Neuregulin improves response to glucose tolerance test in control and diabetic rats

Iliana López-Soldado,1,2,3 Katrin Niisuke,1 Catarina Veiga,1 Anna Adrover,2 Anna Manzano,4 Vicente Martínez-Redondo,1 Marta Camps,1,3,5 Ramon Bartrons,4 Antonio Zorzano,1,2,3 and Anna Gumà1,3,5

1Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Barcelona, Spain; 2Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain; 3Center for Biomedical Investigation in Net of Diabetes and Associated Metabolic Pathologies, Madrid, Spain; 4Department of Physiological Sciences II, Faculty of Medicine, University of Barcelona-IDIBELL: Bellvitge Institute for Biomedical Research, Barcelona, Spain; and 5Institute of Biomedicine from the University of Barcelona, Barcelona, Spain

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López-Soldado I, Niisuke K, Veiga C, Adrover A, Manzano A, Martínez-Redondo V, Camps M, Bartrons R, Zorzano A, Gumà A. Neuregulin improves response to glucose tolerance test in control and diabetic rats. Am J Physiol Endocrinol Metab 310: E440–E451, 2016. First published December 29, 2015; doi:10.1152/ajpendo.00226.2015.—Neuregulin (NRG) is an EGF-related growth factor that binds to the tyrosine kinase receptors ErbB3 and ErbB4, thus inducing tissue development and muscle glucose utilization during contraction. Here, we analyzed whether NRG has systemic effects regulating glycemia in control and type 2 diabetic rats. To this end, recombinant NRG (rNRG) was injected into Zucker diabetic fatty (ZDF) rats and their respective lean littermates 15 min before a glucose tolerance test (GTT) was performed. rNRG enhanced glucose tolerance without promoting the activation of the insulin receptor (IR) or insulin receptor substrates (IRS) in muscle and liver. However, in control rats, rNRG induced the phosphorylation of protein kinase B (PKB) and glycogen synthase kinase-3 (GSK-3) in muscle and liver. In liver, rNRG increased ErbB3 tyrosine phosphorylation and its binding to phosphatidylinositol 3-kinase (PI3K), thus indicating that rNRG activates the ErbB3/PI3K/PKB signaling pathway. rNRG increased glycogen content in liver but not in muscle. rNRG also increased the content of fructose-2,6-bisphosphate (Fru-2,6-P2), an activator of hepatic glycolysis, and lactate both in liver and muscle. Increases in lactate were abrogated by wortmannin, a PI3K inhibitor, in incubated hepatocytes. The liver of ZDF rats showed a reduced content of ErbB3 receptors, entailing a minor stimulation of the rNRG-induced PKB/GSK-3 cascade and resulting in unaltered hepatic glycogen content. Nonetheless, rNRG increased hepatic Fru-2,6-P2 and augmented lactate both in liver and in plasma of diabetic rats. As a whole, rNRG improved response to the GTT in both control and diabetic rats by enhancing hepatic glucose utilization.

neuregulin; ErbB3; liver; glucose tolerance; protein kinase B

NRG for neuronal and cardiac development and function (19, 32, 36).

and the stress-inducible p38 MAPK, to regulate cell proliferation, survival, migration, and differentiation (54). Transgenic mice for NRG or its ErbB receptors highlight the relevance of NRG for neuronal and cardiac development and function (19, 32, 36).

Expressed by cells of endothelial, mesenchymal, and neuronal origin, most NRG isoforms contain one transmembrane domain (16). The NRG EGF-like domain, which is required for ErbB receptor activation, is located extracellularly and released to the extracellular matrix by controlled proteolysis (22, 34, 37, 46) to bind ErbB receptors.

NRG acts locally in an autocrine and/or paracrine manner. However, this growth factor is also present in the blood, and it has been proposed as a biomarker for cardiovascular pathologies such as chronic heart failure (31), coronary artery disease, and ischemia (20) as well as for acute lung injury (15) in humans. In addition, in vivo administration of a bioactive recombinant NRG has been used therapeutically not only in mouse models of muscular dystrophy (30), spinal cord injury (52), Parkinson’s disease (5), diabetic cardiomyopathy (33), and myocardial infarction (1, 27) but also in patients with chronic heart failure (18, 28). In all cases, this treatment was observed to ameliorate such pathological stages by protecting cells from degeneration and inducing proliferation. Recently, it has been proposed that ErbB2, acting as a coreceptor for NRG-1, is required for cardiomyocyte regeneration after myocardial infarction in mice (12).

