Even after 5 days in culture, no tubules were formed and only rudimentary branching of the ureter bud was seen. The corresponding sense and non-sense phosphothionate oligonucleotides did not affect NGFR expression, or growth and differentiation of the kidneys. Removal of the anti-sense oligonucleotides after 2 days in the cultures restored normal nephric differentiation (*19*). Thus, depletion of NGFR specifically perturbed epithelial differentiation of the nephrons. Although the ureter bud did not express NGFR (Fig. 1), its branching was inhibited by anti-sense oligonucleotides, but not by control oligonucleotides. Therefore, inhibition of NGFR expression in nephrogenic tissue also perturbs epithelio-mesenchymal tissue interactions that govern ureter bud branching.

The results show that NGFR expression is required for the formation of epithelial kidney tubules. NGFR is widely distributed in embryonic tissues derived from all germ layers (*7, 8*), so signal transduction by NGFR may be important in differentiation of a variety of organs, as well as of the nervous system.

REFERENCES AND NOTES

1. Y.-A. Barde, *Neuron* **2**, 1525 (1989); J. Leibrock *et al.*, *Nature* **341**, 149 (1989); P. Ernfors *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5454 (1990); A. Hohn, J. Leibrock, K. Bailey, Y.-A. Barde, *Nature* **344**, 339 (1990); P. C. Maisonpierre *et al.*, *Science* **247**, 1446 (1990); A. Rosenthal *et al.*, *Neuron* **4**, 767 (1990); H. Thoenen, *Trends Neurosci.* **14**, 165 (1991).
2. A. Rodriquez-Tébar, G. Dechant, Y.-A. Barde, *Neuron* **4**, 487 (1990).
3. M. Taniuchi, H. B. Clark, E. M. Johnson, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4094 (1986); Q. Yan and E. M. Johnson, *J. Neurosci.* **8**, 3481 (1988).
4. P. G. Chesa, W. J. Rettig, T. M. Thomson, L. J. Old, M. J. Melamed, *J. Histochem. Cytochem.* **36**, 383 (1988). Mesangial cells express NGFR in the adult kidney. Neurotrophin 3 is expressed in glomeruli, which suggests an autocrine-paracrine function for neurotrophin 3 [P. Enfors *et al.*, *Neuron* **5**, 511 (1990)].
5. U. Otten, P. Erhard, R. Peck, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10059 (1989).
6. H. Persson *et al.*, *Science* **247**, 704 (1990); P. Ernfors *et al.*, *Neuron* **1**, 983 (1988).
7. P. Ernfors *et al.*, *Int. J. Dev. Neurosci.* **9**, 57 (1991).
8. J. G. Heuer, S. Fatemie-Nainie, E. F. Wheeler, M. Bothwell, *Dev. Biol.* **137**, 287 (1990).
9. C. J. Grobstein, *Exp. Zool.* **130**, 319 (1955).
10. L. Saxén, *Organogenesis of the Kidney* (Cambridge Univ. Press, Cambridge, 1987), pp. 1–173.
11. H. Sariola, P. Ekblom, S. Henke-Fahle, *Dev. Biol.* **132**, 271 (1989).
12. A. Sutter, R. J. Riopelle, R. M. Harris-Warrick, E. M. Shooter, *J. Biol. Chem.* **254**, 5972 (1979).
13. N. R. Woodruff and K. E. Neet, *Biochemistry* **25**, 7956 (1986).
14. D. R. Kaplan *et al.*, *Science* **252**, 554 (1991); D. R. Kaplan *et al.*, *Nature* **350**, 158 (1991); B. L. Hemstead *et al.*, *ibid.*, p. 678 (1991); R. Klein *et al.*, *Cell* **65**, 189 (1991); A. R. Nebreda *et al.*, *Science* **252**, 558 (1991).
15. S. H. Green, R. E. Rydel, J. L. Connolly, L. A. Greene, *J. Cell Biol.* **102**, 830 (1986).
16. P.-M. Grob, A. H. Ross, H. Koprowski, H. J. Bothwell, *J. Biol. Chem.* **260**, 8044 (1985).
17. S. O. Meakin and E. M. Shooter, *Neuron* **6**, 153 (1991).
18. B. Bowen, J. Steinberg, U. K. Laemmli, H. Weintraub, *Nucleic Acids Res.* **8**, 1 (1980).
19. H. Sariola *et al.*, unpublished results.
20. D. G. Wilkinson, G. Peters, C. Dickson, A. P. McMahon, *EMBO J.* **7**, 691 (1988). Formamide (60%) was used in the hybridization mixture. Biotin-streptavidin immunoperoxidase kit was obtained from Zymed Laboratories, and MAb against NGFR from Oncogene Science (diluted 1:10 and incubated overnight at 4°C).
21. A.-M. Partanen and I. Thesleff, *Dev. Biol.* **120**, 186 (1987). Microsurgically separated kidneys were incubated in iodinated βNGF (5 ng/ml) (Amersham) for 90 min at room temperature, washed at 4°C, fixed in 3% paraformaldehyde, processed for autoradiography, and exposed for 12 days.
22. N. V. Costrini and R. A. Bradshaw, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3242 (1979).
23. M. Hosang and E. M. Shooter, *J. Biol. Chem.* **260**, 655 (1985).
24. A. A. Karavanov, *Experientia (Basel)* **39**, 1015 (1983). The nitrocellulose strip was incubated in 5 ml of binding buffer (150 mM NaCl, 1 mM EDTA, 10 mM tris-HCl, pH 7.5, 0.3% bovine serum albumin, 0.05% Ficoll, 0.02% polyvinyl pyrrolidone) with 5 nM iodinated βNGF. In competition experiments, 1000-fold excess unlabeled βNGF was added to the binding medium. The binding step was followed by four 1-hour washes in 100 ml of binding buffer with 0.1% of Triton X-100. Autoradiography was overnight at −70°C with the use of Kodak X-Omatic AR film and intensifying screens.
25. M. Wilkinson, *Nucleic Acids Res.* **16**, 10933 (1988).
26. T. M. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982). The blots were hybridized with ^{32}P-labeled rat NGFR cDNA in a Hybrid-Ease Hybridization Chamber (Hoefer Scientific Instruments), as directed by the manufacturer, washed at 65°C, and exposed in Kodak X-Omatic cassette with intensifying screens at −70°C.
27. B. C. Froehler, P. G. Ng, M. D. Mattencci, *Nucleic Acids Res.* **14**, 5399 (1986); M. Bengtström and L. Paulin, *Nucleic Acids Symp. Ser.*, in press. The medium (Eagle's minimun essential medium with 10% fetal calf serum) and oligonucleotides were changed daily. Phosphothionate anti-sense oligonucleotides complementary to 5′-, middle, and 3′-sequences of NGFR mRNA were first tested. Each of them inhibited expression of NGFR in the kidney cultures, but to a different extent. Complete inhibition of NGFR expression was achieved with the anti-sense oligonucleotide to 3′-sequence; this oligonucleotide was therefore used in all subsequent experiments. Comparison of oligonucleotide sequences to the GenEMBL sequence data bank did not reveal any sequences with greater than 70% homology. For whole mount immunostaining, the cultured kidneys were fixed in cold methanol, stained overnight with a MAb to cytokeratin 8 (Amersham), washed in phosphate-buffered saline, incubated overnight in fluorescein-conjugated secondary antibodies (Dakopatts), and washed and mounted in Elvanol.

