Rat Insulin Turnover *in Vivo**

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ABSTRACT

Zucker lean and obese rats were injected under pentobarbital anesthesia with ^{125}I-labeled insulin; at timed intervals from 30 to 120 sec, blood samples were extracted and used for the estimation of insulin levels by RIA. A group of rats from each series was maintained under a constant infusion of noradrenaline. For each insulin determination, a duplicate blood sample containing the same amount of insulin as that used in the RIA, but without the radioactive label, was used as a blank for insulin measurement. The radioactivity in these tubes was then used for the measurement of insulin label per ml blood. From plasma label decay curves and insulin concentrations, the in-

sulin pool size, half-life, and rate of degradation were calculated. Obese rats had higher insulin levels (2.43 nM) and showed less effect of noradrenaline than their lean counterparts, in which insulin distribution volume shrank with noradrenaline treatment. The half-life of plasma insulin was similar in all groups (range, 226–314 sec). Pool size and overall degradation rates were higher in obese (198 femtokatals) than in lean rats (28 femtokatals). It is postulated that obese rats synthesize and cleave much more insulin than lean controls despite their higher circulating levels of insulin. (*Endocrinology* **136**: 3871–3876, 1995)

CIRCULATING insulin has, necessarily, a short half-life (1), because of its fast response to hyperglycemic challenges (2), a consequence of its fundamental role in the maintenance of glycemia (3). The insulin response to increases in circulating glucagon (4), catecholamines (5), or amino acids (6–8) is practically immediate. The rate of removal of insulin by the liver is high (9–11), as is the ability of peripheral tissues to inactivate most of the remaining circulating insulin (10, 12, 13). In target tissues, insulin is inactivated by internalization after interaction with specific receptors (14). The main insulin-cleaving agent is insulin:glutathione transhydrogenase (EC 1.8.4.2) (12, 15), probably in addition to other enzymes (16, 17). The overall capacity of the mammal for insulin removal and inactivation is considerable, as is the ability of the endocrine pancreas to release large amounts of insulin on demand. All of these factors combine to establish a fast insulin turnover, subject to wide oscillations depending on physiological condition and environment.

Adequate knowledge of insulin turnover may be important for the study of insulin responses to different stimuli as well as in the understanding of the mechanisms involved in the development of late-onset diabetes, insulin resistance, and obesity, situations in which insulin release and the physiological response to insulin are altered (18–20).

The direct estimation of insulin turnover is complex, and only approximate estimates are available (10, 21–23). The low circulating levels of insulin complicate its direct estimation. This is further compounded by the wide differences in blood insulin concentration within different vessels (2), a hindrance requiring that blood sample sources be as uniform as pos-

sible (*i.e.* arterial blood). The scarce data available on insulin turnover *in vivo* were obtained using kinetic methods and arterio-venous differences to estimate insulin synthesis (22–26), a approach not suitable for many experimental situations.

The rates of removal of insulin, labeled with radioactive iodine, from blood may be used to obtain an approximate estimation of insulin turnover. However, this approach poses the problem of distinguishing between intact insulin and other labeled products of insulin cleavage presumably present in plasma. The most common method of measurement of insulin concentration in blood plasma is radioimmunoanalysis (27), a procedure that makes use of radioactive iodine as a labeling agent bound to insulin tyrosinyl residues. The use of this approach thwarts any further attempt to measure insulin levels, through RIA, in animals previously injected with labeled insulin.

The method presented here has been devised for the short term estimation of insulin turnover *in vivo* by measuring the rate of disappearance of radioiodine-labeled insulin injected into the bloodstream combined with a standard RIA procedure.

Materials and Methods

Materials and animals

Pure recombinant human insulin (Humulin, Sigma Chemical Co., St. Louis, MO) was labeled with ^{125}I [sodium iodide; specific radioactivity, 80.5 gigabecquerels (GBq)/μmol; Amersham, Aylesbury, UK] at initial proportions of 37 megabecquerels (MBq) ^{125}I and 0.4 μg chloramine-T (Sigma)/μg (222 MBq/nmol) insulin (28). Iodination was stopped with sodium metabisulfite (0.4 μg/μg insulin). Labeled insulin was purified through a column of Bio-Gel P6DG (Bio-Rad Laboratories, Richmond, CA) (28). An insulin preparation with a specific radioactivity of 55 MBq/nmol was obtained.