NRG is also involved in the regulation of glucose metabolism and insulin sensitivity in muscle (24). It rapidly induces glucose uptake in skeletal muscle cell cultures and tissue in an insulin-independent manner by recruiting glucose transporters to the plasma membrane (49). NRG is released by muscle during contraction in a calcium- and metalloprotease-dependent manner (3). This effect of NRG appears to be local since an acute bout of exercise does not change serum NRG-1β levels for 30 min (38). The inhibition of muscle ErbB4 receptors by blocking antibodies abrogates 50–75% of the contraction-induced glucose uptake in skeletal muscle and also impedes the recovery of glycogen and ATP content (3). These observations thus indicate the physiological relevance of muscle NRG on the rapid metabolic adaptation to contraction.

Type 2 diabetes is characterized by hyperglycemia, a condition caused by insulin resistance of the tissues targeted by this hormone, namely muscle, liver, and adipose tissue (13). Many efforts have been devoted to elucidating this complex pathology as well
as finding effective therapies to bypass defects in insulin action. A recent report (51) indicates that NRG-4, which is expressed mainly by brown and white adipose tissue, has distal effects on hepatic metabolism, reducing lipogenesis and protecting against diet-induced insulin resistance. Here, we studied the systemic effects of in vivo NRG administration in control and diabetic rats. We report that a bioactive recombinant NRG rapidly improves body response to a glucose tolerance test without altering the activation of the insulin receptor in skeletal muscle or in liver of control and diabetic rats. In control rats, NRG increases hepatic glucose utilization by inducing the PI3K/PKB pathway, which is unaltered in skeletal muscle, whereas diabetic rats show resistance to NRG action.

**METHODS**

**Materials.** A recombinant NRG 1 (rNRG) isoform, rHeregulin-β1-(177–244), MW 7884, containing the bioactive EGF domain, was initially donated by Genentech [South San Francisco, CA (currently a member of the Roche Group)]. Anti-phosphorytrosine (cat. no. 610000), anti-IR chain (cat. no. 611277), and anti-GSK-3 (cat. no. 610201) antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-PKCζ (C-20; cat. no. sc-216), anti-ErbB1/EGF receptor (1005; cat. no. sc-03), and anti-ErbB3 (c-17; cat. no. sc-285) antibodies were supplied by Santa Cruz Biotechnology (Dallas, TX). Anti-PKB (cat. no. 9272), anti-phospho-Thr308-PKB (cat. no. 9275), anti-phospho-Ser473-PKB, phospho-Thr308-PKB, total PKB, phospho-Ser473-PKB, total PKC (cat. no. 9336) antibodies were purchased from Cell Signaling Technology (Dallas, TX). Anti-IR (cat. no. 9227), anti-phospho-Thr308-PKB (cat. no. 9275), anti-phospho-Ser373-PKB, anti-phospho-Thr410/416, PKCζ/α (cat. no. 9378), and anti-phospho-Ser3-GSK-3 (cat. no. 9366) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-IRS-1 (insulin receptor substrate-1; cat. no. 06-248), anti-IRS-2 (cat. no. 06-506), and anti-p85 subunit of PI3K (cat. no. 06-195) antibodies were obtained from Millipore Corp (Bedford, MA). Anti-heresulin antibody (EGF-like domain) (cat. no. RB-276) was from Thermo Scientific (Waltham, MA). Anti-α-tubulin antibody (cat. no. T5168) and wortmannin (cat. no. W1628) were from Sigma-Aldrich (St. Louis, MO). All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad (Hercules, CA). The BCA Protein Assay Reagent Kit was supplied by Pierce (Rockford, IL). Immobilon polyvinylidene difluoride (PVDF) was from Millipore (Bedford, MA). ECL reagents were purchased from Amersham Biosciences (Piscataway, NJ).