28. We thank L. Saxén and S. Gilbert for their comments on the manuscript, A. Tuomi and B. Tjäder for technical assistance, and L. Paulin for synthesis of the oligonucleotides. Supported by grants of Sigrid Jusélius Foundation and Finnish Cultural Foundation.

29 April 1991; accepted 1 August 1991

Quantitation of Hepatic Glycogenolysis and Gluconeogenesis in Fasting Humans with ^{13}C NMR

DOUGLAS L. ROTHMAN, INGER MAGNUSSON, LEE D. KATZ, ROBERT G. SHULMAN, GERALD I. SHULMAN*

The rate of net hepatic glycogenolysis was assessed in humans by serially measuring hepatic glycogen concentration at 3- to 12-hour intervals during a 68-hour fast with ^{13}C nuclear magnetic resonance spectroscopy. The net rate of gluconeogenesis was calculated by subtracting the rate of net hepatic glycogenolysis from the rate of glucose production in the whole body measured with tritiated glucose. Gluconeogenesis accounted for 64 ± 5% (mean ± standard error of the mean) of total glucose production during the first 22 hours of fasting. In the subsequent 14-hour and 18-hour periods of the fast, gluconeogenesis accounted for 82 ± 5% and 96 ± 1% of total glucose production, respectively. These data show that gluconeogenesis accounts for a substantial fraction of total glucose production even during the first 22 hours of a fast in humans.

