Glycine Normalizes Hepatic Triglyceride-Rich VLDL Secretion by Triggering the CNS in High-Fat Fed Rats

Jessica T.Y. Yue, Patricia I. Mighiu, Mark Naples, Khosrow Adeli, Tony K.T. Lam

Rationale: Dysregulation of hepatic triglyceride (TG)-rich very low-density lipoproteins (VLDL-TG) in obesity and type 2 diabetes contributes to the dyslipidemia that leads to cardiovascular morbidity. The central nervous system (CNS), particularly the hypothalamus, regulates hepatic lipid metabolism. Although the underlying neurocircuitry remains elusive, glycine has been documented to enhance CNS N-methyl-D-aspartate (NMDA) receptor-mediated transmission.

Objective: We tested the hypothesis that glycine regulates hepatic VLDL-TG secretion by potentiating NMDA receptor-mediated transmission.

Methods and Results: Using 10-hour fasted male Sprague-Dawley rats implanted with stereotactic cannulae into an extrahypothalamic region termed the dorsal vagal complex (DVC) and vascular catheters to enable direct DVC infusion and blood sampling, respectively, the rate of hepatic VLDL-TG secretion was measured following tyloxapol (an inhibitor of lipoprotein lipase) injection. Direct DVC infusion of glycine lowered VLDL-TG secretion, whereas NMDA receptor blocker MK-801 fully negated glycine’s effect. NRI subunit of NMDA receptor antagonist 7-chlorokynurenic acid, adenosine injection of NRI short hairpin RNA (shRNA), and hepatic vagotomy also nullified glycine’s effect. Finally, DVC glycine normalized the hypersecretion of VLDL-TG induced by high-fat feeding.

Conclusions: Molecular and pharmacological inhibition of the NRI-containing NMDA receptors in the DVC negated the ability of glycine to inhibit hepatic secretion of VLDL-TG in vivo. Importantly, the hypersecretion of VLDL-TG from the liver induced by a model of high-fat feeding was restored by the hepatic lipid control of CNS glycine sensing. These findings collectively suggest that glycine or glycine analogues may have therapeutic benefits in lowering plasma lipid levels in diabetes and obesity by triggering the CNS. (Circ Res. 2012;110:00-00.)

Key Words: glycine ■ NMDA receptors ■ CNS ■ hepatic lipid metabolism ■ obesity

Hypertriglyceridemia and overproduction and secretion of VLDL-TG are key contributors to the metabolic syndrome and atherogenic dyslipidemia. Although circulating insulin and free fatty acids (FFA) regulate hepatic VLDL-TG secretion, the underlying regulatory mechanisms remain unclear. Given that diabetes and obesity are associated with hypertriglyceridemia and increased hepatic secretion and production of VLDL-TG, it is important to elucidate the regulatory mechanisms of hepatic VLDL-TG secretion in a normal setting with the hope to discover novel therapeutic molecules to lower blood lipid levels in obesity and diabetes.

In addition to the array of circulating factors that directly regulate hepatic lipid metabolism, the hypothalamic region of the brain senses circulating nutrients and hormones to regulate peripheral lipid homeostasis and blood pressure. For example, nutrients such as glucose signal within the hypothalamus to inhibit VLDL-TG secretion through the hepatic vagus. Consistent with the fact that nutrients in the brain lowers appetite through the suppression of neuropeptide Y, direct administration of neuropeptide Y into the hypothalamus conversely stimulates VLDL-TG secretion. Central neuropeptide Y signaling also negates the ability of circulating insulin to inhibit VLDL-TG secretion, whereas hormones such as insulin, ghrelin, melanocortins, and leptin trigger hypothalamic signaling cascades to modulate peripheral lipid profiles. In spite of the fact that the

Original received March 2, 2012; revision received March 26, 2012; accepted March 27, 2012. In February 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.77 days.

From the Toronto General Research Institute (J.T.Y.Y., P.I.M., T.K.T.L.), University Health Network; Departments of Medicine (J.T.Y.Y., T.K.T.L.), Physiology (P.I.M., T.K.T.L.), and Biochemistry (K.A.), University of Toronto; Department of Molecular Structure and Function (M.N., K.A.), Research Institute, The Hospital for Sick Children; Banting and Best Diabetes Centre (T.K.T.L.), University of Toronto; Toronto, ON, Canada. Jessica T.Y. Yue conducted and designed the experiments, performed data analyses, and wrote the manuscript. Patricia I. Mighiu and Mark Naples assisted in experiments. Khosrow Adeli contributed to discussion and edited the manuscript. Tony K.T. Lam supervised the project, designed the experiments, and edited the manuscript.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.268276/-/DC1.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.112.268276

© 2012 American Heart Association, Inc.
hypothalamic region, has been documented to relay ascending projections from the gut to regulate energy balance and glucose homeostasis. In addition, direct activation of DVC NMDA receptors is sufficient and necessary for hypothalamic nutrient sensing to regulate hepatic glucose production. However, it is currently unknown whether hepatic lipid metabolism is regulated by neuronal transmission in the DVC.

Given that glycine is a coagonist of the NMDA receptor and binds to the NR1 subunit of the NMDA receptor to potentiate its activation, we here investigated, using molecular, pharmacological, and surgical approaches, whether potentiating DVC NMDA receptor by glycine is sufficient to regulate VLDL-TG secretion through the hepatic vagus in normal and high-fat feeding conditions (Figure 1A).

