Continuous 24-h nicotinic acid infusion in rats causes FFA rebound and insulin resistance by altering gene expression and basal lipolysis in adipose tissue

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[1]*Department of Physiology and Biophysics, University of Southern California Keck School of Medicine, Los Angeles, California;* [2]*Department of Biochemistry and Molecular Biology, Kyung Hee University School of Medicine, Seoul;* [3]*Department of Molecular Science and Technology, Ajou University, Suwon, Korea; and* [4]*Department of Kinesiology, University of Southern California, Los Angeles, California*

Oh YT, Oh KS, Choi YM, Jokiaho A, Donovan C, Choi S, Kang I, Youn JH. Continuous 24-h nicotinic acid infusion in rats causes FFA rebound and insulin resistance by altering gene expression and basal lipolysis in adipose tissue. *Am J Physiol Endocrinol Metab* 300: E1012–E1021, 2011. First published March 8, 2011; doi:10.1152/ajpendo.00650.2010.—Nicotinic acid (NA) has been used as a lipid drug for five decades. The lipid-lowering effects of NA are attributed to its ability to suppress lipolysis in adipocytes and lower plasma FFA levels. However, plasma FFA levels often rebound during NA treatment, offsetting some of the lipid-lowering effects of NA and/or causing insulin resistance, but the underlying mechanisms are unclear. The present study was designed to determine whether a prolonged, continuous NA infusion in rats produces a FFA rebound and/or insulin resistance. NA infusion rapidly lowered plasma FFA levels (>60%, $P < 0.01$), and this effect was maintained for ≥5 h. However, when this infusion was extended to 24 h, plasma FFA levels rebounded to the levels of saline-infused control rats. This was not due to a downregulation of NA action, because when the NA infusion was stopped, plasma FFA levels rapidly increased more than twofold ($P < 0.01$), indicating that basal lipolysis was increased. Microarray analysis revealed many changes in gene expression in adipose tissue, which would contribute to the increase in basal lipolysis. In particular, phosphodiesterase-3B gene expression decreased significantly, which would increase cAMP levels and thus lipolysis. Hyperinsulinemic glucose clamps showed that insulin's action on glucose metabolism was improved during 24-h NA infusion but became impaired with increased plasma FFA levels after cessation of NA infusion. In conclusion, a 24-h continuous NA infusion in rats resulted in an FFA rebound, which appeared to be due to altered gene expression and increased basal lipolysis in adipose tissue. In addition, our data support a previous suggestion that insulin resistance develops as a result of FFA rebound during NA treatment. Thus, the present study provides an animal model and potential molecular mechanisms of FFA rebound and insulin resistance, observed in clinical studies with chronic NA treatment.

hypolipidemic drug; free fatty acids; perilipin; phosphodiesterase; triglyceride synthesis; insulin resistance; nicotinic acid receptor; microarray analysis

NICOTINIC ACID (NA; or niacin) is a B-group vitamin. In addition to its function as a vitamin, NA, in high doses, has been used as a lipid drug for five decades (6, 21); it produces very desirable effects such as decreasing plasma triglycerides (TG), VLDL, and LDL-cholesterol levels and increasing HDL-cholesterol levels (14, 38). Major clinical trials have demonstrated that NA treatment reduces the progression of atherosclerotic cardiovascular disease (9, 18). The lipid-lowering effects of NA have traditionally been attributed to its antilipolytic effect in adipocytes (10). NA binds to and stimulates a G protein-coupled receptor [i.e., GPR109A or HM74A (37, 41)] in the plasma membrane of adipocytes to decrease cAMP and lipolysis, which in turn reduces the plasma levels of FFA, substrates for hepatic TG synthesis, and VLDL formation. However, this classic view may need revision, because circulating FFA levels, which initially decrease during treatment, rebound after long-term NA treatment (see below), whereas its lipid-lowering effects persist (38). When this occurs, the hypolipidemic effects of NA cannot be explained by reduced FFA delivery to the liver. There is evidence that NA directly inhibits diacylglycerol *O*-acyltransferase-2 in the liver to decrease TG synthesis and VLDL formation (20). Moreover, NA may lower circulating lipid levels by increasing the lipoprotein lipase activity, thereby enhancing the TG-VLDL removal rate (8, 14). Thus, despite a long history of clinical use, the precise mechanism by which NA lowers circulating lipids remains unclear.

Acute NA administration in humans (39) or rodents (29) results in a rapid decrease in the plasma FFA level followed by a rebound and an overshoot above preinfusion levels. The rebound or overshoot of the plasma FFA level is probably due to the combination of waning of antilipolytic effects of ingested or injected NA and stimulation of lipolysis by lipolytic hormones, such as epinephrine and cortisol, which increase following NA administration (28, 29, 40). In contrast, chronic treatment with NA is associated with increased basal (or fasting) FFA levels (32, 33). This may occur as a result of an overshoot of plasma FFA following the last NA administration the previous evening (42). However, when type 2 diabetic patients were treated extensively (i.e., with high and frequent dosages) for 3 days with acipimox, a long-acting antilipolytic drug derived from NA, plasma FFA levels remained suppressed during the treatment period, but mean FFA levels gradually increased from *day 1* to *day 3* (42). These data suggest that slowly developing changes occur in adipocytes that increase lipolysis during repetitive or continuous exposure to acipimox (or NA). Consistent with this idea, fasting plasma FFA levels decreased in healthy subjects during the first week of NA treatment but rebounded to control levels after 2 wk (2),

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and increased fasting FFA levels have been observed after 2 wk or more of NA treatment (32, 33). Because FFA rebound may offset some of the lipid-lowering effects of NA and/or cause insulin resistance (2, 22, 32, 42), it is important to understand the underlying mechanisms.

The mechanisms underlying FFA rebound during chronic NA treatment have been unclear, in part due to a lack of appropriate animal models. One goal of the present study was to develop an animal model of FFA rebound during continuous NA administration. Our recent study (12) showed that the ability of NA to lower plasma FFA was maintained for up to 7 h when NA was constantly infused in rats. In the present study, we extended this infusion to 24 h and tested whether this prolonged exposure to NA would cause an FFA rebound. In addition, we evaluated the molecular mechanisms underlying this phenomenon. Our recent study (12) demonstrated that NA exerts widespread effects to alter gene expression in major tissues involved in lipid metabolism, including adipose tissue. Therefore, we were particularly interested in assessing changes in gene expression in adipose tissue after 24-h NA infusion using a microarray analysis. Finally, we investigated whether the FFA rebound was accompanied by impaired insulin's action to suppress lipolysis and/or to stimulate glucose metabolism.