HEPATIC GLUCONEOGENESIS AND glycogenolysis are essential processes for maintaining plasma glucose during fasting. The relative contributions of these processes to glucose production have been difficult to quantify in humans. The rate of gluconeogenesis has been estimated by measuring the incorporation of isotopically labeled gluconeogenic precursors into plasma glucose or by measuring net splanchnic uptake of gluconeogenic substrates (*1*). Both methods rely on multiple assumptions of uncertain validity (*2*).

The rate of net hepatic glycogenolysis in humans during a 68-hour fast was measured by means of ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The advantage of ^{13}C NMR spectroscopy over existing techniques is that hepatic glycogen concentration can be measured noninvasively at multiple time points (*3*). We calculated the rate of net hepatic glycogenolysis by multiplying the

D. L. Rothman and I. Magnusson, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06510.
L. D. Katz, Department of Diagnostic Radiology, Yale University School of Medicine, New Haven, CT 06510.
R. G. Shulman, Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06510.
G. I. Shulman, Departments of Internal Medicine and Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06510.

*To whom correspondence should be addressed at the Department of Internal Medicine.

Table 1. Metabolic and hormone data. All units expressed in mean ± SEM (n = 7).

Item	15 hours	40 hours	64 hours
Body mass (kg)	72 ± 2†		69 ± 2**
Liver volume (liter)	1.49 ± 0.13†		1.12 ± 0.08**
Glucose (mmol/liter)	4.7 ± 0.1	3.7 ± 0.2*	3.5 ± 0.1*
Insulin (μU/ml)	11 ± 1	7 ± 1	8 ± 1
Glucagon (pg/ml)	75 ± 17	139 ± 13*	172 ± 21*
Cortisol (μg/dl)	19 ± 2	20 ± 2	21 ± 3
Urinary nitrogen (mmol/24 hours)	1012 ± 156	1066 ± 50	829 ± 88
RQ	0.89 ± 0.02	0.80 ± 0.02	0.79 ± 0.02*
Glycogen (μmol/ml)	251 ± 30	66 ± 9*	42 ± 9*

*p < 0.05 compared with t = 15 hours by means of analysis of variance in conjunction with the Student–Newman-Kuel test. **p < 0.05 compared with t = 0.5 hours by means of paired t test. †Measured at t = 0.5 hours. ‡Measured at t = 67.5 hours.

change in liver glycogen concentration for a given time interval by the volume of the liver. We obtained the net rate of whole body gluconeogenesis by subtracting the glycogenolytic rate from the rate of whole body glucose production measured by [6-^{3}H]glucose turnover.

Seven healthy volunteers (six male and one female, aged 20 to 26 years; body mass index ± SEM; 22.7 ± 0.5 kg/m^2) were fed a standard high carbohydrate diet (40 to 45 kcal/kg per day; 60% carbohydrate, 20% protein, 20% fat) for 3 days before the fast. At 5:00 p.m. [time (t) = 0] on day 3, they ingested a liquid meal consisting of 650 kcal (60% carbohydrate, 20% protein, 20% fat) in less than 5 min. The subjects then fasted for 68 hours, during which period they were given unlimited access to drinking water. The ^{13}C NMR measurements of glycogen concentration in the liver were performed after 4, 7, 10, 13, 16, 22, 28, 40, 46, 52, and 64 hours (*4*). The volume of the liver was measured with magnetic resonance imaging on all subjects after 0.5 and 67.5 hours (*5*). Rates of total glucose production were assessed after administration of a primed continuous infusion of [6-^{3}H]glucose at 22, 43, and 67 hours of fasting (*6*). After the 68 hours of fasting, subjects were given unlimited access to food (mean intake was ~2000 kcal; 47% carbohydrate, 23% protein, 30% fat). We obtained the final measurement of glycogen concentration in the liver 3 hours later to estimate rates of liver glycogen repletion. During the fast, the plasma glucose concentration as well as the respiratory quotient (RQ) decreased, reflecting an increase in fat use (Table 1). Liver volume also decreased by 23 ± 4% (± SEM) after 67 hours of fasting.