Methods
An expanded Methods section is provided in the online-only Data Supplement.

Experimental Animals
Nine-week-old male Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada) initially weighing 280 to 300 g were used and individually housed. Rats had free access to drinking water and fed ad libitum with standard rat chow (Teklad Global 18% protein, Harlan Laboratories, Madison, WI). Bilateral, 26-gauge, stainless steel guide cannulae (HRS Scientific, Montreal, QC, Canada) were stereotaxically implanted into the DVC targeting the ventral subdiaphragmatic vagal trunk (shRNA NR1) or a mismatch sequence (MM) as a control in one group of rats with the same brain and vascular surgeries, recovery, and fasting as described above. Rats were subjected to a peripheral intracarotid injection of glycine (50 nmol/kg) or an equal volume of isotonic saline injection (0.3 mL) followed by an intravenous injection of tyloxapol (Sigma-Aldrich, St. Louis, MO; 600 mg/kg, dissolved in saline, over 1 minute). This dose of tyloxapol inhibits endogenous lipoprotein lipase and thus blocks the clearance of nascent VLDL particles. As such, the rate of secretion of VLDL-TG is proportional to the rise in concentrations of VLDL-TG over time (Figure 1F, G). Blood samples were subsequently obtained every 15 minutes until the end of the experiment at t = 150 minutes at which DVC tissue wedges and liver were carefully obtained, frozen in liquid nitrogen, and stored at −80°C until used for analysis. Plasma was separated by centrifugation and stored at −20°C until assayed. Packed red blood cells were resuspended in 0.2% heparinized saline and reinfused into the rat.

Peripheral Glycine Experiments
In a separate group of rats with the same brain and vascular surgeries, recovery, and fasting as described above, rats were subjected to a peripheral intracarotid injection of glycine (50 nmol/kg) or an equal volume of isotonic saline injection (0.3 mL) after basal blood samples were taken at t = −10 minutes. This dose of glycine and route of administration was used previously in experiments that measured brain uptake of glycine. Blood was then collected at t = 0, 15, and 150 minutes to monitor plasma glycine concentrations, and DVC tissue was collected as above for tissue glycine analysis.

Adenovirus Infection in the DVC
We injected an adenovirus expressing the shRNA to the NR1 subunit of the NMDA receptor to knockdown the glycine-activated subunit (shRNA NR1) or a mismatch sequence (MM) as a control in one group of rats as previously described. Immediately after stereotaxic surgery while anesthetized, 5 µL of adenovirus were injected over 30 seconds into each of the cannulae with microsyringes. We have verified that this adenoviral NR1 shRNA knockdown procedure decreased NR1 protein levels specifically in the region DVC. Seven days after DVC cannulation and adenoviral injection, vascular catheterization was performed as described above. On recovery, virus-injected rats underwent VLDL-TG experiments with DVC glycine infusions as described above.

Hepatic Branch Vagotomy
Hepatic branch vagotomy was performed in another group of rats as previously described. Briefly, on the day of vascular catheterization, the stomach and lower esophagus were exposed and gently protracted from the abdominal cavity. The hepatic branch of the ventral subdiaphragmatic vagal trunk was transected, and the hepatic-oesophageal omentum was severed to remove tissue connections between the liver and esophagus. Transection of the hepatic vagus nerve disrupts neural communication between the brain and liver and slightly decreases innervation to the gut. Sham-operated rats underwent the same procedure except for transection of the nervous tissue. After a 5-day recovery, rats underwent VLDL-TG experiments with DVC glycine or saline infusions as explained above.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Non-standard Abbreviations and Acronyms</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7CKNA</td>
<td>7-chlorokynurenic acid</td>
</tr>
<tr>
<td>apolipoprotein B (apoB)</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DVC</td>
<td>dorsal vagal complex</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>HFD</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>MM</td>
<td>mismatch</td>
</tr>
<tr>
<td>mtp</td>
<td>microsomal transfer protein</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>scd-1</td>
<td>stearoyl-CoA denaturase-1</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonuclease acid</td>
</tr>
<tr>
<td>srebp-1</td>
<td>sterol regulatory element binding protein-1</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>VLDL-TG</td>
<td>triglyceride-rich very-low-density lipoproteins</td>
</tr>
</tbody>
</table>
High-Fat Feeding

Another group of rats were fed a palatable, lard-oil enriched, high-fat diet (TestDiet #57IR, Purina Mills, Richmond, IN) for 3 days. Compared with regular chow, this high-fat diet had greater total calorie content (5.14 versus 3.83 kcal/g), higher fat content (33 versus 17%), and lower protein and carbohydrate content (22% versus 31% and 45% versus 52%, respectively). Two days after vascular catheterization, regular chow was replaced with high-fat diet, and food intake was measured for 3 days of high-fat feeding leading to the experimental day. Rats were similarly subjected to a ~10-hour fast on the night prior to the VLDL-TG experiment with DVC glycine or glycine + MK-801 or saline.

