only transiently affects p70-S6K phosphorylation, and rapamycin does not lead to hyperphosphorylation of Akt. These findings suggest that Nrg1β activates mTORC1 and protein synthesis without inducing the negative feedback loop that is perceived as one of the causes of insulin resistance.

Our study also demonstrates that Nrg1β increases glucose uptake. Nrg1β was reported to enhance glucose uptake in cardiomyocytes only in one earlier study, in which it was shown that the endothelium generates both Nrg1α and Nrg1β, but that only Nrg1β causes ErbB2 tyrosine phosphorylation with functional consequences such as increased glucose uptake (11). Whereas that study did not analyze the underlying pathways, our study now shows that Nrg1β increases glucose uptake via ErbB2/ErbB4 heterodimers and enhanced signaling via PI3Kα, Akt, and AS160. Given the well-established function of Akt/AS160, our data suggest that the increase in glucose uptake after Nrg1β stimulation is a consequence of GLUT4 translocation to the sarcolemma (36). Thus, whereas others have linked Akt activation by Nrg1β to pro-survival pathways (16, 22, 23, 66, 78), here we provide evidence that Nrg1β also triggers glucose uptake via this kinase.
This novel ErbB2/ErbB4-specific mechanism of glucose uptake may be particularly important under conditions of acute stress such as ischemic events, when the heart has to rapidly respond to maintain performance and survival of contractile cells. Whereas fatty acid and glucose oxidation are tightly regulated in the healthy heart to optimally provide it with the high amounts of energy needed for contraction, substrate use for ATP production changes under hypoxic conditions associated with, e.g., ischemia or hypertrophy. A shift from predominant fatty acid oxidation to increased carbohydrate use and glycolysis ensures continued ATP production under conditions of oxygen deficiency (31, 39). Ischemia has previously been associated with the translocation of glucose transporters to the sarcolemma (5, 46, 75). At the early stages of ischemia, the glucose allows a better adaptation and survival (5). In support of this concept, cardiac deletion of GLUT4 leads to a lower tolerance to ischemic events associated with a higher rate of ATP depletion (65). Indeed, ischemic insults acutely caused the rapid release of Nrg1β from microendothelial cells and phosphorylation of ErbB4 (15, 36), and Nrg1β ablation in endothelial cells aggravated the harmful consequences of ischemia (38), whereas intravenous injections of the EGF-like domain of Nrg1β or GGF2 improved ventricular function in rat and swine models of myocardial infarction (18, 27, 41). Our present findings suggest that an increase in glucose uptake is one of the protective mechanisms induced by Nrg1β in these conditions.

The ability of Nrg1β to stimulate glucose uptake has been reported previously for a skeletal muscle cell line (8, 64), and consistent with those in vitro studies, acute infusion of GGF2 or Nrg1β was recently shown to lower blood glucose in swine (18), rats (6), and a mouse model of type 2 diabetes (14). Whereas, in L6E9 myotubes PKCζ was implicated in the glucose uptake response, here we show that in cardiomyocytes Akt activation is implicated, because Nrg1β increases AS160 phosphorylation, and the Akt inhibitor VIII abolishes this as well as the glucose uptake response. Another new finding of our study is that Nrg1β activates mTORC2 because it phosphorylated the mTORC2-specific site Ser473 of Akt in a PP242-sensitive manner. However, consistent with our recently published in vivo data in adult mice (63), Akt-p-Ser473 phosphorylation appears to not be needed for phosphorylation of substrates involved in glucose uptake. This conclusion is supported by our observation that rictor knockdown efficiently reduces Akt-p-Ser473 without having any effects on AS160 phosphorylation and glucose uptake. Thus, whereas a role has previously been attributed to mTORC2 in skeletal muscle (35, 40, 57), fat, and liver (24), our own data demonstrate that mTORC2 is not implicated in glucose uptake of the heart. In this setting, it should be mentioned that PI3K was recently reported to directly phosphorylate Akt at Ser473 and induce glucose transporter translocation (70), and thus mTORC2-dependent Ser473 phosphorylation of Akt appears to be dispensable in this pathway.