The ^{13}C NMR spectra of the C1 position of liver glycogen obtained from one of the subjects during the fast are shown in Fig. 1. The SD in the ^{13}C NMR glycogen concentration measurement due to spectral noise was 13 μmol/ml (*4*). (Throughout this paper, the glycogen concentration is given in micromoles of glycogen per milliliter of liver volume.) Four hours after the subjects ingested the standard meal (t = 4), the average concentration of glycogen in the liver was 396 ± 29 μmol/ml, which is comparable to values obtained by analyses of liver biopsies (*7*). During the fast, liver glycogen concentration decreased at an almost linear rate for the first 22 hours (Fig. 2). Subsequently the rate of glycogenolysis declined, and average glycogen concentrations were 66 ± 9 μmol/ml after 40 hours and 42 ± 9 μmol/ml after 64 hours. After 68 hours, subjects were refed, and the liver glycogen increased over the next 3 hours in each subject to a mean value of 188 ± 19 μmol/ml, yielding an approximate mean net glycogenic rate of 0.8 ± 0.1 μmol/ml per minute (range 0.4 to 1.2 μmol/ml per minute or 14 ± 2 μmol/kg per minute). (Throughout this paper, rates given in micromoles per kilogram refer to kilograms of body mass.)

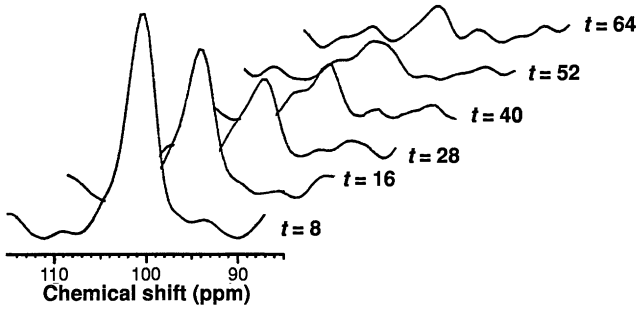

Fig. 1. Staggered plot of the ^{13}C NMR spectra of the C1 position of hepatic glycogen obtained at the indicated hours from one of the subjects during 64 hours of fasting.

The mean rate of glycogenolysis was relatively constant during the first 22 hours of fasting in the present study (4.0 ± 1.2 μmol/kg per minute or 0.19 ± 0.06 μmol/ml per minute from 4 to 13 hours and 4.3 ± 0.6 μmol/kg per minute or 0.20 ± 0.02 μmol/ml per minute from 4 to 22 hours) (Table 2). At t = 22 hours, we calculated that the rate of net hepatic glycogenolysis accounted for only 36% (range 19 to 54%) of total glucose production (*8*). Assuming the same rate of overall glucose production during the initial 4 to 13 hours of the fast, the fractional contribution of glycogenolysis to overall glucose production was the same even for this earlier interval. As the fast progressed, the rate of glycogenolysis decreased, and after 42 hours gluconeogenesis accounted for essentially all of the glucose production. The initial glycogenolytic rate was somewhat lower than the rate of 0.30 μmol/ml per minute determined in repetitive liver biopsies (*7*). In that study, the glycogenolytic rate measurements were performed only during the first 4 hours after an overnight fast and may therefore differ from our measurements, which were performed every 3 to 6 hours during the first 24 hours of fasting. Furthermore, release of epinephrine during biopsy measurements might increase the glycogenolytic rate.

The contribution of gluconeogenesis to total net glucose production that we observed in the early phase of a fast are in contrast to those from earlier studies in which gluconeogenesis was found to contribute less than 35% of glucose production at 12 to 14 hours of fasting (*7, 9, 10*). It is possible that the relatively small size of the evening meal (650 kcal) before the fast in our study contributed to the higher gluconeogenic estimate in our studies. To the extent that glucose cycling occurs in the liver and kidney, our measurements may overestimate rates of net hepatic and renal glucose production, which would cause us to overestimate the fractional contribution of gluconeogenesis to total net glucose production. Studies in conscious dogs found rates of glucose appearance measured with [3-^{3}H]glucose that were 30% higher than rates of net hepatic glucose production measured with arteriovenous-difference techniques (*11*). Even if we adjusted our calculated rates of glucose appearance by this amount, gluconeogenesis would still account for 50% of overall glucose production during the initial 4 to 22 hours (and 4 to 13 hours) of fasting. However, our rates of glucose production (12.2 ± 0.9 μmol/kg per minute) agree well with rates of splanchnic glucose production measured with arteriovenous techniques (13.3 ± 0.2 μmol/kg per minute) (*9*), suggesting insignificant

Table 2. Mean rates of total glucose production, net hepatic glycogenolysis, and gluconeogenesis. All units expressed in mean ± SEM (n = 7). Ranges shown in parentheses.