**Animals.** Animal studies were approved by the Ethics Committee of the University of Barcelona. Six-week-old male fa/fa Zucker diabetic fatty (ZDF) rats and their respective Zucker control lean (ZCL) fa/+ littermates were purchased from Charles River Laboratories (Wilmington, MA). Rats received Teklad Diet 2014 from Harlan (Indianapolis, IN) and had access to water ad libitum. The animals were kept in an environmentally controlled room under a 12-h light-dark cycle.

At 10 to 12 wk of age, ZDF rats were more obese than ZCL control counterparts (346 ± 6 vs. 291 ± 3 g body wt, respectively) and showed basal hyperglycemia under fasted conditions (6.4 ± 0.4 vs. 3.4 ± 0.2 mmol/l, respectively). Similarly, ZDF animals presented hyperinsulinemia under fasted conditions compared with ZCL rats (638 ± 104 vs. 20 ± 2 pmol/l, respectively). For the in vivo NRG treatment, rats were fasted overnight and then injected intraperitoneally (ip) with a solution of rNRG (17.8 μg/ml) in saline at a dose of 25 μg/kg rat body wt. The dose of NRG administered was in the range of that used in other studies in rodents (1–10 μg/kg) (28, 30, 33) and reached a concentration of 3–8 mM considering a mixing distribution space in fluids and tissues of ≤0.3–1 ml/g, to which in vitro effects on muscle glucose uptake were reported previously (49). Untreated control groups received an equivalent amount of saline.

For the glucose tolerance test (GTT), a solution of glucose in saline (30 g/100 ml) was injected ip into fasted rats 15 min after rNRG/saline administration at a dose of 2 g/kg. Blood was extracted before (0 min) and after the glucose injection (15, 30, 60, and 120 min). At 15 min, muscle and liver were dissected from rats anesthetized with isoflurane to analyze rNRG effects on signaling pathways and metabolism.

For insulin tolerance tests (ITTs), rats fasted for 6 h were injected ip with insulin (ZCL: 1.5 U/kg; ZDF: 2.0 U/kg) 15 min after rNRG/saline administration, and glycemia was measured from tail blood taken at the indicated times after injection (15, 30, and 60 min).

**Cell incubation.** Suspensions of isolated hepatocytes from 24-h-starved ZCL and ZDF rats (12 wk of age) were prepared as indicated (35). Cells were suspended in Krebs-Ringer bicarbonate buffer (pH 7.4) in the presence or absence of 20 mM glucose at 37°C with continuous shaking and gassed with carbogen (O2:CO2, 19:1) throughout the incubation period. Hepatocytes (6 ml, 2.5 × 107 cells/ml) were incubated in the absence or presence of wortmannin [2 μM in dimethyl sulfoxide (DMSO)] for 15 min. Control cells were incubated in 0.2% (vol/vol) DMSO. Corresponding cells were then treated with recombinant neuregulin (3 nM) for 30 min. At the end of the experiments, the content of the incubated vials was centrifuged at 3,000 g for 1 min, and cell pellets were immediately frozen.

**Measurement of blood metabolites.** Blood glucose was determined in an Accu-Chek glucose monitor (Roche Diagnostics). Plasma was obtained from the extracted blood by centrifugation (1,650 g, 20 min) at 4°C and stored at −20°C until assayed. Plasma insulin was determined with the Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden), and plasma lactate was determined spectrophotometrically using a commercial kit (Horiba, Montpellier, France).

**Obtention of total lysates from muscle, liver, and incubated hepatocytes.** For total lysates, pellets of incubated hepatocytes or 100 mg of frozen gastrocnemius or liver samples were homogenized as described before (4, 49).

**Immunoprecipitation and Western blots.** To identify the tyrosine phosphorylation levels of IRζ, IRS-1, IRS-2, ErbB3, and ErbB1 and the levels of IRS-1, IRS-2, and ErbB3 bound to the p85 regulatory subunit of the PI3K, total lysates were immunoprecipitated by conjugating 30 μl of protein G-Sepharose beads with 2–5 μg of the corresponding polyclonal antibody for 1 h at 4°C. The beads were then washed twice in lysis buffer (4, 49) and incubated with 0.5–1 mg of protein lysate for 90 min with constant shaking at 4°C. After brief centrifugation, the supernatant was discarded. The pellet was washed several times with the lysis buffer and boiled for 5 min with 50 μl of Laemmli sample buffer for Western blot analysis using a monoclonal anti-phosphotyrosine antibody or polyclonal p85-PI3K antibody. To identify the threonine phosphorylation levels of PKCζ, total lysates were immunoprecipitated with beads conjugated to total PKCζ antibody, and the Western blot analysis was performed with an anti-phospho-Thr410/416-PKCζ antibody. For Western blot assays, immunoprecipitated samples were subjected to SDS-PAGE and transferred to PVDF membranes, as described previously (4). Total lysates were used for immunoblot detection of IRζ, IRS-1, IRS-2, p85-PI3K, total PKB, phospho-ser373-PKB, phospho-Thr308-PKB, total PKCζ, total GSK-3, and phospho-ser473-GSK-3.