METHODS

Animals and Catheterization

Male Wistar rats weighing 280–300 g were obtained from Simonsen (Gilroy, CA) and studied at least 5 days after arrival. Animals were housed under controlled temperature ($22 \pm 2°C$) and lighting (12-h light, 6 AM-6 PM; 12-h dark, 6 PM-6 AM) with free access to water and standard rat chow. At least 4 days before the experiment, the animals were placed in individual cages with tail restraints as previously described (11, 12, 24), which was required to protect tail blood vessel catheters during the experiments. The animals were free to move about and were allowed unrestricted access to food and water. One tail vein infusion catheter was placed the day before the experiment, and one tail artery blood sampling catheter was placed the morning of the experiment (~7 AM). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Experimental Protocols

Short (5-h) infusions. Experiments were conducted in the conscious state after an overnight fast; food was removed at 5 PM on the day before the experiment. Animals received a constant infusion of saline or NA (30 µmol/h) for 5 h starting at 1 PM. NA infusate (60 mM) was prepared in saline, and the pH was adjusted to 7.4. During the NA infusion, blood samples were collected at 10-min intervals for immediate measurement of the plasma glucose level. Exogenous glucose was infused at varying rates to maintain plasma glucose at basal levels (~5.5 mM). Small amounts of glucose needed to be infused during the first 2 h of the NA infusion by exogenous glucose infusion (12). Additional blood samples were collected at various times for measurements of plasma insulin and FFA.

24-h infusions. Saline or NA (30 µmol/h) was infused for 24 h starting at ~1 PM on *day 1* and continuing until the same time on *day 2*. Food was removed at 5 PM on *day 1*. Blood samples were collected at the end of the 24-h infusions for measurements of plasma glucose, insulin, FFA, glycerol, catecholamine, and corticosterone levels. After the blood sampling, the animals were anesthetized with pentobarbital sodium, and epididymal adipose tissue samples were rapidly dissected

out, frozen immediately using liquid N_2 cooled aluminum blocks, and stored at $-80°C$ for later analysis.

24-h NA infusion followed by 3-h saline infusion. In separate experiments, the 24-h NA (30 µmol/h) infusion was followed by a 3-h saline infusion. The purpose of this experiment was to evaluate effects of the washout of NA on plasma FFA and glycerol levels. A control group continued to receive the NA infusion during the 3-h follow-up period. Blood samples were collected at various times before (2 h) and during the 3-h follow-up period for measurements of plasma FFA and glycerol levels.

Hyperinsulinemic euglycemic clamp. In separate experiments, the 24-h saline and NA infusions were followed by a 2-h hyperinsulinemic euglycemic clamp. To accomplish this, insulin was infused at a constant rate of 1 or 5 mU·kg^{-1}·min^{-1}, and blood samples were taken at 10-min intervals for immediate measurement of the plasma glucose level. Exogenous glucose was infused at varying rates to maintain the plasma glucose at basal levels. The glucose infusion rate (G_{INF}) required to clamp plasma glucose during insulin infusion was used as an index of insulin's ability to suppress hepatic glucose production and to increase peripheral glucose uptake. Additional blood samples were taken at various time points for measurements of plasma insulin and FFA levels.

Gene Expression Microarray Analysis

Total RNA was extracted from frozen adipose tissue samples using Tri Reagent obtained from the Molecular Research Center (Cincinnati, OH) according to the manufacturer's instructions. The RNA samples ($n = 3$ for each group) were subjected to DNA microarray analysis. First-strand cDNA was synthesized from 3 µg of total RNA using T7-oligo(dT) primer and SuperScript II (Invitrogen Life Technologies), while second-strand cDNA was synthesized with second-strand buffer (Invitrogen Life Technologies), DNA polymerase I (New England Biolabs), DNA ligase (NEB), and RNase H (Invitrogen Life Technologies). The cDNA was used as a template, and in vitro transcription was conducted using an RNA Transcript Labeling Kit (Enzo Diagnostics) to produce hybridizable biotin-labeled RNA targets. After in vitro transcription, cRNA was purified using an RNeasy Mini Kit (Qiagen). The cRNA was fragmented by incubation at 94°C for 35 min, after which it was applied to the Affymetrix GeneChip Rat Gene 1.0 ST Array and hybridized at 40°C for 16 h. The GeneChip was then washed several times and stained with streptavidin-conjugated phycoerythrin in a GeneChip Fluidics Station 400 (Affymetrix). Finally, the chips were scanned using an Agilent Scanner (Agilent Technologies) and analyzed with GeneChip Analysis Suite 5.0 (Affymetrix).

Western Blot Analysis

Frozen tissues (~100 mg) were homogenized using a Tekmar homogenizer (Cincinnati, OH) at half-maximum speed (1 min, on ice) in 500 µl of buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1% NP-40, 2% protease inhibitor cocktail) (11). Tissue lysates were further solubilized by incubation at 4°C for 1 h with continuous rotation, after which they were centrifuged at 14,000 *g* and 4°C for 30 min. The supernatants (25 µg of protein) were then resolved by SDS-PAGE followed by electrophoretic transfer of the proteins onto nitrocellulose membranes (Bio-Rad). Next, the membranes were probed with antibodies against hormone-sensitive lipase (HSL, rabbit polyclonal; a generous gift from Dr. Andrew Bicknell, University of Reading, UK), phospho-HSL (Ser^{563}, rabbit polyclonal; Cell Signaling Technology, Beverly, MA), diacylglycerol acyltransferase (DGAT2, rabbit polyclonal; GeneTex, San Diego, CA), phosphodiesterase (PDE)-3B (rabbit polyclonal; kindly provided by Dr. Vincent Manganiello at NIH/NHLBI), and perilipin (goat polyclonal; AbCam, Cambridge, MA). After incubation with the primary antibodies, the membranes were incubated with Alexa 680-labeled goat
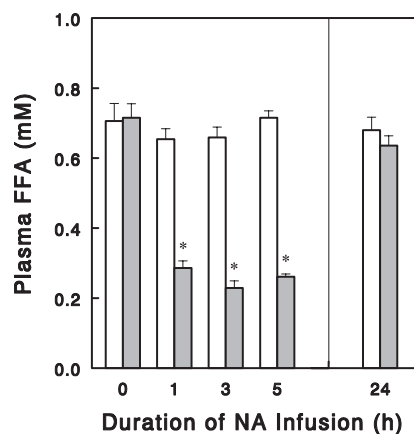
Fig. 1. Plasma FFA levels during saline (open bars) or nicotinic acid (NA) (30 μmol/h; gray bars) infusion in overnight-fasted rats. Effects of short (≤5 h) vs. 24-h infusion were determined in different rats such that plasma FFA levels were measured in similar fasting (18–24 h) states. Data are means ± SE ($n = 5$ for short and $n = 7$ or 9 for 24-h infusions). *$P < 0.05$ vs. saline control.

anti-rabbit or Alexa 680-labeled donkey anti-goat secondary antibody (Molecular Probes). The signals were then detected and quantitated on an Odyssey Infrared Imaging System (Li-COR, Lincoln, NE).