Item	0 to 22 hours	22 to 46 hours	46 to 64 hours
Glucose production	12.2 ± 0.9	8.8 ± 0.5*	8.7 ± 0.4*
(μmol/kg per minute)	(8.6 to 16.1)	(6.6 to 10.5)	(6.9 to 10.4)
Hepatic glycogenolysis	4.3 ± 0.6	1.7 ± 0.5*	0.3 ± 0.6*
(μmol/kg per minute)	(2.2 to 7.5)	(0.4 to 4.3)	(0.0 to 0.5)
Gluconeogenesis	7.9 ± 1.0	7.1 ± 0.5	8.3 ± 0.5
(μmol/kg per minute)	(5.0 to 12.7)	(5.0 to 8.5)	(6.4 to 10.4)
Glucose production (%)	64 ± 5	82 ± 5*	96 ± 1*
from gluconeogenesis	(46 to 81)	(54 to 95)	(94 to 100)

*p < 0.05 compared with 0 to 22 hours by means of analysis of variance.

glucose production by the kidney and minimal loss of the tritium label in [6-^{3}H]glucose because of recycling.

In the repetitive biopsy study, glycogenolysis was calculated to account for between 57 and 66% of assumed total glucose production (7); however, liver volume was not measured. If the earlier results are recalculated with the average liver volume measured initially in this study, which was 20% smaller than the assumed liver volume (1.8 liters) (7), the glycogenolytic contribution to glucose production in the whole body is 47 to 56%, which is similar to our findings.

A study of the uptake of gluconeogenic substrates across the splanchnic bed indicated that gluconeogenesis contributed only 20% of total glucose production during the first 12 to 14 hours of fasting (*9*). This estimate may have been low for several reasons: (i) Release of gluconeogenic substrate from the gut during a fast would not be detected by the splanchnic catheterization technique. However, studies in fasting dogs (*12*) and humans (*13*) in which portal vein blood was obtained suggest that there is relatively little gluconeogenic substrate release from the gut. (ii) Glucose production from renal gluconeogenesis (*14*) would not be detected by the splanchnic catheterization technique, resulting in an underestimation of the rate of total gluconeogenesis. However, renal contribution to wholebody glucose production in humans is minor in the overnight fasted state and only becomes important during prolonged fasting (*15*). (iii) The splanchnic catheterization method does not take into consideration intrahepatic proteolysis, which might contribute substrates to gluconeogenesis. The 23% decrease in liver volume that we observed during 64 hours of fasting (Table 1) might reflect loss in volume due to intrahepatic proteolysis as well as glycogenolysis.

The rate of gluconeogenesis has also been quantified (*10*) in humans with an isotopic method (*2*) in which researchers infused [2-^{14}C]acetate into subjects to monitor gluconeogenesis and allow calculation of Krebs cycle carbon exchange. They calculated that gluconeogenesis accounted for only 28 ± 2% of total glucose production after an overnight fast (*10*). However, labeled acetate has been shown to be an unsuitable substrate for quantitation of gluconeogenesis in vivo because acetate is metabolized in extrahepatic tissues (*2*). Also, the method does not take into consideration dilution of label by intrahepatic proteolysis, and both these factors could result in an underestimation of the rate of gluconeogenesis.

We found that, in contrast to other studies, gluconeogenesis contributed substantially to overall glucose production in humans even during the initial 22 hours of a fast. During refeeding after an overnight fast, approximately half of the newly synthesized liver glycogen is replenished by gluconeogenesis (*16*). Together, these results suggest that hepatic gluconeogenesis is always operating at an appreciable rate in humans.

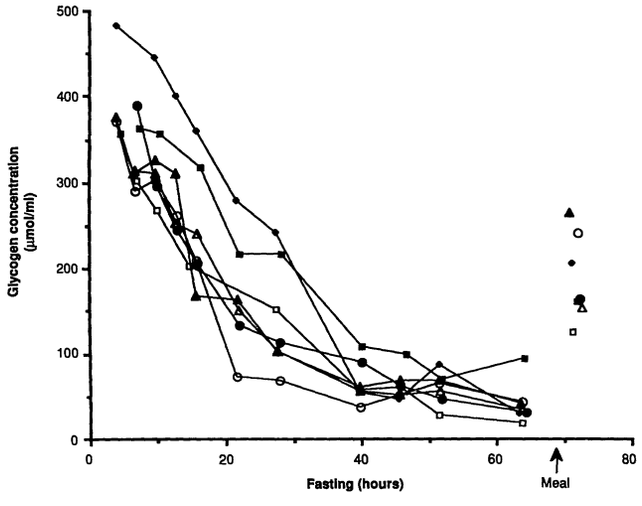

Fig. 2. The ^{13}C NMR measurements of liver glycogen concentration in seven subjects during 64 hours of fasting and 3 hours after refeeding with a standard meal (t = 68 hours).