**Determination of lactate from muscle, liver, and incubated hepatocytes.** The intracellular concentration of lactate was measured from perchloric acid extracts of muscle and liver and in pellets of incubated rat hepatocytes using a spectrophotometric commercial kit method (Horiba).

**Measurement of muscle and liver glycogen content.** Tibialis muscle (20–30 mg) was hydrolyzed in 250 μl of 1 mol/l HCl by heating at 95°C for 2 h. The solution was then neutralized with 250 μl of 2 mol/l NaOH. Liver samples (100 mg) were hydrolyzed in 30% KOH by heating at 100°C for 10 min and then precipitated with ethanol and neutralized with 5 N H3SO4. In both cases, the resulting free glycosyl units were assayed spectrophotometrically using a hexokinase-dependent assay, as described previously (21).

**Measurement of glucose 6-phosphate in liver.** The intracellular concentration of glucose 6-phosphate (G-6-P) was measured from perchloric acid extracts of livers using a fluorometric method, as described previously (55).
Analysis of fructose-2,6-bisphosphate levels in liver extracts. For fructose-2,6-bisphosphate (Fru-2,6-P₂) analysis, frozen livers were homogenized in five volumes of 50 mmol/l NaOH. The homogenates were heated at 80°C for 10 min, cooled, and adjusted to a pH of 6–7. After centrifugation at 15,000 g for 10 min, Fru-2,6-P₂ was quantified in the supernatants as described by Van Schaftingen et al. (50).

RNA extracts from rat liver and quantitative RT-PCR. Total RNA was isolated from rat liver tissue with Trizol reagent and purified with RNaseasy kit columns (Qiagen). RNA from each sample was then reverse-transcribed using SuperScript III reverse transcriptase (Super-Script First-Strand Synthesis System for RT-Q-PCR; Invitrogen, Barcelona, Spain). Quantitative real-time PCR (qPCR) tests were performed, following the standard protocol of the ABI Prism 7900 Detection System. The following primers were used: ErbB1 forward primer 5'-GCTGTCGGATTAGCAACAA-3', reverse primer 5'-GGACAGTCTGGATCACATT-3'; ErbB2 forward primer, 5'-CGAGAGGAAGATGTTGAT-GTA-3', reverse primer 5'-CAAGGAGGAGGGCCTAG-3'; ErbB3 forward primer 5'-TGGATCTAGGTGCCAAG-3', reverse primer 5'-TTGCTACTCAACACCTCTGC-3'; ErbB4 forward primer 5'-CTCTAACCCCTAGTGGCACA-3', reverse primer 5'-GCCAGGGGAGCTGTGCACG-3', reverse primer 5'-GCTACGTCCTTCTTACCTC-3'. Data were analyzed with the 2ΔΔCT method, with Pia as used as endogenous control.

Statistical analysis. Data are presented as means ± SE. Unpaired or paired t-tests were used to compare two groups and two-way ANOVA, and Tukey’s post hoc test was used to compare more than two groups.

RESULTS

NRG improved body response to the GTT. To determine whether the in vivo administration of NRG affects the GTT, rNRG was injected ip into ZCL and ZDF rats 15 min before the test was performed. The large increase in blood glucose observed 15 min after glucose administration was significantly reduced by rNRG in control rats (Fig. 1A). The area under the curve (AUC) corresponding to the GTT of ZCL rats was significantly lower (17% decrease in AUC) in the NRG-treated animals (Fig. 1A). When ZCL rats were treated with rNRG without glucose being administered, basal glycemia was not altered (data not shown). rNRG also improved the response to a GTT (18% decrease in AUC) in ZDF rats (Fig. 1A). Paralleling the effects on glycemia, the plasma insulin concentration was also reduced by rNRG in control rats (Fig. 1B); however, no significant differences were found in ZDF rats, a model in which hyperinsulinemia is a characteristic feature (Fig. 1B). Next, we analyzed the effects of NRG under an ITT to reach high insulin levels (Fig. 1C). NRG significantly reduced glycemia during the ITT in control rats, whereas in ZDF rats the decrease in glycemia did not reach significance (Fig. 1C).