RT-PCR

Semiquantitative RT-PCR was performed to measure PDE-3B mRNA expression in adipose tissue, using Takara Ex Taq Polymerase (Takara, Japan). Primer sequences used were as follows: PDE-3B, F 5′-GAG GAA GGA CGA GCG CGA GC-3′, R 5′-CTC AGC AGC GTC CGA AGC CC-3′; GAPDH F 5′-AAT GCA TCC TGC ACC ACC AAC TGC-3′, R 5′-GGA GGC CAT GTA GGC CAT GAG GTC-3′. Thermal cycling was first carried out with a denaturation phase of 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 52°C, and 45 s at 72°C, with a final extension cycle of 5 min at 72°C. Amplification was carried out in a Veriti 96-well Thermal Cycler (Applied Biosystems). The final products were resolved on 1% agarose gel stained with ethidium bromide.

Other Assays

Plasma glucose was analyzed using the glucose oxidase method on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma insulin was measured using a radioimmunoassay kit from Linco Research (St. Charles, MO). Plasma FFA was measured using an acyl-CoA oxidase-based colorimetric kit (Wako Chemicals, Richmond, VA) and plasma glycerol using Glycerol Reagent from Sigma-Aldrich (St. Louis, MO). Plasma corticosterone was analyzed using an AssayMax ELISA kit from AssayPro (St. Charles, MO). Plasma epinephrine and norepinephrine were measured using a single-isotope derivative radioenzymatic assay (31).

Statistical Analysis

All data are expressed as means ± SE. The significance of differences in the mean values between groups was assessed by Student's *t*-test. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Effects of Continuous NA Infusion on Plasma FFA Levels

NA infusion rapidly (<1 h) lowered plasma FFA levels, and this effect (>60%, $P < 0.01$) was maintained for at least 5 h (Fig. 1). However, when this infusion was extended to 24 h,

plasma FFA levels rebounded to the levels of saline-infused control rats. Thus, plasma FFA levels were no longer lowered by the NA infusion. We next tested whether the apparent lack of NA effect on plasma FFA was due to a downregulation of NA action or a compensatory increase in lipolysis after the 24-h NA infusion. To address this issue, the NA infusion was stopped after 24 h, and changes in plasma FFA and glycerol levels were observed during the following 3-h period. Plasma FFA levels rapidly increased more than twofold upon cessation of the NA infusion (Fig. 2). Plasma glycerol levels also increased in parallel to the increases in plasma FFA levels. These data suggest that after the 24-h infusion NA was still able to suppress lipolysis and thus plasma FFA levels, but this effect was masked by an increase in basal lipolysis.

Effects of 24-h NA Infusion on Gene (mRNA) Expression in Adipose Tissue

Microarray analysis was conducted to determine whether the FFA rebound that occurred after the 24-h NA infusion was associated with changes in mRNA expression for proteins involved in lipolysis or its regulation. Lipolysis-TG degradation to FFA and glycerol involves three lipases: adipose triglyceride lipase (ATGL or PNPLA2), HSL (or LIPE), and monoglyceride lipase (MGLL). HSL expression decreased by 24% ($P = 0.043$), whereas ATGL and MGLL expressions were not significantly altered (Table 1). In addition, the expression of adiponutrin (PNPLA3), another lipase with a function that has not been well defined, decreased by 54% ($P = 0.023$). These changes in lipase expression cannot explain the increase in lipolysis (or FFA rebound) that occurred after 24 h of NA infusion. Interestingly, the expression of the lipid droplet-associated protein perilipin (PLIN1) decreased by 39% ($P = 0.006$), which can potentially explain some of the
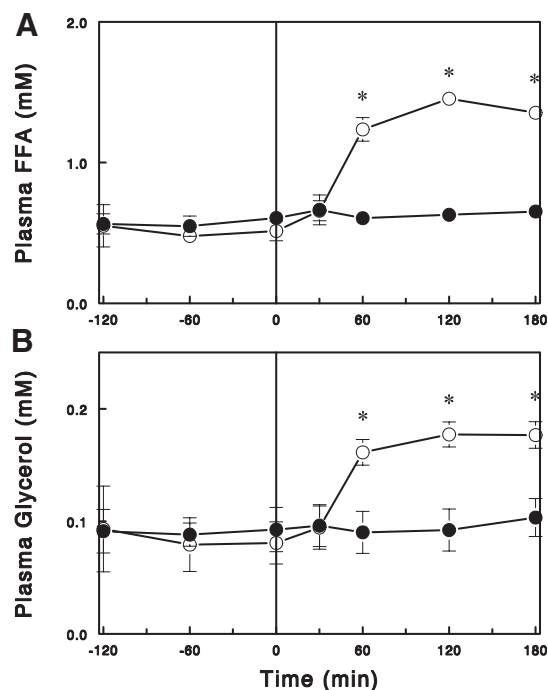

Fig. 2. Plasma FFA (*A*) and glycerol (*B*) levels during a saline (○) or NA (●) infusion (0–180 min) following 24-h NA infusion. Data are means ± SE ($n = 5$). *$P < 0.05$ vs. control.

Table 1. *Effects of 24-h NA infusion on gene expression in adipose tissue detected by microarray analysis*