REFERENCES AND NOTES

1. E. Weinman, E. Strisower, I. Chaikoff, *Physiol. Rev.* **37**, 252 (1952); G. Hetenyi, Jr. in *Hormonal Control of Gluconeogenesis*, N. Kraus-Freidman, Ed. (CRC Press, Boca Raton, FL, 1986), pp. 79–96; A. D. Cherrington and M. Vranic, *ibid.*, pp. 15–37.
2. H. A. Krebs, R. Hems, M. J. Weidemann, R. N. Speake, *Biochem. J.* **101**, 242 (1966); E. Strisower, G. Kohler, I. Chaikoff, *J. Biol. Chem.* **198**, 115 (1952); G. Hetenyi, Jr., and C. Ferrarotto, *Biochem. Med.* **29**, 372 (1983); J. Katz, *Am. J. Physiol.* **248**, R391 (1985); W. C. Schumann *et al.*, *J. Biol. Chem.* **266**, 6985 (1991).
3. T. Jue *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4489 (1989).
4. Spectra were obtained with a 9-cm circular ^{13}C observation coil and a 12 cm by 14 cm coplanar butterfly ^{1}H-decoupler coil placed rigidly over the lateral aspect of the liver in the supine subject. We determined initial coil placement by percussing the borders of the liver. The magnetic field homogeneity was optimized with the water signal obtained from the decoupling coil. We confirmed the position of the coil over the liver by imaging the liver from the surface coil with a multislice gradient echo image [A. Haase *et al.*, *J. Magn. Reson.* **67**, 258 (1986)]. Localized ^{13}C NMR liver spectra were obtained with a modified one-dimensional inversion-based sequence [R. J. Ordidge, A. Connelly, J. A. B. Lohman, *ibid.* **66**, 283 (1986)]. We applied a 4-ms phase-swept hyperbolic secant inversion pulse (frequency bandwidth, 4000 Hz, μ = 3) [M. S. Silver *et al.*, *Nature* **310**, 681 (1984)] in a gradient parallel to the coil axis at the sample surface and used the pulse to invert the signal from tissues within 2.5 cm of the surface. We eliminated the surface signal by adding scans obtained with an inversion pulse to scans obtained without the inversion. On the basis of ^{1}H-density profiles of the sequence, less than 15% of the glycogen signal could arise from tissues within 2.5 cm of the surface of the subject, assuming equal glycogen concentration throughout the volume of observation. In each subject, all skin and muscle in the coil field of view was within 2.5 cm of the surface, as assessed by the image. Signal excitation was obtained with a 500-W excitation pulse set to a 180° pulse angle in the plane of the coil. The pulse angle at coil center was determined from a 2-cm-diameter sphere containing [^{13}C]formic acid (99% enriched) as a standard. Spectra were obtained with a 150-ms repetition rate, which is approximately twice the longitudinal relaxation time for the C1 resonance of glycogen [L. H. Zang, M. R. Laughlin, D. L. Rothman, R. G. Shulman, *Biochemistry,* **29**, 6815 (1990)]. We applied ^{1}H single-frequency decoupling at 15 W to the C1 proton resonance during the 25.6-ms signal acquisition period. Each measurement required 30 min of signal averaging. Heating from decoupling radio frequency was calculated with a magnetic vector potential model [P. A. Bottomley, C. J. Hardy, P. B. Roemer, O. M. Mueller, *Magn. Reson. Med.* **12**, 348 (1989)] and was <4 W per kilogram of tissue, which was below Food and Drug Administration limits for local heating. Spectra were processed with a mild 30-Hz Lorentzian to Gaussian filter and a 500-Hz convolution difference. After we processed the glycogen linewidth, it was 70 to 90 Hz. We measured resonance intensity by integrating over a bandwidth of ±120 Hz. We determined the glycogen concentration of liver by comparing the signal intensity obtained with the localized spectroscopy sequence in

vivo with the signal intensity obtained from a volume selected at the same coordinates relative to the coil in a rectangular phantom containing a solution of KCl (50 mM) and glycogen (150 mM). The phantom was raised 2.5 cm above the surface of the coil to simulate liver depth. Spectrometer sensitivity was normalized between phantom and in vivo measurements with the signal from the formate sphere as a calibration standard. To correct for the liver size being smaller than the coil field of view in the y dimension, we obtained an xy image of the pulse-sequence signal volume with a 9-cm ^{1}H surface coil on a phantom filled with water. The liver outlines determined from in vivo imaging and the glycogen phantom outline were superimposed on the pulse-sequence image, and we used the relative integrals to obtain a correction factor. The mean correction factor was 23%, with a range of 3 to 47%. We assessed reproducibility of the glycogen concentration measurement by obtaining 11 pairs of glycogen concentration measurements at 45-min intervals on four subjects. Each of the subjects had fasted between 6 and 12 hours. Subjects were removed from the magnet and probe between measurements. The coefficient of variation between the pairs of measurements was 7%.