NRG action on muscle and liver insulin signaling. Given that skeletal muscle is the major site of glucose disposal during the absorptive state (14), we initially tested whether rNRG activates the insulin receptor (IR)-dependent cascade in skeletal muscle of control and diabetic rats under absorptive conditions (15 min after glucose administration). rNRG (30 min) did not induce IR or IRS-1 tyrosine phosphorylation or the association of IRS-1 with the p85 subunit of PI3K in gastrocnemius muscles of lean and diabetic rats compared with saline-treated animals (Fig. 2A).

Since rNRG did not induce the IR/IRS1/PI3K axis in skeletal muscle under absorptive conditions, we analyzed whether rNRG targets this pathway in the liver, an organ that also has a major role in blood glucose homeostasis. Again, rNRG had no effect on the tyrosine phosphorylation of the IR or IRS-1 in the livers of control or diabetic rats (Fig. 2B). However, rNRG significantly reduced tyrosine phosphorylation of IRS-2 as well as the level of p85-PI3K bound to IRS-1 and IRS-2 in the livers of lean and diabetic rats (Fig. 2B).

In accord with the lack of rNRG effect on the IR/IRS/PI3K pathway, rNRG did not increase PKB phosphorylation in skeletal muscle (Fig. 3A), although it significantly increased Thr410/PKB activity in the livers of diabetic rats (Fig. 3A). However, the PKB activity in the livers of control rats was not affected by rNRG (Fig. 3A). Thus, rNRG did not alter the PKB activity in the livers of control or diabetic rats (Fig. 3A). This result suggests that PKB activation is not affected by rNRG in the livers of diabetic rats.

Metabolic effects of NRG in the liver. To determine whether rNRG stimulates glucose utilization in the liver, we analyzed the levels of G-6-P and the hepatic 6-phosphofructo 1-kinase (PFK-1) allosteric activator Fru-2,6-P₂ (44, 53). rNRG did not induce G-6-P accumulation (Fig. 6A), but it induced significant increases in Fru-2,6-P₂ levels in the livers of both control and diabetic rats.
Fig. 1. In vivo administration of neuregulin (NRG) improved the response to the glucose tolerance test. A: blood glucose concentration and area under the curve (AUC) graphs in Zucker control lean (ZCL) and Zucker diabetic fatty (ZDF) rats after an intraperitoneal (ip) glucose challenge in the absence (● and open bars) or presence (● and black bars) of recombinant NRG (rNRG). B: plasma insulin concentration and AUC graphs in ZCL and ZDF rats after an ip glucose challenge in the absence or presence of rNRG. C: blood glucose concentration and AUC graphs for control ZCL and diabetic ZDF rats after an ip insulin challenge in the absence or presence of rNRG. Data are expressed as means ± SE (n = 4–6). Statistical differences (*P < 0.05 and **P < 0.01) by unpaired t-test between absence and presence of rNRG; #statistical differences (P < 0.05) by unpaired t-test between ZCL and ZDF rats.

(Fig. 6B). We also explored whether rNRG increases glycogen content in liver. Indeed, rNRG had rapid effects, doubling the amount of liver glycogen in control rats. In contrast, no significant increases were observed in liver of diabetic rats, maintaining a large amount of glycogen (25- to 50-fold higher than in control rats) even after 16 h of fasting (Fig. 6C). Skeletal muscle glycogen content was not affected by the rNRG treatment in control ZCL or ZDF rats (Fig. 6C). We measured lactate levels in liver and muscle. rNRG increased lactate content in the liver of control and diabetic rats but did not alter its levels in skeletal muscle (Fig. 6D). Although no increase in plasma lactate was observed in rNRG-treated control rats, a significant increase was detected in diabetic ZDF rats after rNRG injection. This observation may indicate an increase in glucose flux through glycolysis in diabetic rats compared with the control ones (Fig. 6D). These rNRG effects occurred without the expression of the glucose transporter GLUT2 being altered in the liver (data not shown). Studies in incubated hepatocytes indicated that the increased lactate content induced by rNRG was abrogated by wortmannin (Fig. 6E).