Symbol	Description	Saline	NA	%Δ	P Value
		Hybridization/Intensity			
	Lipases and lipid droplet-associated proteins				
PNPLA2	adipose triglyceride lipase (desnutrin)	6,335 ± 191	6,540 ± 124	↑ 3%	0.419
LIPE	hormone sensitive lipase	3,121 ± 120	2,374 ± 225	↓ 24%	0.043
MGLL	monoglyceride lipase	1,672 ± 195	1,701 ± 34	↑ 2%	0.890
PNPLA3	adiponutrin	121 ± 18	55 ± 5	↓ 54%	0.023
PLIN1	perilipin 1	1,950 ± 76	1,181 ± 119	↓ 39%	0.006
M6PRBP1	TIP47, perilipin 3	85 ± 9	82 ± 4	↓ 3%	0.769
LPL	lipoprotein lipase	6,273 ± 181	5,491 ± 112	↓ 12%	0.021
	Enzymes of triglyceride synthesis				
AGPAT2	1-acylglycerol-3-phosphate *O*-acyltransferase-2	2,750 ± 248	1,655 ± 147	↓ 40%	0.019
AGPAT3	1-acylglycerol-3-phosphate *O*-acyltransferase-3	1,244 ± 102	606 ± 30	↓ 51%	0.004
AGPAT9	1-acylglycerol-3-phosphate *O*-acyltransferase-9	580 ± 39	256 ± 35	↓ 56%	0.004
DGAT1	diacylglycerol *O*-acyltransferase-1	1,003 ± 119	582 ± 37	↓ 42%	0.028
DGAT2	diacylglycerol *O*-acyltransferase-2	1,057 ± 102	535 ± 73	↓ 49%	0.014
GPAM	glycerol-3-phosphate acyltransferase	1,458 ± 105	1,082 ± 168	↓ 26%	0.131
GPD1	glycerol-3-phosphate dehydrogenase-1 (soluble)	5,401 ± 323	2,799 ± 347	↓ 48%	0.005
PCK1	phospho*enol*pyruvate carboxykinase 1	6,229 ± 500	2,894 ± 91	↓ 54%	0.003
	cAMP regulators				
ADCY6	adenylate cyclase 6	630 ± 52	540 ± 34	↓ 14%	0.222
ADCY4	adenylate cyclase 4	151 ± 8	192 ± 14	↑ 27%	0.070
PDE-3B	phosphodiesterase 3B	1,627 ± 175	707 ± 77	↓ 57%	0.009
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type IIβ	1,845 ± 136	1,095 ± 32	↓ 41%	0.006
NIACR1	nicotinic acid receptor,	1,149 ± 93	428 ± 103	↓ 63%	0.007
PLA2G16	AdPLA, phospholipase A2, group XVI	1,604 ± 62	1,268 ± 75	↓ 21%	0.026
	Other major proteins of adipocytes				
ADIPOQ	adiponectin	6,616 ± 95	6,200 ± 316	↓ 6%	0.276
LEP	leptin	453 ± 61	592 ± 46	↑ 31%	0.141
FABP4	fatty acid binding protein-4	4,749 ± 155	4,305 ± 122	↓ 9%	0.087
FASN	fatty acid synthase	954 ± 395	715 ± 128	↓ 25%	0.596
GADPH	glyceraldehyde-3-phosphate dehydrogenase	5,219 ± 705	4,947 ± 67	↓ 5%	0.720
ACTB	β-actin	4,890 ± 285	5,421 ± 126	↑ 11%	0.164
	Transcription factors				
PPARG	PPARγ	775 ± 30	562 ± 73	↓ 27%	0.055
CEBPA	C/EBPα	786 ± 60	601 ± 63	↓ 24%	0.101
CEBPB	C/EBPβ	97 ± 3	81 ± 2	↓ 17%	0.008
CEBPG	C/EBPγ	108 ± 10	315 ± 25	↓ 23%	0.026

NA, nicotinic acid.

increase in lipolysis (see DISCUSSION). Another lipid droplet-associated protein, TIP47 (perilipin 3 or M6PRBP1), showed no significant change in gene expression.

Enzymes directly involved in TG synthesis include 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT), diacylglycerol *O*-acyltransferase (DGAT), and glycerol-3-phosphate acyltransferase (GPAM). In addition, glycerol-3-phosphate dehydrogenase (GPD1) and phospho*enol*pyruvate carboxykinase-1 (PCK1 or PEPCK1) contribute to TG synthesis by providing glycerol 3-phosphate, which is required for TG synthesis (15). With the exception of GPAM, all of these genes showed substantially (40–56%) decreased expression after 24 h of NA infusion ($P <$ 0.05). These changes occurred without significant changes in the expression of major genes of adipocytes, such as adiponectin, leptin, fatty acid-binding protein-4, fatty acid synthase, glyceraldehyde-3-phosphate dehydrogenase, and β-actin ($P >$ 0.05 for all).

cAMP is an important regulator of lipolysis in adipocytes, and its level is determined by the balance of the activities of adenylate cyclase, which synthesizes cAMP, and PDE, which degrades cAMP. Adenylate cyclase expression was not significantly altered. In contrast, PDE-3B (but not PDE-3A or PDE-4) expression decreased by 57% (P = 0.009). Moreover, cAMP-dependent protein kinase (PRKAR2B) expression decreased by 41% (P = 0.006). Interestingly, nicotinic acid receptor (NIACR1) expression decreased by 63% (P = 0.007), and adipose-specific phospholipase A$_2$ (AdPLA or PLA2G16) expression decreased by 21% (P = 0.026) (see DISCUSSION). Finally, all of the transcription factors involved in adipocyte differentiation, such as C/EBPα, C/EBPβγ, C/EBPγ, and PPARγ (43), showed decreased expression, although only the changes in C/EBPβ and C/EBPγ gained statistical significance.

Effects of 24-h NA Infusion on Protein Expression and Phosphorylation in Adipose Tissue

We also examined changes in protein expression in adipose tissue after the 24-h NA infusion. HSL, perilipin, and DGAT2 protein contents decreased by 26, 31, and 39%, respectively (Fig. 3*A*), similar to the corresponding changes in mRNA
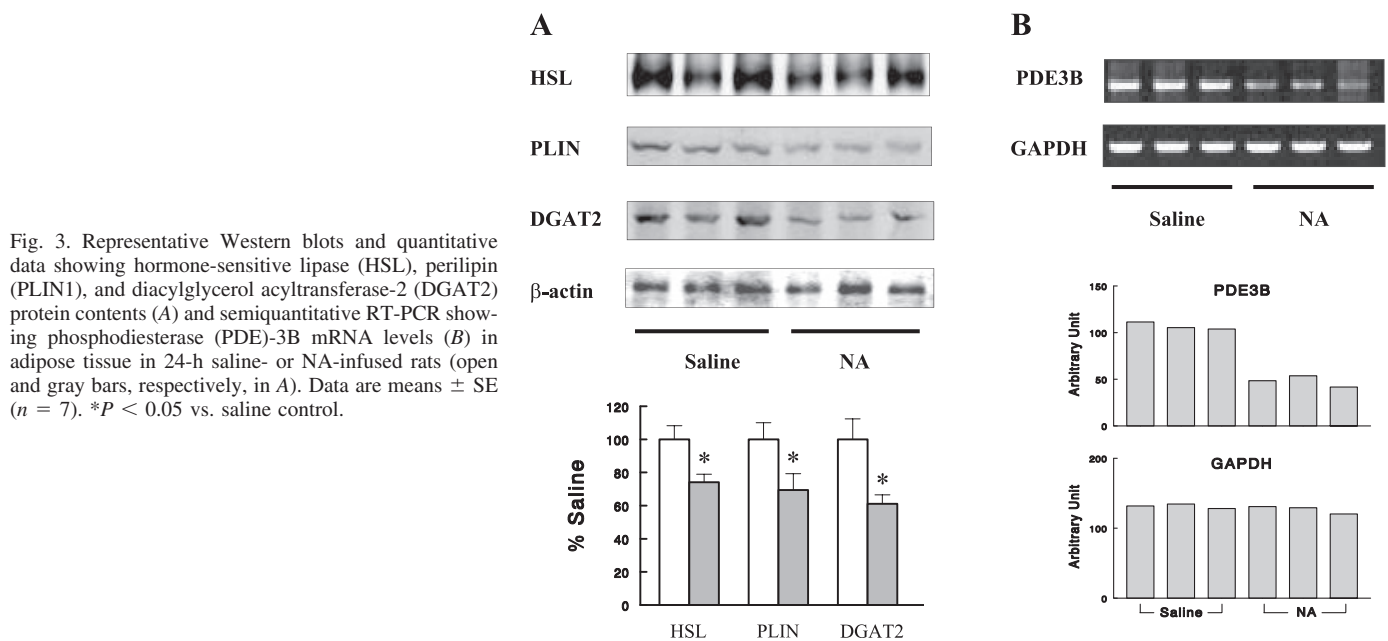
Fig. 3. Representative Western blots and quantitative data showing hormone-sensitive lipase (HSL), perilipin (PLIN1), and diacylglycerol acyltransferase-2 (DGAT2) protein contents (*A*) and semiquantitative RT-PCR showing phosphodiesterase (PDE)-3B mRNA levels (*B*) in adipose tissue in 24-h saline- or NA-infused rats (open and gray bars, respectively, in *A*). Data are means ± SE ($n = 7$). *$P < 0.05$ vs. saline control.