5. Clinical imaging of all patients was performed on a 1.5-T magnet (General Electric, Milwaukee, WI). Multiecho axial scanning was performed [total echo time, 20 ms/80 ms (and repetition time, 2 s)]. The data were transferred to an independent work station (I.S.G. Technologies, Mississauga, Canada) and processed with the CAMRA S200 Program (I.S.G. Technologies), which provided three-dimensional reconstruction and volume calculations. Accuracy of the measurement was assessed with water-filled phantoms of known volume and determined to be ±5% with a coefficient of variation of ±1%.
6. Rates of total glucose production were determined at t = 22, 43, and 67 hours by a primed (25 μCi)–continuous (0.25 μCi/min) infusion of [6-^{3}H]glucose for 2.5 hours into a deep ante decubital vein. Blood samples were obtained every 10 min during the last 40 min of infusion from a retrodorsal vein in the hand, which was warmed to 70°C to arterialize the blood samples. Plasma [^{3}H]glucose specific activity was determined after isolation of glucose by ion-exchange chromatography as described [R. Kreisberg, A. Siegal, W. Owen, *J. Clin. Endocrinol. Metab.* **34**, 876 (1972)]. The rate (R_a) of total glucose production was calculated from the equation: R_a = infusion rate of tracer (disintegrations per minute per kilogram per minute)/[6-^{3}H]glucose specific activity (disintegrations per minute per milligram). The protocol was approved by the Yale University Human Investigation Committee and informed consent was obtained from each subject.
7. L. Nilsson and E. Hultman, *Scand. J. Clin. Lab. Invest.* **32**, 325 (1973).
8. We calculated rates of glycogenolysis for each individual by finding the best fit of the glycogen concentration to a line at three intervals: t = 0 to 22, t = 22 to 46, and t = 46 to 64 hours. This slope was multiplied by liver volume, which was determined by magnetic resonance imaging (*5*). This method assumes 100% NMR visibility of the glycogen molecule; if the glycogen molecule is not 100% NMR detectable, the rate of glycogenolysis will be underestimated. However, studies of extracted liver glycogen and excised liver tissue have found the glycogen molecule to give an NMR signal equivalent to the sum of the glucose moieties in the molecule [L. O. Sillerud and R. G. Shulman, *Biochemistry* **22**, 1987 (1983); W. E. Hull, M. Zerfowski, P. Bannasch, Abstracts of the *Sixth Society of Magnetic Resonance in Medicine Meeting*, New York, 17 to 21 August 1987 (Society of Magnetic Resonance in Medicine, Berkeley, CA, 1987), p. 488; R. A. Shalwitz *et al.*, *Magn. Reson. Med.* **5**, 462 (1987); C. L. Jackson and R. G. Bryant, *Biochemistry* **28**, 5024 (1989)]. In addition, two studies that compare ^{13}C NMR measurements of muscle glycogen concentration with biochemical measurements from tissue obtained by biopsy in rabbits [R. Gruetter, T. Prolla, R. G. Shulman, *Magn. Reson. Med.* **20**, 327 (1991)] and humans (R. Taylor, T. Price, D. L. Rothman, R. G. Shulman, G. I. Shulman, in preparation) have found excellent agreement between the two methods. This rate of glycogenolysis represents a net rate because it is possible that a small amount of glycogen synthesis may be simultaneously occurring under these conditions [G. I. Shulman *et al.*, *J. Biol. Chem.* **263**, 5027 (1988)].
9. J. Wahren, P. Felig, E. Cerasi, R. Luft, *J. Clin. Invest.* **51**, 1870 (1972).
10. A. Consoli, F. Kennedy, J. Miles, J. Gerich, *ibid.* **80**, 1301 (1987).
11. B. Adkins, S. Myers, R. Stevenson, P. Williams, A. Cherrington, *ibid.* **79**, 557 (1987).
12. N. N. Abumrad *et al.*, *Am. J. Physiol.* **242**, E398 (1982).
13. O. Björkman, L. S. Eriksson, B. Nyberg, J. Wahren, *Diabetes* **39**, 747 (1990).
14. H. A. Krebs, in *Renal Gluconeogenesis*, vol. 1 of *Advances in Enzyme Regulation*, G. Weber, Ed. (Pergamon, New York, 1963), pp. 385–399.
15. O. Owen, P. Felig, A. P. Morgan, J. Wahren, G. F. Cahill, Jr., *J. Clin. Invest.* **48**, 574 (1969); O. Björkman and P. Felig, *Diabetes* **31**, 516 (1982).
16. J. D. McGarry and J. Katz, *J. Clin. Invest.* **74**, 1901 (1984); I. Magnusson *et al.*, *ibid.* **80**, 1748 (1987); G. I. Shulman *et al.*, *Am. J. Physiol.* **259**, E335 (1990).
17. We thank E. Barrett and A. D. Cherrington for discussions; T. Nixon, R. Mellancamp, M. Luffburrow, and B. Jucker for technical assistance; R. Green and M. Merriam for assistance with magnetic resonance imaging; and the staff of the Yale–New Haven Hospital General Clinical Research Center and metabolic kitchen. Supported in part by NIH grants DK-40936, DK-34576, and MO1-RR-00125-26.