Figure 1. A, Schematic representation of working hypothesis: glycine, a coagonist of the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor, potentiates the activation of NMDA receptors in the dorsal vagal complex (DVC) and lowers the secretion of hepatic triglyceride-rich very low-density lipoproteins (VLDL-TG). NMDA receptor ion channel blocker, MK-801, negates the ability of glycine to lower hepatic VLDL-TG. B, Experimental protocol. Day 0: Stereotaxic placement of cannulae in the DVC, targeting the NTS, in male Sprague Dawley rats (~280–300 g). Day 7: Indwelling catheters were surgically implanted in the left carotid artery and right jugular vein for blood sampling and infusion, respectively. Day 12: Rats were given 5 days to recover from vascular surgery prior to tyloxapol experiments. During tyloxapol experiments, DVC infusions at 0.33 L/h of saline (n=5), MK-801 (0.06 ng/min; n=7) glycine (10 μmol/L; n=12), or glycine (10 μmol/L) + MK-801 (0.06 ng/min; n=7) were commenced at t=−10 minutes after basal blood samples were obtained. Time 0 minutes blood samples were obtained after 10 minutes of DVC infusions, followed by an intravenous injection of tyloxapol (600 mg/kg). Blood samples were subsequently obtained every 15 min until the end of the experiment at t=150 min. C, DVC infusion of 10 μmol/L glycine for 150 min resulted in an increase of DVC tissue glycine levels as compared with DVC saline infusion. Peripheral (i.v. intravessel) injection of 50 nmol/kg glycine (n=7) increased: (D) plasma glycine levels and (E) DVC tissue glycine levels 160 min postinjection as compared with iv saline (n=6). Following tyloxapol administration, DVC glycine: (F) lowered plasma triglyceride (TG) and (G) decreased the rate of appearance of VLDL-TG in plasma compared with saline. Coinfusion of NMDA receptor blocker MK-801 fully reversed the effects of glycine. H, DVC glycine reduced the hepatic mRNA expression of stearoyl-CoA desaturase-1 (scd-1) as compared with vehicle, and simultaneous administration of DVC MK-801 reversed glycine’s effect to lower the expression of this gene. White (□), saline; light gray (■), MK-801 only; black (■■), glycine; dark gray (■■■), glycine + MK-801. Crossed-hatched bars represent iv saline or glycine administration. White with light gray diagonal stripes represents a vehicle control group (n=7). Data are presented as mean±SEM. *P<0.05 glycine vs all other treatment groups.
infusions as described above. Rats that did not overeat were excluded from the study.

Lipid, Glycine, and Hormone Analyses
Plasma triglycerides were measured using a commercially available kit (Roche Diagnostics, Indianapolis, IN). For apolipoprotein B (apoB) measurements, plasma samples were diluted 100 X in water prior to being prepared for SDS-PAGE according to the method of Laemmli.22 Each lane contained equal volumes of denatured plasma (20 μL). Samples were resolved by SDS-PAGE and detected by chemiluminescent immunoblotting using a commercially available antihuman apoB antibody (Midland BioProducts, Boone, IA). Plasma levels of glycine (ELISA; IMMUNODIAGNOSTIK, BENSHEIM, GERMANY), insulin, leptin, and adiponectin (RIA; LINCO RESEARCH, Inc., ST. CHARLES, MO/MILLIPORE CORPORATION, BILLERICA, MA), and free fatty acids (FFA; WAKO DIAGNOSTICS, Richmond, VA) were measured using commercially available kits. Frozen DVC were weighed and homogenized in 100 μL buffer containing 2 mmol/L Tris-HCl and 1 mmol/L EDTA at pH 7. Samples were centrifuged at 3000 rpm for 3 minutes, and supernatant was used in the ELISA kit to determine glycine levels. DVC glycine measurements were normalized for tissue weight.

Hepatic RNA Extraction and cDNA Synthesis
Total RNA was extracted following homogenization of frozen liver samples using a commercially-available RNA isolation kit (RNeasy Plus Mini kit, Qiagen). Prior to cDNA synthesis, total RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). To ensure the quality of RNA, aliquots of denatured RNA were denatured and separated on MOPS-buffered 1% agarose gel. RNA was deemed to be of acceptable quality if both the 28S rRNA and 18S rRNA were visible as clear, sharp bands under UV light after staining with ethidium bromide. Following quantification, 0.4 μg of total RNA from each animal was converted to single-stranded cDNA using TaqMan Reverse Transcription (RT) Reagents (ABI). Random hexamers were used to prime the RT reaction, allowing the expression of 18S rRNA to be used as an internal reference during subsequent PCR reactions. The RT reaction was carried out in a Mastercycler gradient (Eppendorf) as follows: samples were preincubated at 25°C for 10 minutes, RT performed at 37°C for 120 minutes, and, finally, the reverse transcriptase was inactivated by heating the samples at 85°C for 5 minutes. Following RT, cDNA was stored at −20°C.