expression, supporting the reliability of the microarray analysis. NA is known to decrease lipolysis by inhibiting adenylate cyclase and lowering cAMP. The finding of 57% decreased PDE-3B mRNA expression after 24-h NA infusion suggests that the initial decrease in cAMP by NA (due to reduced cAMP synthesis) may be reversed by reduced cAMP degradation arising from decreased PDE-3B activity, explaining the FFA rebound that occurred after 24-h of NA infusion. To test this idea, we measured the phosphorylation status of HSL (Ser[563]), which reflects cAMP-dependent protein kinase [i.e., protein kinase A (PKA)] activity and thus the cAMP level. As expected, phospho-HSL normalized to total HSL was profoundly decreased by a short (1.5-h) NA infusion, but after a 24-h NA infusion it rebounded to a level not different from controls (Fig. 4). These data suggest that decreases in PDE-3B gene expression may be a major mechanism responsible for FFA rebound after 24-h NA infusion. The effect of NA infusion on PDE-3B expression was not tested at the protein level because of technical problems with antibodies. However, using semiquantitative RT-PCR with gene-specific primers, we confirmed that PDE-3B mRNA levels indeed decreased after the 24-h NA infusion (Fig. 3*B*). The decrease was quantified to be 55% ($P < 0.01$), very similar to the 57% decrease detected by the microarray analysis.

Effects of Continuous NA Infusion on Insulin Action

Hyperinsulinemic euglycemic clamps were conducted in 24-h saline or NA-infused rats to assess insulin's actions to suppress lipolysis and to stimulate glucose metabolism. First, the clamp was performed by infusing insulin at a rate of 1 mU·kg^{-1}·min^{-1} while the saline or NA infusion was continued. During the insulin infusion, plasma insulin levels were raised to similar levels of ~17 µU/ml in both groups (Fig. 5). Plasma FFA levels were lowered by insulin similarly in both groups. The G_{INF} required to maintain plasma glucose, which reflects insulin's action to suppress hepatic glucose production and to increase peripheral glucose uptake, was significantly

higher during the second hour of the clamp in the NA-infused rats compared with the saline-infused control rats (average G_{INF}: 2.44 ± 0.25 vs. 1.48 ± 0.29 mg/min, $P < 0.05$). In separate experiments, we conducted a hyperinsulinemic clamp in which the NA infusion was stopped at the beginning of the clamp. In this experiment, plasma FFA rebounded above basal levels due to waning of the NA effect to suppress lipolysis (Fig. 2). To suppress the FFA rebound/overshoot, insulin was infused at a higher rate of 5 mU·kg^{-1}·min^{-1}, which raised
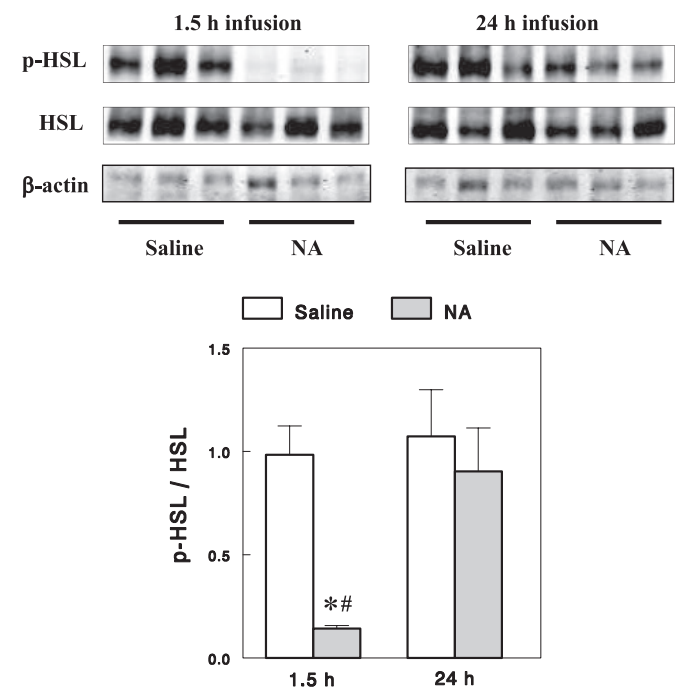

Fig. 4. Representative Western blots for total and phosphorylated HSL and quantitation of the ratio of phosphorylated to total HSL. Data are means ± SE ($n = 5$ for 1.5-h and 7 for 24-h infusions). *$P < 0.05$ vs. saline control; #$P < 0.05$ vs. 24-h infusion.