13 March 1991; accepted 30 July 1991

Cloning and Expression of a Cocaine-Sensitive Dopamine Transporter Complementary DNA

SHOICHI SHIMADA, SHIGEO KITAYAMA, CHIEN-LIANG LIN, AMRAT PATEL, ELIZABETH NANTHAKUMAR, PAUL GREGOR, MICHAEL KUHAR, GEORGE UHL*

A rat dopamine (DA) transporter complementary DNA has been isolated with combined complementary DNA homology and expression approaches. The DA transporter is a 619–amino acid protein with 12 hydrophobic putative membrane-spanning domains and homology to the norepinephrine and γ-aminobutyric acid transporters. The expressed complementary DNA confers transport of [^{3}H]DA in *Xenopus* oocytes and in COS cells. Binding of the cocaine analog [^{3}H]CFT {[^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane} to transfected COS cell membranes yields a pharmacological profile similar to that in striatal membranes.

DOPAMINE TRANSPORTERS TERMINATE dopaminergic neurotransmission by Na^+- and Cl^--dependent reaccumulation of DA into presynaptic neurons (*1*). Cocaine and related drugs bind to these transporters in a fashion that correlates well with their behavioral reinforcing and psychomotor stimulant properties; these transporters are thus the principal brain "cocaine receptors" related to drug abuse (*2*).

To find cDNAs that encode members of this neurotransmitter transporter family and that are expressed in brain regions rich in dopaminergic neurons, 500,000 plaques of a size-selected λ-Zap II rat ventral midbrain cDNA library were screened with a radiolabeled oligonucleotide complementary to conserved segments of the norepinephrine (NE) and γ-aminobutyric acid (GABA) transporters and products of polymerase chain reaction (PCR) amplification of brain cDNA with transporter-specific oligonucleotides (*3–5*). Messenger RNA transcribed from one of the 27 hybridization-positive clones, DAT1, conferred consistent cocaine-blockable accumulation of [^{3}H]DA in the *Xenopus* oocyte uptake assay that was more than ten times background levels (*6, 7*).

pDAT1 contains a 3.4-kb cDNA insert with a 1857-bp open reading frame. Assignment of the first ATG as the translation initiation site, on the basis of resemblances to consensus sequences for translational initiation (*8*), results in a protein of 619 amino acids with a nonglycosylated molecular weight of 69,000 (Fig. 1). Hydrophobicity analysis reveals 12 hydrophobic segments long enough to form transmembrane domains. The predicted DAT1 protein lacks an identifiable signal sequence. It displays four potential sites for N-linked glycosylation (Fig. 1). This sequence shows 67% amino acid identity and 81% similarity with the human NE transporter (*3*), 45% identity and 67% similarity with the rat GABA

S. Shimada, S. Kitayama, C.-L. Lin, P. Gregor, Laboratory of Molecular Neurobiology, Addiction Research Center, National Institute on Drug Abuse, Baltimore, MD 21224.
A. Patel and M. Kuhar, Laboratory of Molecular Pharmacology, Addiction Research Center, National Institute on Drug Abuse, Baltimore, MD 21224.
E. Nanthakumar, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224.
G. Uhl, Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21224 and Laboratory of Molecular Neurobiology, Addiction Research Center, National Institute on Drug Abuse, Baltimore, MD 21224.

*To whom correspondence should be addressed.