Real-Time Quantitative RT-PCR Analysis
Messenger RNA (mRNA) levels were assessed by real-time quantitative RT-PCR using an ABI Prism 7900 sequence detector. All PCR reactions were performed in a total volume of 50 μL and included the following components: cDNA derived from 20 ng of total RNA (or 5 ng for 18S), 400 mmol/L each of forward and reverse primers, RNase-free water, and 25 μL of Power SYBR Green PCR Master Mix (ABI), an optimized buffer system containing AmpliTaq Gold DNA polymerase and dNTPs. All PCR reactions were performed in duplicate (n=4–12). Cycling parameters were as follows: after an initial denaturation step for 10 minutes at 95°C, 40 subsequent cycles were performed in which samples were denatured at 15 s at 95°C followed by primer annealing and elongation at 60°C for 1 minute. Relative quantities of mRNA were calculated from Ct values using the comparative Ct method (ΔΔCt) using 18S RNA as an internal reference. Primer pairs for real-time PCR were designed using Primer3 software and sequence information obtained from NCBI. The following forward/reverse primer sets were used: sterol regulatory element binding protein-1 (srebp-1), 5′-actacatgcctcttcaccagcc-3′/5′-gctcttgctcttcgctcc-3′; stearoyl-CoA desaturase-1 (sd-1), 5′-acagtgtgctagctaaag-3′/5′-gtttcctggaaacctc-3′; microsomal transfer protein (mtp) 5′-attgaaggtctgtatcagc-3′/5′-gccctggccttcgctcc-3′; 18S, 5′-taatcctgcctcttatancaaca-3′/5′-gatggaggctcattacaac-3′. To ensure specificity of amplification during real-time PCR, a dissociation curve was generated, and the final amplified products were subjected to agarose gel electrophoresis and stained with ethidium bromide. Each experiment was run in triplicate, and the mean and SD were determined. Differences between groups were calculated using a two-tailed t test.

Table 1. Plasma Insulin (ng/mL), Leptin (ng/mL), and Adiponectin (mg/mL) Concentrations at Basal (–10 min) and After (150 min) Tyloxapol Administration

<table>
<thead>
<tr>
<th></th>
<th>Basal (–10 min)</th>
<th>Vehicle</th>
<th>Glycine</th>
<th>MK-801 + Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.66±0.08</td>
<td>0.60±0.09</td>
<td>0.55±0.09</td>
<td>0.50±0.08</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>1.34±0.11</td>
<td>1.28±0.17</td>
<td>1.10±0.13</td>
<td>1.11±0.18</td>
</tr>
<tr>
<td>Adiponectin (mg/mL)</td>
<td>2.35±0.25</td>
<td>2.53±0.39</td>
<td>2.15±0.29</td>
<td>2.65±0.39</td>
</tr>
</tbody>
</table>

The vehicle group includes saline and MK-801 antagonist only groups. Data are presented as mean±SEM.

Statistical Analyses
Statistical analysis was performed using a 2-tailed t test. Unless otherwise stated, significance was deemed when P<0.05. Sample sizes for groups presented in Figures are indicated in the Figure Legends or otherwise indicated in Results. Data are presented as mean±SEM.

Results
Glycine Lowers Secretion of Hepatic VLDL-TG Through the Activation of DVC NMDA Receptors and Inhibition of Hepatic Scd-1
To examine whether direct administration of glycine into the DVC affects the secretion of VLDL-TG in 10-hour fasted normal rats with comparable body weights (Online Table 1), we inhibited lipoprotein lipase with tyloxapol and monitored the rate of plasma TG appearance as a function of time (Figure 1A and 1B).

DVC infusion of 10 μmol/L glycine for 160 minutes (10 minutes to 150 minutes) increased DVC tissue glycine levels 1.4-fold (Figure 1C). Peripheral injection of 50 mmol/kg glycine significantly elevated plasma glycine levels (Figure 1D) and likewise led to a 1.4-fold increase in DVC tissue glycine levels 160 minutes following injection (Figure 1E). These findings illustrate that the current DVC glycine infusion protocol (Figure 1A and 1B) elevates tissue glycine levels in the DVC to a similar extent as when plasma glycine is physiologically elevated by peripheral injection.

Table 2. Plasma Free Fatty Acid (FFA; Mm) Concentrations Before (0 min) and After (150 min) Tyloxapol Administration

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>150 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.820±0.060</td>
<td>1.050±0.082</td>
</tr>
<tr>
<td>MK-801</td>
<td>0.931±0.073</td>
<td>0.999±0.084*</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.983±0.099</td>
<td>1.501±0.154*</td>
</tr>
<tr>
<td>Glycine+MK-801</td>
<td>0.838±0.073</td>
<td>1.107±0.071*</td>
</tr>
<tr>
<td>7CKNA</td>
<td>0.925±0.105</td>
<td>0.817±0.129*</td>
</tr>
<tr>
<td>Glycine+7CKNA</td>
<td>0.983±0.099</td>
<td>1.501±0.154*</td>
</tr>
<tr>
<td>MM+glycine</td>
<td>0.836±0.096</td>
<td>1.268±0.157*</td>
</tr>
<tr>
<td>NR1 shRNA+glycine</td>
<td>0.866±0.044</td>
<td>1.034±0.095*</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. *P<0.05 150 min vs 0 min.
DVC glycine administration was not only able to elevate DVC tissue glycine levels (Figure 1C) but importantly also diminished the rise in plasma TG levels in tyloxapol-injected rats (Figure 1F). This hypolipidemic effect induced by DVC glycine corresponded to an inhibition of the rate of VLDL-TG secretion as compared to DVC saline (Figure 1G). DVC 100 and 1000 mol/L, but not 1 mol/L, glycine also diminished the rise in plasma TG and the rate of VLDL-TG secretion (Online Figure IA and 1B). Hence, DVC glycine at a dose of 10 mol/L was the minimal dose tested that achieved a significant hypolipidemic effect and was used in all subsequent experiments.

To begin delineating the hepatic mechanisms responsible for the reduction of VLDL-TG secretion, gene expression of several proteins (scd-1, mtp, srebp-1) that regulate hepatic lipid metabolism were assessed using livers that were obtained immediately following the DVC glycine-infused experiments. DVC glycine significantly inhibited hepatic scd-1 mRNA expression (Figure 1H), but not mtp or srebp-1 (Online Figure IC and 1D). DVC glycine did not significantly reduce (although had a strong tendency; \( P=0.06 \)) the tyloxapol-induced apoB48 secretion rate (Online Figure IE, and 1F). However, the rate of ApoB 48 secretion was only assessed by measuring plasma apoB48 levels over time.