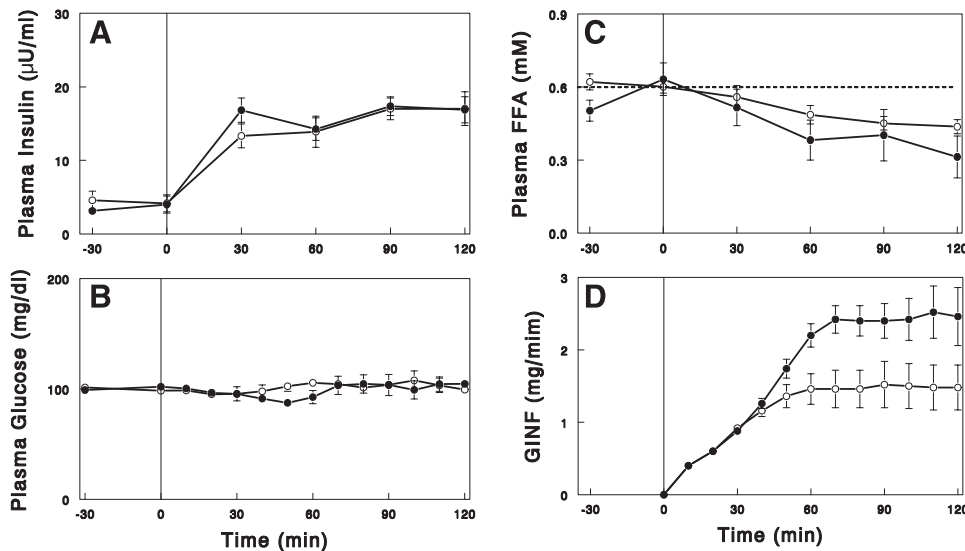Fig. 5. Plasma insulin (*A*), glucose (*B*), and FFA (*C*) levels and glucose infusion rate (G_{INF}; *D*) during hyperinsulinemic euglycemic clamp (insulin infusion = 1 mU·kg^{-1}·min^{-1}) following 24-h saline (○) or NA (●) infusion. In the NA group, NA infusion was continued during the clamp. Data are means ± SE (*n* = 5).

plasma insulin to similar levels of ~80 μU/ml in both groups (Fig. 6). Even with the increased insulin infusion, plasma FFA levels were not fully suppressed in NA-infused rats; plasma FFA levels were similarly decreased by insulin at 30 min in both groups, but while this effect was maintained throughout the clamp in control rats, plasma FFA levels rebounded after 30 min in the 24-h NA-infused rats. G_{INF} during the first hour of the clamp was similar or slightly greater in the 24-h NA-infused rats than in the control rats. G_{INF} showed a tendency for further increases during the second hour in control rats. In contrast, in NA-infused rats, G_{INF} showed a tendency for decreases, presumably due to the rebound of plasma FFA (see DISCUSSION), resulting in slightly but significantly lower G_{INF} toward the end of the clamps ($P < 0.05$).

Effects of 24-h NA Infusion on Plasma Corticosterone and Catecholamine Levels

Previous human studies have shown that NA raised plasma epinephrine levels at resting states (28, 40). In addition, NA infusion in rats significantly increased plasma corticosterone

levels (29). These hormones are known to increase lipolysis in adipocytes and are potentially responsible for FFA rebound. We measured plasma levels of corticosterone and catecholamines in the 24-h saline- or NA-infused rats. There were tendencies for these hormones to increase after the 24-h NA infusion, but the changes were not statistically significant ($P > 0.05$ for all; Fig. 7).

DISCUSSION

In the present study, we found that a 24-h continuous NA infusion in rats resulted in FFA rebound; while NA infusion lowered plasma FFA levels for the initial 5 h, plasma FFA levels rebounded to preinfusion levels when the NA infusion was extended to 24 h. This was not due to a downregulation of NA action, because when the NA infusion was stopped, plasma FFA levels rapidly increased more than twofold, demonstrating that NA was still able to suppress lipolysis. Microarray analysis revealed that the FFA rebound was accompanied by many changes in gene expression in adipocytes that would increase lipolysis. In particular, PDE-3B gene expression decreased
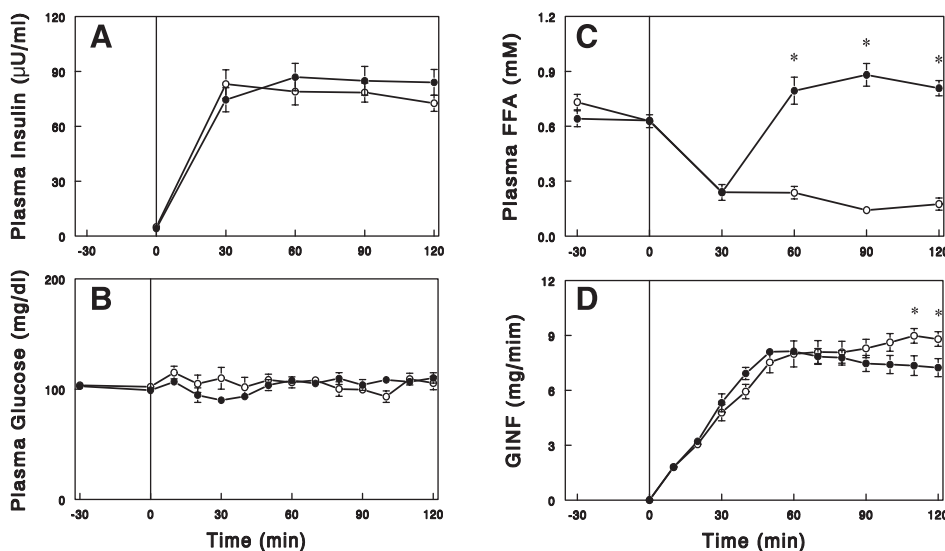

Fig. 6. Plasma insulin (*A*), glucose (*B*), and FFA (*C*) levels and G_{INF} (*D*) during hyperinsulinemic euglycemic clamp (insulin infusion = 5 mU·kg^{-1}·min^{-1}) following 24-h saline (○) or NA (●) infusion. During the clamps, NA infusion was stopped. Data are means ± SE (*n* = 7 or 9). *$P < 0.05$, saline vs. NA.
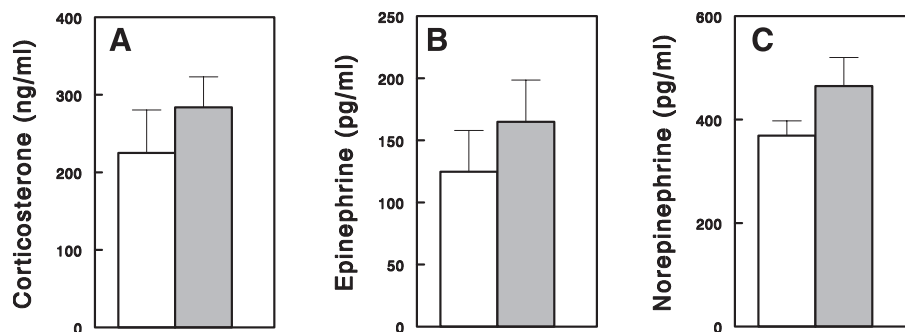
Fig. 7. Plasma corticosterone (*A*), epinephrine (*B*), and norepinephrine (*C*) levels in 24-h saline- (open bars) or NA (gray bars)-infused rats. Data are means ± SE (*n* = 6 or 8).

significantly, which would increase cAMP levels and thus lipolysis. In addition, the expression of several key enzymes of triglyceride synthesis was markedly decreased, which would also contribute to the FFA rebound (see below). Thus, the present study provides an animal model and potential molecular mechanisms for the FFA rebound observed in clinical studies with chronic NA treatment.