DISCUSSION

Here, we show that the in vivo administration of rNRG rapidly improves glucose tolerance in response to both the
Fig. 2. Effect of NRG on early steps of the insulin-signaling pathway in skeletal muscle and liver of control and diabetic rats. Liver and gastrocnemius muscle were dissected 15 min after glucose administration from rats treated or not with rNRG (25 μg/kg, 30 min). Total protein lysates were obtained and analyzed by Western blot. Representative images and quantification graphs of insulin receptor (IR) and insulin receptor substrate (IRS) tyrosine phosphorylation, phosphatidylinositol 3-kinase (PI3K) bound to IRS, total IR, IRS, and PI3K abundance are shown for gastrocnemius muscle (A) and liver extracts (B) from ZCL and ZDF rats. α-Tubulin was used as a protein loading control. Open bars indicate the absence of rNRG and black bars the presence of rNRG. Data are expressed as means ± SE (n = 5). *Statistical differences (P < 0.05) by 2-way ANOVA between the absence and presence of rNRG in ZCL rats and ZDF rats; #statistical differences (P < 0.05) by 2-way ANOVA between ZCL and ZDF rats.
Fig. 3. Effect of NRG on late steps of the insulin-signaling pathway in skeletal muscle and liver of ZCL and ZDF rats. Liver and gastrocnemius muscle were dissected 15 min after glucose administration from rats treated or not with rNRG. Total protein lysates were obtained and analyzed by Western blot. Representative images and quantification graphs of PKB phosphorylation, total PKB, GSK-3 phosphorylation, total GSK-3, PKCζ phosphorylation, and total α-tubulin abundance are shown for gastrocnemius muscle (A) and liver extracts (B) from ZCL and ZDF rats. α-Tubulin was used as a protein-loading control. In the graphs, open bars indicate absence of rNRG and black bars the presence of rNRG. Data are expressed as means ± SE (n = 5). *Statistical differences (P < 0.05) by 2-way ANOVA between the absence and presence of rNRG in ZCL and ZDF rats; #statistical differences (P < 0.05) by 2-way ANOVA between ZCL and ZDF rats.
GTT and ITT in control rats. At the doses used in our study (25 μg/kg), rNRG did not alter basal glycemia in rats, although in swine it has been described that higher doses (2 mg/kg) may induce a transient hypoglycemic effect (17). Diabetic rats also showed an improved GTT profile in response to acute rNRG action without the characteristic hyperinsulinemic state being altered. This observation indicates that rNRG alleviates insulin resistance or alternatively acts through an insulin-independent pathway. Here, we show that rNRG did not cause any change in IR phosphorylation in muscle or liver. The lack of an effect of rNRG on the IR has been reported previously in in vitro studies with muscle cells and tissue (3, 4). The potent rNRG