To the best of our knowledge, our study is the first to identify Nrg1β as an activator of PI3K/Akt-mediated glucose uptake in cardiac cells. We show unique features of this Nrg1β-induced pathway, such as its Das sensitivity, which was not observed in IGF-1-stimulated cells. Consistently, Nrg1β, but not IGF-I or insulin, induced phosphorylation of FAK at Tyr397. LY did not block FAK phosphorylation, whereas Das and PP2 blocked phosphorylation of FAK and Akt as well as the glucose uptake response, suggesting that Src family kinases and FAK may be implicated either upstream of or in parallel to PI3K, consistent with previous studies in the heart (9) and noncardiac cells (42). Whether or not Src-dependent FAK-Tyr397 phosphorylation is implicated in transmitting the signal from ErbB to PI3K remains to be proven. Others have demonstrated that ErbB activation may also directly stimulate PI3K in cardiomyocytes (70).

Our observation that Nrg1β rapidly increases FAK-p-Tyr397 is consistent with a previous study on adult cardiomyocytes (36). In contrast to that study, phosphorylation of c-Src at Tyr416 was high in our unstimulated NRVMs, and Nrg1β did not increase this further. Das decreased c-Src-p-Tyr416, suggesting that it was already active under basal conditions in our
model. On the other hand and consistent with Kuramochi et al. (36), Nrg1β increased the signal detected with an antibody to Src-p-Tyr215. PP2 and Das, but not PP3, blocked this increase as well as the glucose uptake response. However, our observation that this phosphorylation happens later than the Akt phosphorylation excludes its role in the specific activation by ErbB2/ErbB4. We conclude that the presence of a PP2/Das-sensitive kinase, but most likely not c-Src, is important for the phosphorylation of FAK at Tyr861 and the glucose uptake response to Nrg1β, although the causal relationship between FAK-p-Tyr861 and Akt remains to be proven.

Interestingly, we found that phosphorylation of FAK at Tyr861 by Nrg1β does not depend on FAK phosphorylation at Tyr397, a site that autophosphorylates upon integrin stimulation and leads to additional phosphorylation events after Src family kinase recruitment to SH2 domains. Our conclusion is supported by the observation that PF reduces integrin-related FAK-p-Tyr397 and paxillin-p-Tyr118 but not the Nrg1β-induced phosphorylation of FAK at Tyr861. Differential inhibition by PF of these two phosphorylation events has also been reported for lung and breast cancer cells (28, 33). Thus, Nrg1β induces FAK phosphorylation independently of the classical integrin pathway.

A limitation of our own as well as other studies with different cell types and hormonal stimuli (32, 54, 71) is that pharmacological inhibition was used to implicate Src family kinases in glucose uptake responses. Since PP2 and Das, besides inhibiting multiple Src family kinases, have recently been described to have off-target effects, further studies are needed to determine which kinases inhibited by PP2 and Das are responsible for FAK-Tyr861 phosphorylation and the glucose uptake response to Nrg1β. It also remains possible that although the time course and PP2/Das sensitivity of FAK-Tyr861 phosphorylation parallels that of Akt and AS160 phosphorylation, FAK is not causally implicated in the glucose uptake. Moreover, it is also possible that ErbB directly activates PI3K (55).

Taken together, we show that Nrg1β enhances glucose uptake via ErbB2/ErbB4, PI3Kα, Akt, and AS160 (Fig. 9) and that these effects are sensitive to PP2 and Das. These novel insights provide a basis for future experimental and clinical studies in which this pathway may be exploited to increase glucose uptake, especially in states of insensitivity to insulin.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.P., P.H., S.L., C.M., and L.X. performed experiments; L.P., P.H., C.M., and M.B. analyzed data; L.P., P.H., and M.B. interpreted results of experiments; L.P., P.H., and M.B. prepared figures; L.P. drafted manuscript; L.P., P.H., S.L., C.M., L.X., and M.B. edited and revised manuscript; L.P., P.H., S.L., C.M., L.X., and M.B. approved final version of manuscript.

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