The hypolipidemic effect of DVC glycine at 160 minutes was independent of changes in plasma insulin, leptin, adiponectin, and glucose levels (Table 1; Online Figure IG). However, plasma FFA was increased by DVC glycine as compared to controls (Table 2).

To assess whether the potentiation of NMDA receptors in the DVC mediates the effect of glycine, DVC glycine was coininfused with NMDA receptor ion channel blocker MK-801 (Figure 1A and 1B). The hypolipidemic effect of DVC glycine was fully reversed by DVC MK-801, whereas DVC MK-801 alone had no effect (Figure 1F and 1G). DVC MK-801 also negated the glycine-induced decrease of hepatic scd-1 gene expression (Figure 1H) but did not reverse the tendency of glycine to lower apoB48 (Online Figure IE and 1F). DVC coinfusion of MK-801 with glycine also reversed the increase of plasma FFA levels (Table 2), whereas plasma insulin, leptin and adiponectin levels were unchanged (Table 1).

These findings collectively indicate that direct administration of glycine into the DVC lowers the secretion of VLDL-TG via the activation of NMDA receptors and reduction of hepatic scd-1 mRNA expression.
Glycine Activates the NR1 Subunit of the NMDA Receptor in the DVC to Lower VLDL-TG

NMDA receptors are composed of NR1 and NR2 subunits, which are activated by the coagonist glycine and glutamate or aspartate, respectively. To determine whether glycine lowers VLDL-TG via the NR1 subunit of the DVC NMDA receptor, we first inhibited the NR1 subunit with 7CKNA, an antagonist to the NR1 subunit (Figure 2A and 2B). Following tyloxapol injection, DVC infusion of 7CKNA alone had no effect on VLDL-TG secretion profiles (Figure 2C and 2D) as compared to saline controls (Figure 1C and 2D). However, coinfusion of 7CKNA (30 μmol/L) with glycine negated the ability of glycine to lower VLDL-TG secretion (Figure 2C and 2D). Of note, DVC 7CKNA at 10 μmol/L did not negate the effect of glycine to lower the rate VLDL-TG secretion (0.042 ± 0.003 mmol/L; 7CKNA (10 μmol/L) + glycine (10 μmol/L), n = 5). DVC 7CKNA also reversed the reduction of hepatic scd-1 mRNA expression by glycine (Figure 2E) but did not reverse the tendency of glycine to lower apoB48 (Online Figure IIC and IID). Plasma insulin, leptin, and adiponectin levels (Table 1) were again unaffected, whereas DVC 7CKNA reversed the increase in plasma FFA by DVC glycine (Table 2).

To alternatively test whether activation of the DVC NR1 subunit of the NMDA receptor inhibits VLDL-TG secretion, we infused serine, an amino acid that also binds to the NR1 subunit, with or without coinfusion of 7CKNA. DVC serine (10 μmol/L) reduced plasma TG and the rate of secretion of VLDL-TG (Online Figure IIA, B) to a similar extent as DVC glycine (Figure 1F and 1G). DVC 7CKNA blocked this effect of serine (Online Figure IIA).

We next knocked down NR1 in the DVC using an adenoviral vector expressing the shRNA of the NR1 subunit as previously described (Figure 3A, and 3B). Following tyloxapol injection, DVC glycine lowered VLDL-TG via the hepatic vagus.

Glycine Activates the NR1 Subunit of the NMDA Receptor in the DVC to Lower VLDL-TG

Secretion and Inhibit Hepatic Scd-1

NMDA receptors are composed of NR1 and NR2 subunits, which are activated by the coagonist glycine and glutamate or aspartate, respectively. To determine whether glycine lowers VLDL-TG via the NR1 subunit of the DVC NMDA receptor, we first inhibited the NR1 subunit with 7CKNA, an antagonist to the NR1 subunit (Figure 2A and 2B). Following tyloxapol injection, DVC infusion of 7CKNA alone had no effect on VLDL-TG secretion profiles (Figure 2C and 2D) as compared to saline controls (Figure 1C and 2D). However, coinfusion of 7CKNA (30 μmol/L) with glycine negated the ability of glycine to lower VLDL-TG secretion (Figure 2C and 2D). Of note, DVC 7CKNA at 10 μmol/L did not negate the effect of glycine to lower the rate VLDL-TG secretion (0.042 ± 0.003 mmol/L; 7CKNA (10 μmol/L) + glycine (10 μmol/L), n = 5). DVC 7CKNA also reversed the reduction of hepatic scd-1 mRNA expression by glycine (Figure 2E) but did not reverse the tendency of glycine to lower apoB48 (Online Figure IIC and IID). Plasma insulin, leptin, and adiponectin levels (Table 1) were again unaffected, whereas DVC 7CKNA reversed the increase in plasma FFA by DVC glycine (Table 2).

To alternatively test whether activation of the DVC NR1 subunit of the NMDA receptor inhibits VLDL-TG secretion, we infused serine, an amino acid that also binds to the NR1 subunit, with or without coinfusion of 7CKNA. DVC serine (10 μmol/L) reduced plasma TG and the rate of secretion of VLDL-TG (Online Figure IIA, B) to a similar extent as DVC glycine (Figure 1F and 1G). DVC 7CKNA blocked this effect of serine (Online Figure IIA).