Previous studies in humans and rodents had demonstrated a characteristic pattern of change in plasma FFA levels following acute administration of NA, a rapid decrease followed by a rebound and an overshoot above pretreatment levels that occurs within a few hours (29, 39). The rebound may largely reflect waning of the initial NA effect following an injection or oral administration of NA; NA has a short half-life in blood [e.g., 1 h in rats (30)], and its effect may last for only a few hours. On the other hand, the overshoot above basal levels may be due to increases in lipolytic hormones such as epinephrine, glucocorticoids, and/or growth hormone induced by acute NA administration (28, 29, 40). Previous studies in rats had demonstrated that the overshoot could be completely prevented by the combination of adrenalectomy and hypophysectomy, but not by either treatment alone, suggesting that both the adrenal gland and pituitary hormones are involved. However, when NA (or long-acting derivatives) is administered in high and frequent doses, the plasma FFA level can be maintained for an extended period without a rebound or an overshoot (40, 42). In this case, the inhibitory effect of NA on lipolysis may dominate acute influences of lipolytic hormones. In our studies, continuous NA infusion in rats maintained its ability to lower plasma FFA for at least 5 h (present study) or 7 h (12). However, when this NA infusion was extended to 24 h, plasma FFA rebounded to control levels, suggesting that there was a slowly developing process that caused the FFA rebound. This was not due to a downregulation of NA action, because when the NA infusion was stopped after 24 h the plasma FFA level rapidly increased. The ratio of the average plasma FFA level with NA to that without NA was 0.46 during the 3-h follow-up period after 24-h NA infusion (Fig. 2), which was similar to the value of 0.36 observed during the initial hours of NA infusion (Fig. 1), indicating that the ability of NA to suppress lipolysis was not greatly affected. Thus, our data suggest that the FFA rebound occurred not because of a downregulation of NA action but because of an increase in the basal rate of lipolysis.

Microarray analysis revealed that the FFA rebound after 24-h NA infusion was associated with many changes in gene expression in adipocytes. Among these changes, the decrease in PDE-3B gene expression is of particular interest. This change was very specific, as genes encoding other isoforms of

PDE (i.e., PDE-3A, PDE-4A–D) were not affected (data not shown). PDE-3B degrades cAMP, a major regulator of lipolysis in adipocytes, and decreased PDE-3B helps increase the cAMP level and thus lipolysis (27). The mechanism by which NA inhibits lipolysis in adipocytes is well established (9, 10). NA binds to and activates the NA receptor, a G protein (G_i)-coupled receptor, to inhibit adenylate cyclase and lower the cAMP level. Low cAMP levels in turn decrease PKA activity and the phosphorylation (and activity) of proteins involved in lipolysis (e.g., HSL and perilipin). Thus, cAMP levels are expected to be reduced (to suppress lipolysis) during the initial hours of NA infusion, but this change may be reversed after 24-h NA infusion due to decreased PDE-3B that will, in turn, decrease cAMP degradation. To test this idea, we first measured cAMP concentrations in adipose tissue and found that it may not well reflect changes in adipocytes. For example, a short (i.e., 1.5-h) NA infusion decreased tissue cAMP concentration, but this effect was small (14%) and statistically insignificant ($P > 0.05$). This may be due to the fact that adipose tissues contain cells other than adipocytes, which may contribute more than adipocytes to the tissue cAMP pool. Therefore, we chose to measure the phosphorylation levels of HSL (Ser^{563}), which reflect PKA activity and cAMP levels. As expected, phosphorylation levels of HSL were profoundly decreased after 1.5-h NA infusion. It is important to note that most of the decrease was reversed after 24-h NA infusion, supporting the concept that the short-term effects of NA to decrease cAMP and lipolysis were reversed by the effects of decreased PDE-3B to increase cAMP after 24 h of exposure to NA. These data suggest that decreased PDE-3B expression may be a major mechanism underlying the FFA rebound after a prolonged exposure to NA.

When plasma FFA levels rise after cessation of NA infusion (Fig. 2), cAMP levels are expected to rise in adipocytes, and this would increase phosphorylation of HSL. Interestingly, we did not see any significant increase in phospho-HSL (Ser^{563}) (p-HSL) above the levels seen after 24-h NA infusion (data not shown). These data may suggest that cAMP levels did not rise after cessation of NA infusion, contrary to our expectation. However, this is unlikely, based on the rapid increase of lipolysis after cessation of NA infusion. Alternatively, it is possible that cAMP levels rose but p-HSL did not increase. Previous studies have demonstrated that a rapid increase in lipolysis in adipocytes leads to an activation of AMP-dependent protein kinase (AMPK) (16), and increased AMPK activity inhibits phosphorylation of HSL (Ser^{563}) (3). Therefore, it may be conceivable that the rapid increase in lipolysis after cessation of NA infusion, arising from increased cAMP levels,

activates AMPK activity to inhibit phosphorylation of HSL (Ser^{563}), resulting in no significant increase in p-HSL with an increase in cAMP level, which can increase lipolysis by acting on other molecules, such as adipose triglyceride lipase or perilipin.

Other changes in gene expression that might also contribute to the FFA rebound after a 24-h NA infusion were the 40–60% decreases in the expression of key enzymes of TG synthesis, including AGPAT, DGAT, GPD1, and PEPCK1. The fatty acyltransferases AGPAT and DGAT are directly involved in TG synthesis (or FFA reesterification). In addition, GPD1 and PEPCK1 contribute to TG synthesis by providing glycerol 3-phosphate required for FFA reesterification (15). FFA release from the adipose tissue is determined by the balance between lipolysis and FFA reesterification, and decreased FFA reesterification helps increase the net rate of TG degradation into FFA and glycerol. For example, decreased DGAT2 activity decreases diacylglycerol conversion to TG, thereby increasing the availability of diacylglycerol for lipolysis to monoacylglycerol and to FFA and glycerol. Previous studies established a role of PEPCK1, the key enzyme involved in glyceroneogenesis in adipocytes (15), in the regulation of FFA reesterification. Glucocorticoids are known to decrease the expression of PEPCK1 in adipocytes, and this may contributes to the action of the hormone to promote FFA release from adipocytes (15). Also, INFγ was shown to promote FFA release from adipose tissue by decreasing PEPCK expression without affecting lipolysis (23). Thus, our novel finding of the coordinated decreases in gene expression of the enzymes of TG synthesis suggests that the FFA rebound after a 24-h NA infusion may result, at least in part, from decreased TG synthesis or FFA reesterification in adipocytes.