Fig. 4. NRG-signaling cascade in liver. Liver and gastrocnemius muscle were dissected 15 min after glucose administration from rats treated or not with rNRG. A: quantitative real-time PCR showing relative mRNA level of ErbB1, ErbB2, ErbB3, and ErbB4 in the livers of ZCL and ZDF rats treated or not with rNRG. Open bars indicate ZCL rats in the absence of rNRG, dark gray bars ZCL rats in the presence of rNRG, black bars ZDF rats in absence of rNRG, and light gray bars ZDF in the presence of rNRG. B: total protein lysates were obtained and analyzed by Western blot. Representative images and quantification graphs of ErbB3 and ErbB1 tyrosine phosphorylation and PI3K bound to ErbB3, as well as ErbB3, PI3K, ErbB1, and NRG abundance, were measured in liver total protein extracts from ZCL and ZDF rats. α-Tubulin was used as a protein-loading control. Open bars indicate ZCL rats and black bars ZDF rats. Graph data are expressed as means ± SE (n = 5). *Statistical differences (P < 0.05) by 2-way ANOVA between the absence and presence of rNRG in ZCL and ZDF rats; #statistical differences (P < 0.05) by unpaired t-test between ZCL and ZDF rats.
effect on PKB phosphorylation, observed selectively in the liver, contrasts with the lack of action on PKB observed in skeletal muscle and confirms a previous report in incubated muscle (4). This observation may explain the scarce capacity of this growth factor to increase glucose uptake in resting muscle when compared with contracting muscle, a situation in which NRG is clearly involved (4). Such a distinct effect of rNRG on PKB in liver and muscle could result from differences in the early events of the NRG pathway in each tissue. Indeed, these two tissues show differences in the expression pattern of NRG receptors. Although ErbB4 is essentially involved in NRG promotion of glucose uptake during skeletal muscle contraction (3), its expression is barely detected in liver, where ErbB3 is the main NRG receptor (2, 9). Compared with ErbB4, ErbB3 has limited, if any, tyrosine kinase activity (47), and thus the presence of a coreceptor is essential to drive intracellular signaling. Although ErbB2 is the preferred dimerization partner for ErbB3, it is lost during hepatic development, being undetectable in adult liver (2, 10). However, the increase in ErbB1 expression during the postnatal period renders a new partner for ErbB3 (10). Our studies confirmed the prevalence of ErbB1 and ErbB3 expression, both at the mRNA and protein level, in adult liver. Therefore, our results showing that rNRG induces hepatic ErbB3 tyrosine phosphorylation may indicate that, in response to rNRG, ErbB3 heterodimerizes and activates ErbB1 tyrosine kinase, whereas ErbB1 is not phosphorylated, as expected by the null-ErbB3 kinase activity.

In liver, rNRG administration generated a decrease in PI3K association with IRS-1 and IRS-2, which was concomitant with increased PI3K binding to phosphorylated ErbB3. Thus com-

Fig. 5. Effects of wortmannin on NRG-induced PKB phosphorylation in incubated hepatocytes. Total protein lysates were obtained from hepatocytes incubated in the absence (A) or presence (B) of glucose and then analyzed by Western blot. Representative images and quantification graphs of PKB phosphorylation in Ser473 and Thr308. α-Tubulin was used as a protein-loading control. Data are from ZCL rats (open bars) in the presence or absence of wortmannin and NRG and ZDF rats (black bars) in the presence or absence of wortmannin and NRG. Graph data are expressed as means ± SE (n = 3). *Statistical differences (P < 0.05) by paired t-test between the absence and presence of rNRG in ZCL and ZDF rats; #statistical differences (P < 0.05) by paired t-test between the absence and presence of wortmannin in ZCL and ZDF rats; &statistical differences (P < 0.05) by unpaired t-test between ZCL and ZDF rats.
Fig. 6. In vivo NRG effect on liver glucose 6-phosphatase (G-6-P) and fructose-2,6-bisphosphate (Fru-2,6-P2) liver and muscle glycogen and lactate and plasma lactate. Wortmannin effect on NRG action in incubated hepatocytes. Blood, liver, and tibialis anterior muscle samples were dissected 15 min after glucose administration from rats treated or not with rNRG to measure liver G-6-P concentration (A), liver Fru-2,6-P2 concentration (B), total glycogen content in liver and muscle (C), lactate content in liver, muscle, and plasma (D), and lactate content in hepatocytes in the presence or absence of wortmannin and NRG (E). Data from ZCL and ZDF rats in the absence (open bars) or presence (black bars) of rNRG are shown. Graph data are expressed as means ± SE (n = 5). *Statistical differences (P < 0.05) by 2-way ANOVA (A–D) or paired t-test between the absence and presence of rNRG in ZCL and ZDF rats; #statistical differences (P < 0.05) by 2-way ANOVA (A–D) or unpaired t-test (E) between ZCL and ZDF rats; &statistical differences (P < 0.05) by paired t-test between the absence and presence of wortmannin in ZCL and ZDF rats.
The activation of PKB is involved in the hepatic metabolic utilization of glucose. Thus, mammalian target of rapamycin complex 2 is responsible for PKB Ser473 phosphorylation. PI3K, which is in accord with the above-mentioned observations. Adult rat liver shows a circadian rhythm of ErbB3 expression and to a minor extent also of ErbB1, which increases by the end of the resting nonfeeding period when insulinemia reaches its lowest level, just before the animals become active and feed (10). Moreover, hepatic ErbB3 expression increases substantially in two models of in vivo insulin resistance. Here, we provide the first evidence indicating that NRG activates a PI3K/PKB signaling pathway in the liver, it is feasible that, in a physiological context, neither effector acts simultaneously but that they compensate one another. At the expression level, it has been observed that insulin reduces the action of NRG in the liver. Whereas NRG increases ErbB3 protein synthesis (9), insulin suppresses ErbB3 expression (8, 9) in cultured rat hepatocytes. It is interesting to note that our diabetic model, characterized by sustained hyperinsulinemia, showed a lower protein content of both ErbB1 and ErbB3 receptors in the liver, which is in accord with the above-mentioned observations.