We next knocked down NR1 in the DVC using an adenoviral vector expressing the shRNA of the NR1 subunit as previously described (Figure 3A, and 3B). Following tyloxapol injection, DVC glycine lowered VLDL-TG via the hepatic vagus.

Hepatic Innervation Is Required for Glycine to Lower VLDL-TG Secretion

We next delineated the neuronal circuit downstream of the effect of DVC glycine by repeating the tyloxapol experiments in rats that received hepatic branch vagotomy (Figure 4A and 4B). Hepatic vagotomy alone did not affect VLDL-TG secretion profiles (Figure 4C and 4D) but reversed the ability of glycine to lower VLDL-TG secretion (Figure 4C and 4D). Thus, DVC glycine lowers VLDL-TG via the hepatic vagus.
Glycine Normalizes VLDL-TG Secretion in High-Fat Feeding Through the Activation of DVC NMDA Receptors

Short-term high-fat feeding in rats and mice increases hepatic VLDL-TG secretion,12,31 and we here tested whether glycine can trigger the NMDA receptors in the DVC to lower VLDL-TG following high-fat feeding (Figure 5A, B). Tyloxapol and DVC glycine experiments were performed in rats subjected to 3 days of high-fat feeding (Figure 5B). Caloric intake in rats fed with high-fat diet (HFD) was approximately 2.5-fold greater than in their regular chow-fed counterparts (Online Table II), demonstrating a model of marked overfeeding. Overfeeding with a high-fat diet augmented the rate of VLDL-TG secretion by approximately 20% following tyloxapol administration as compared to a diet of regular chow (reg chow/saline versus HFD/saline, \( P < 0.03 \)) (Figure 5C and 5D). Importantly, DVC glycine normalized the VLDL-TG secretion rate in HFD rats to that of control rats on a diet of regular chow, and this hypolipidemic effect of glycine was prevented by coinfusion of MK-801 (Figure 5C and 5D).

Discussion

Overproduction of VLDL-TG by the liver is prominent sequela of insulin resistance, obesity, and diabetes1,2,32,33 and leads to dyslipidemia and cardiovascular disease.32,34 We here report, for the first time to our knowledge, that glycine lowers hepatic VLDL-TG secretion via the central nervous system (CNS). Glycine is the smallest nonessential amino acid and a coagonist of the NMDA receptor along with glutamate.29,30 NMDA receptors in the CNS are important for neurotransmission and have critical roles in mechanisms of synaptic plasticity and network synchronization.29 Our current study demonstrates that glycine-induced potentiation of NR1-containing NMDA receptors in the DVC is sufficient to trigger the hepatic vagus and lower VLDL-TG secretion in normal rats.

The NR1 subunit is an obligatory subunit that forms a functional NMDA receptor when combined with either NR2 or NR3 subunits.29 A typical NMDA receptor is a tetramer that most often consists of 2 glycine-binding NR1 subunits and 2 glutamate-binding NR2 subunits.25,29,35 In the current study, glycine was administered into the DVC approximately 1.7-fold higher than the concentration of glycine in the cerebrospinal fluid (6 \( \mu \)mol/L)36 and DVC glycine elevated DVC tissue glycine levels by 1.4-fold. The fact that direct molecular and pharmacological inhibition of NR1-containing NMDA receptors in the DVC alone did not alter VLDL-TG secretion but was sufficient to negate the hypolipidemic effect of glycine illustrates that NR1 subunit in the DVC are not saturated under basal conditions. This is likely due to the activity of high capacity glycine transporters 1 and 2 that maintain low glycine concentrations at synaptic clefts proximal to NMDA receptors in the DVC.36,37 Of note, although it remains to be assessed whether the activation of the NR2 subunit of the NMDA receptors in the DVC lowers VLDL-TG, it is unlikely that the nonconventional NMDA receptors that comprised of NR1 and NR3 subunits (which are resistant...
to MK-801\textsuperscript{35,38} are involved since DVC MK-801 negated the hypo lipemic effect of glycine.

Scd-1 is an enzyme that catalyzes the conversion of stearyl-CoA to oleyl-CoA in the liver. Presently, we demonstrate that the suppressive effect of DVC glycine on hepatic VLDL-TG secretion is associated with an inhibition of hepatic scd-1 gene expression. This is supported by previous findings in which hypothalamic glucose metabolism reduced hepatic scd-1 mRNA and inhibited hepatic VLDL-TG secretion,\textsuperscript{12} whereas hypothalamic neuropeptide Y signaling increases VLDL-TG secretion and scd-1 mRNA.\textsuperscript{5} Although the mechanistic links between hepatic scd-1 and the alteration of VLDL-TG induced by the CNS remain elusive, changes in the formation of hepatic oleyl-CoA may be a potential mediator.\textsuperscript{12}