There were other changes in gene expression that might also have contributed to the increase in lipolysis after a 24-h NA infusion. First, perilipin mRNA and protein expression decreased by 39 and 31%, respectively. Perilipin coats lipid droplets in adipocytes and suppresses lipolysis by reducing lipase accessibility to the surface of lipid droplets (36). Perilipin knockout mice were lean, had elevated basal lipolysis, and were resistant to diet-induced obesity (1, 26). Therefore, the 31% decrease in perilipin protein content after 24 h of NA likely contributed to the increase in lipolysis. Second, there was a 21% decrease in adipose-specific phospholipase A$_2$ (AdPLA or PLA2G16) gene expression. AdPLA is a recently identified, membrane-associated, calcium-dependent phospholipase that is expressed exclusively in adipocytes and is the major phospholipase in adipocytes. AdPLA suppresses lipolysis by producing prostaglandin E$_2$, which decreases cAMP via EP receptors (19). AdPLA-null mice showed elevated lipolysis in adipocytes and were resistant to diet-induced obesity (35). These data suggest that the small (21%) but significant decrease in AdPLA gene expression might have contributed to the increase in lipolysis that occurred after 24-h NA infusion (assuming that a similar change in protein level occurred). Finally, NA receptor gene expression decreased by 63% after 24-h exposure to NA. However, as discussed above, the NA effect to suppress lipolysis was not significantly affected, suggesting that signaling through the NA receptor was still strong enough to suppress lipolysis under our experimental conditions, possibly due to spare receptors and/or high plasma NA levels. It is conceivable, however, that such a change in

nicotinic acid receptor expression, which would decrease NA signaling and action, may account for some of FFA rebound in clinical situations when plasma NA levels are not too high.

What are the mechanisms by which these changes in gene expression in adipose tissue were brought about by NA? NA lowers cAMP and thus PKA activity in adipocytes. Therefore, one possibility is that the cAMP/PKA-dependent pathways mediated some gene expression changes via regulation of the activity of transcription factors such as CREB and CREM (17), which bind to cAMP response elements of gene promoters. Alternatively, NA may regulate gene expression by decreasing the plasma levels of FFAs, which are endogenous ligands of the PPARs. Although plasma FFA levels rebounded after the 24-h NA infusion, some of the changes in gene expression might be a delayed response to earlier decreases in plasma FFA levels. On the other hand, as discussed above, NA has been shown to increase plasma epinephrine and glucocorticoid levels (28, 29, 27). We found that NA infusion significantly increased plasma levels of both epinephrine and corticosterone at 1 h (data not shown) although these effects were not statistically significant at 24 h (Fig. 7). Therefore, some gene expression changes may have arisen from increases in these hormones early during NA infusion. Interestingly, the 24-h NA infusion decreased the expression of transcription factors involved in adipocyte differentiation, such as C/EBPβ, C/EBPγ, and PPARγ (43), suggesting that NA may affect differentiation/dedifferentiation status. However, major adipocyte-specific genes such as adiponectin, leptin, fatty-acid synthase, and fatty-acid-binding protein showed no significant changes in gene expression, suggesting that adipocyte differentiation/dedifferentiation might not play a role. Thus, there could be several different mechanisms by which gene expression changes were induced. Future studies are warranted to elucidate the underlying mechanisms.

The present rat model of FFA rebound with 24-h NA infusion may not be directly relevant to clinical situations with chronic treatments with NA or its derivatives. This is because the mode, dose, and duration of NA administration used in the present study are different from those used in clinical studies. Moreover, we studied the phenomenon in normal metabolic states, whereas clinical studies involve patients with dyslipidemia. Nonetheless, our study provided potential molecular mechanisms for FFA rebound during prolonged exposure to NA, such as gene-expression changes in adipocytes, which can be tested in clinical studies using fat biopsy samples taken before and after chronic NA treatment. If similar changes occur in patients during chronic NA treatment, our 24-h NA infusion model would be an excellent model with which to further study molecular mechanisms underlying the changes.

The 24-h NA infusion improved insulin's action to stimulate glucose metabolism as assessed with continued NA infusion, presumably due to decreased plasma FFA levels that were present during a significant portion of the 24-h infusion period. Our additional data show that the effect of NA to lower plasma FFA level was well maintained after 12-h NA infusion (data not shown). Therefore, although plasma FFA rebounded to control levels after 24-h NA infusion, plasma FFA levels appeared to be lowered during most (>12 h) of the 24-h NA infusion period. Previous studies in humans have demonstrated that lowering plasma FFA using the NA analog acipimox for a short term (hours or days) increased insulin sensitivity (13, 34).

Our preliminary data also show a 37% decrease in hepatic TG concentration ($P < 0.05$, $n = 4$) after 24-h NA infusion despite the FFA rebound, reflecting the effect of earlier decreases in plasma FFA levels, consistent with the effect on insulin sensitivity. However, when NA infusion was stopped, basal lipolysis increased, and insulin was not able to fully suppress lipolysis (or lower plasma FFA), resulting in impaired insulin action. It is well established that the plasma FFA level is a major determinant of insulin's action (4). Although the effect of FFA rebound on insulin action was small (i.e., 18%), this was likely due to a short exposure to elevated plasma FFA. Previous studies have shown that it takes time for elevated FFA levels to decrease insulin's action on glucose uptake. For example, in human studies (5, 7), it took 2–3 h to see any effect on insulin action (i.e., G_{INF}) even with FFA levels elevated higher than those in the present study. In our own study in rats (25), insulin action was inhibited by elevated plasma FFA in a time-dependent manner, i.e., ~10% at 1 h, ~25% at 2 h, and >50% at 4 h. In the present study, NA-infused rats were exposed to elevated plasma FFA levels only during the final 1–1.5 h of the clamp, explaining the small effect (i.e., 18%) of FFA rebound on insulin sensitivity in NA-infused rats. Thus, our data support the previous suggestion that insulin resistance develops as a result of FFA rebound during NA treatment (2, 22, 32, 42).

In conclusion, a 24-h continuous NA infusion in rats resulted in FFA rebound, accompanied by many changes in gene expression and increased basal lipolysis in adipose tissue. In particular, PDE-3B gene expression decreased significantly, which might have led to increased cAMP levels and HSL activity to cause the FFA rebound. In addition, the expression of several key enzymes of triglyceride synthesis was markedly decreased, suggesting the possibility that decreased TG synthesis contributes to the FFA rebound. Thus, the present study provides an animal model and potential molecular mechanisms for the FFA rebound observed in clinical studies with chronic NA treatment. Future studies are warranted to determine whether similar changes occur in humans to account for FFA rebound during chronic NA treatment.

DISCLOSURES

No conflicts of interest are reported by the authors.

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