Since both insulin and rNRG activate a PI3K/PKB signaling pathway in the liver, it is feasible that, in a physiological context, neither effector acts simultaneously but that they compensate one another. At the expression level, it has been observed that insulin reduces the action of NRG in the liver. Whereas NRG increases ErbB3 protein synthesis (9), insulin suppresses ErbB3 expression (8, 9) in cultured rat hepatocytes. It is interesting to note that our diabetic model, characterized by sustained hyperinsulinemia, showed a lower protein content of both ErbB1 and ErbB3 receptors in the liver, which is in accord with the above-mentioned observations. Adult rat liver shows a circadian rhythm of ErbB3 expression and to a minor extent also of ErbB1, which increases by the end of the resting nonfeeding period when insulinemia reaches its lowest level, just before the animals become active and feed (10). Moreover, hepatic ErbB3 expression increases substantially in two models of in vivo insulin deficiency, namely 48-h fasting and experimental streptozotocin-induced diabetes (8). Notably, using antibodies that specifically recognize the characteristic signature of the EGF domains of all NRG isoforms, here we report that the liver endogenously expresses NRG. The finding that NRG expression increased significantly in diabetic rats, although it failed to sustain ErbB receptor expression, points to the alteration of the positive feedback between NRG and ErbB receptors in the liver in diabetes and the involvement of hyperinsulinemia in this alteration. Whether deficient hepatic NRG function contributes to the manifestation of diabetes remains to be determined. It is also unknown whether a higher NRG expression is contributing to the downregulation of IR expression that is observed in liver (48) in insulin resistance.

The activation of PKB is involved in the hepatic metabolic utilization of glucose. Thus, mammalian target of rapamycin complex 2 is responsible for PKB Ser473 phosphorylation. Using a liver-specific knockout, it has been established that PKB contributes to insulin-induced hepatic glycolysis (25). 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFKFB) is responsible for Fru-2,6-P2 synthesis and breakdown. Fru-2,6-P2 allosterically activates PFK-1 and glycolysis (40, 44). The concentration and activity of PFKFB is higher in diabetic than in lean control animals, which might explain the higher concentration of Fru-2,6-P2 observed in the former (41). In the heart, PKB is required for the insulin-triggered activation of PFKFB (43) by inducing specific phosphorylation at Ser483, which increases its kinase activity. Fru-2,6-P2 content, and consequently, lactate concentration (39). The contribution of PKB to Fru-2,6-P2 content in the liver remains to be determined. Furthermore, rNRG activation of the PKB-GSK-3 axis may be relevant for the increase in hepatic glycogen synthesis in control rats, as demonstrated previously for insulin action (11, 45). The deficient NRG-dependent activation of the PKB/GSK-3 axis and already sizable glycogen pool in diabetic rats may explain the unaltered hepatic glycogen content and the increase in plasma lactate levels in these animals. Additionally, diabetic animals treated with NRG continued to show higher levels of Fru-2,6-P2 and lactate in the liver, indicating that hepatic glycolysis was further potentiated, thereby contributing to maintain glucose utilization.

Here, we provide the first evidence indicating that NRG improves the response to the glucose tolerance test in both control and diabetic rats by enhancing hepatic glucose utilization (Fig. 7). Our results also suggest that, although it is an essential factor in the stimulation of glucose uptake during muscle contraction, NRG does not target muscle metabolism during absorptive conditions. The resistance to NRG observed in the liver of diabetic rats may be caused by a variety factors in which hyperinsulinemia may play a relevant role by reducing ErbB3 expression as an initiating factor.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS


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