ApoB lipoproteins are required for the assembly and stabilization of mature VLDL-TG particles, which are subsequently secreted into the peripheral circulation.\textsuperscript{39} ApoB lipoproteins are secreted by the liver and intestine and under post absorptive conditions, the majority of the apoB lipoproteins are derived from the liver. In contrast to humans, the rat liver produces predominantly apoB48 instead of apoB100.\textsuperscript{40} Our present data appear to suggest that changes in hepatic VLDL-TG induced by DVC NMDA receptor activation did not correlate with consistent changes in apoB48 secretion as assessed by plasma apoB48 measurements. Future studies using \textsuperscript{35}S-methionine to measure the rate of apoB secretion are warranted to further clarify whether changes in apoB are associated with changes in VLDL-TG secretion. Although no corresponding changes in plasma insulin, leptin, and adiponectin levels were detected as well, DVC NMDA receptor activation increased plasma FFA levels. Future studies aim to assess the rate of (and the corresponding changes in the expression and activity of enzymes that govern) fatty acid oxidation and uptake in selective tissues will help to clarify the role of FFA in the alteration of VLDL-TG induced by DVC NMDA receptor activation. In addition, in light of our current data and the fact that hepatic vagotomy negates the ability of hypothalamic nutrient sensing in mice\textsuperscript{14} to regulate hepatic VLDL-TG secretion and circulating HDL-cholesterol levels, these observations together indicate that the hepatic innervation between the brain and the liver plays an important role in mediating the peripheral lipid control by the CNS.

Inhibition of hepatic VLDL-TG secretion is one of the therapeutic approaches to lower plasma lipid levels and attenuate the progression of cardiovascular disease in obesity and diabetes.\textsuperscript{41} Consistent with previous findings,\textsuperscript{12,31} we first...
confirmed that short-term high-fat feeding leads to hyperphagia and hypersecretion of VLDL-TG. Importantly, direct activation of NMDA receptor in the DVC by glycine lowers and normalizes VLDL-TG secretion in high-fat fed rats. In the same high-fat fed model, hypothalamic glucose sensing fails to lower hepatic VLDL-TG secretion. However, this central defect of hypothalamic glucose sensing is bypassed when the downstream hypothalamic lactate metabolic pathway is activated. Although the hypolipidemic effect induced by CNS mechanisms remains to be assessed in alternative high-fat diet regimes and obese models, these findings collectively suggest that NMDA-receptor-mediated transmission in the DVC may integrate hypothalamic nutrient signals to lower hepatic VLDL-TG secretion in normal and obese conditions.

In summary, our current study indicates that glycine normalizes high-fat diet-induced hypersecretion of hepatic VLDL-TG by triggering the NR1-containing NMDA-receptor-mediated neuronal transmission in the DVC. Although it remains to be assessed whether CNS glycine sensing can restore peripheral lipid homeostasis in alternative models of the metabolic syndrome, in light of our current findings and the fact that dietary glycine intake has been documented to lower plasma TG levels in rats, these data together suggest that glycine, or a glycine analog, may have therapeutic benefits to lower plasma lipid levels in diabetes and obesity by triggering the CNS.

Acknowledgments

The authors are grateful to Penny Y.T. Wang for her technical assistance in assaying plasma triglycerides, free fatty acids, and hormones as well as plasma and DVC glycine levels.

Sources of Funding

This research was funded by a research grant to Tony K.T. Lam from the Canadian Institutes of Health Research (MOP-50594), Jessica Y. Yue is supported by a postdoctoral fellowship from the University Health Network and Banting and Best Diabetes Centre (BBDC), University of Toronto. Patricia I. Mighiu is supported by an Ontario Graduate Scholarship and a scholarship from the BBDC. Tony K.T. Lam holds the John Kitson McIvor (1915–1942) Endowed Chair in Diabetes Research and the Canada Research Chair in Obesity at the Toronto General Research Institute and the University of Toronto.

Disclosures

None.

References


---

**Novelty and Significance**

**What Is Known?**

- The hypothalamus senses nutrients and hormones to regulate hepatic lipid metabolism.
- Hypothalamic sensing mechanisms fail to regulate hepatic lipid metabolism in diet-induced obese rodents.

**What New Information Does This Article Contribute?**

- Glycine potentiates N-methyl-D-aspartate (NMDA) receptor-mediated transmission in an extrahypothalamic region termed dorsal vagal complex (DVC) to inhibit hepatic triglyceride-rich very low-density lipoproteins (VLDL-TG) via the hepatic vagus.
- Glycine normalizes high-fat diet-induced hypersecretion of VLDL-TG by triggering the NMDA receptors in the DVC.

Dyslipidemia in diabetes and obesity is partly due to an increase of VLDL-TG secretion, but the underlying regulatory mechanisms of VLDL-TG secretion remain elusive. Here, we report that the smallest amino acid, glycine, triggers neurotransmission in the central nervous system to inhibit VLDL-TG secretion in normal rats. More importantly, glycine normalizes the hypersecretion of VLDL-TG induced by high-fat feeding by triggering neurotransmission. These findings highlight the therapeutic potential of dietary glycine or glycine analogues to lower blood lipid concentrations in individuals with obesity and type 2 diabetes.
Glycine Normalizes Hepatic Triglyceride-Rich VLDL Secretion by Triggering the CNS in High-Fat Fed Rats

Jessica T.Y. Yue, Patricia I. Migliu, Mark Naples, Khosrow Adeli and Tony K.T. Lam

Circ Res. published online April 3, 2012;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/04/03/CIRCRESAHA.112.268276

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/04/03/CIRCRESAHA.112.268276.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL
Online Figure I. Dose-response curves demonstrating the effect of various DVC glycine doses (white (□), 1 mmol/L; black (■), 10 mmol/L, n=12; light grey (○), 100 mmol/L, n=4; dark grey (●), 1000 mmol/L, n=3) on: (A) plasma TG and (B) rate of appearance of VLDL-TG following tyloxapol administration. DVC glycine doses at 100 and 1000 mmol/L did not significantly lower the tyloxapol-induced increase in plasma TG or rate of VLDL-TG appearance further than the reduction achieved with 10 mmol/L glycine. DVC glycine (black (■)) did not affect the hepatic mRNA expression of (C) microsomal transfer protein (mtp) or (D) sterol regulatory element-binding protein-1 (srebp1) relative to saline controls (white (□)). (E) Representative immunoblot of plasma apoB48 before (t=0 min) and after (t=150 min) tyloxapol administration. (F) DVC glycine demonstrated a tendency (P=0.06) to lower the tyloxapol-induced increase of plasma apoB48 levels, but co-infusion of MK-801 with glycine did not reverse this trend. White with light grey diagonal stripes represents a vehicle control consisting of saline and MK-801 only groups; black (■), glycine; grey (●), glycine + MK-801. (G) Plasma glucose levels were unchanged 150 min following tyloxapol administration in rats given DVC vehicle (white (□)) or glycine (black (■)). Data are presented as mean ± SEM. *P<0.05 vs DVC glycine 1 mM.
Online Figure II. Following tyloxapol administration at t=0, DVC serine (white □; 10 mmol/L, n=5), a co-agonist of the NR1 subunit of the NMDA receptor: (A) lowered plasma TG and (B) decreased the rate of appearance of VLDL-TG in plasma compared with a group given a simultaneous infusion of serine (10 mmol/L) with NR1 subunit antagonist 7CKNA (30 mmol/L; black ■, n=5). (C) Representative immunoblot of plasma apoB48 before (t=0 min) and after (t=150 min) tyloxapol administration in rats given DVC 7CKNA only (30 mmol/L; white □), glycine (10 mmol/L; black ■), or glycine (10 mmol/L) + 7CKNA (30 mmol/L; grey □). (D) DVC glycine demonstrated a tendency (P=0.08) to lower the tyloxapol-induced increase of plasma apoB48 levels compared to DVC 7CKNA alone, but co-infusion of 7CKNA with glycine did not reverse this trend. Data are presented as mean ± SEM. *P<0.05 DVC serine vs DVC serine+7CKNA.
**Online Table I.** Body weights (g) of experimental animals on the experimental day before any treatments. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>330 ± 19</td>
</tr>
<tr>
<td>MK-801</td>
<td>299 ± 8</td>
</tr>
<tr>
<td>glycine</td>
<td>327 ± 6</td>
</tr>
<tr>
<td>glycine+MK-801</td>
<td>284 ± 13</td>
</tr>
<tr>
<td>7CKNA</td>
<td>297 ± 25</td>
</tr>
<tr>
<td>glycine</td>
<td>327 ± 6</td>
</tr>
<tr>
<td>glycine+7CKNA</td>
<td>293 ± 10</td>
</tr>
<tr>
<td>MM+glycine</td>
<td>288 ± 12</td>
</tr>
<tr>
<td>NR1 shRNA + glycine</td>
<td>287 ± 5</td>
</tr>
<tr>
<td>sham Vx + saline</td>
<td>328 ± 8</td>
</tr>
<tr>
<td>hep Vx + saline</td>
<td>332 ± 7</td>
</tr>
<tr>
<td>sham Vx + glycine</td>
<td>318 ± 6</td>
</tr>
<tr>
<td>hep Vx + glycine</td>
<td>308 ± 12</td>
</tr>
<tr>
<td>HFD + saline</td>
<td>323 ± 9</td>
</tr>
<tr>
<td>HFD + glycine</td>
<td>339 ± 5</td>
</tr>
<tr>
<td>HFD + glycine + MK-801</td>
<td>326 ± 16</td>
</tr>
<tr>
<td>1 uM glycine</td>
<td>325 ± 11</td>
</tr>
<tr>
<td>10 uM glycine</td>
<td>327 ± 6</td>
</tr>
<tr>
<td>100 uM glycine</td>
<td>311 ± 12</td>
</tr>
<tr>
<td>1000 uM glycine</td>
<td>321 ± 18</td>
</tr>
<tr>
<td>serine + 7CKNA</td>
<td>334 ± 15</td>
</tr>
<tr>
<td>serine</td>
<td>344 ± 15</td>
</tr>
</tbody>
</table>
Online Table II. Average caloric intake (kCa) per day during the period of diet change (i.e. 3 d leading to tyloxapol experiments) in rats fed with regular chow (reg chow) or high-fat diet (HFD). Caloric intake was ~2.5 fold greater in both HFD groups as compared to their reg chow counterparts. Data are presented as mean ± SEM. †P<0.05, reg chow + saline vs HFD + saline. ‡P<0.05, HFD + saline vs HFD + glycine. §P<0.05, reg chow + glycine vs HFD + glycine. ¶P<0.05, reg chow + saline vs HFD + glycine + MK-801.

<table>
<thead>
<tr>
<th></th>
<th>average caloric intake per day (kCa/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>reg chow + saline</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>reg chow + glycine</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>HFD + saline</td>
<td>153 ± 13 †</td>
</tr>
<tr>
<td>HFD + glycine</td>
<td>170 ± 6 ‡§</td>
</tr>
<tr>
<td>HFD + glycine + MK-801</td>
<td>124 ± 16 ¶¶</td>
</tr>
</tbody>
</table>