Zucker lean (Fa/?) and obese (fa/fa) male adult rats, weighing 324 ± 12 and 455 ± 24 g, respectively (13–14 weeks old), bred at the Animal Service of the University of Barcelona from heterozygous stock obtained

from Charles River (Wilmington, MA), were used. The animals were housed in individual polypropylene-bottomed cages under standard conditions (lights on from 0800–2000 h; 22–23 C; 70–75% relative humidity) and were fed standard chow pellets (type A04 from Panlab, Barcelona, Spain). A series of six rats was cannulated, under 50 mg/kg BW ip pentobarbital anesthesia) in the left carotid artery (bringing the tip of the cannula just to the heart) and in the right jugular vein with P50 (Clay-Adams, Parsippany, NJ) polyethylene tubes (id, 0.58; od, 0.97 mm). At the end of the experiment, the anesthetized rats were killed by exsanguination. This study was conducted in accordance with the european community principles, guidelines, and procedures for animal experimentation.

The lean and obese groups of rats were further subdivided into two groups; the first was used as control. The animals of the second group received a constant iv infusion of 280 μM noradrenaline (Sigma) through a cannula inserted in the lower cava at a rate of 4 ml/h·kg while under pentobarbital anesthesia. The infusion was performed with a syringe pump and maintained for 10 min. The rats under the effects of noradrenaline infusion were used for the estimation of insulin turnover, as were their untreated controls.

All work on anesthetized animals was carried out in a chamber kept at 32 C and more than 95% relative humidity to avoid the hypothermic effects of anesthesia.

The resistance to a glucose load was investigated in five lean and five obese rats of the same age and weight as those used in the main experiment. The carotid arteries of these rats were chronically cannulated under ether anesthesia using P50 polyethylene cannulas (29). Two days after the surgical procedure, the rats were given an oral load of 1 ml glucose (0.7 g) in water via a plastic stomach cannula. Samples of carotid blood (0.4 ml for lean and 0.2 ml for obese animals) were taken just before and 10, 15, and 30 min after the loading of glucose (0.2 ml for lean and 0.1 ml for obese rats). This blood was used for the measurement of glucose (30) and plasma insulin (31). Figure 1 shows the plasma levels of both.

Insulin turnover measurement

Each rat was injected (in 5–8 sec) through the jugular venous cannula with 18.3 kBq (330 fmol) labeled insulin in 0.1 ml isotonic saline solution. The radioactivity initially present (and that left after the injection) in the syringe was measured with a γ-counter. At timed intervals of 30, 60, 90,
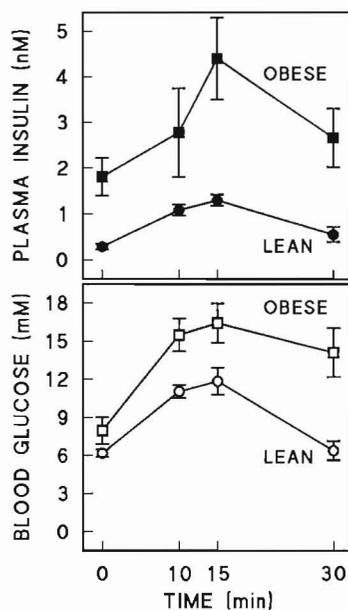
and 120 sec, aliquots of 0.4 ml blood were extracted through the carotid cannula and stored at 4 C in heparinized plastic vials. The blood samples were immediately centrifuged in the cold to separate the plasma samples, which were used directly for labeled insulin estimation.

Plasma insulin levels were estimated by a standard RIA procedure (31) with some minor modifications. Each blood plasma sample was distributed in two tubes; in the first, in addition to the plasma sample (50–100 μl) and 0–50 μl buffer, 100 μl ^{125}I-labeled insulin (74 MBq/nmol; Amersham) solution containing 250 Bq (*i.e.* 3.4 fmol) and 100 μl diluted specific insulin antibody (Amersham) were added. In the remaining tube, the plasma samples (50–100 μl) received 0–50 μl buffer, 100 μl unlabeled insulin (3.4 fmol), and 100 μl of the specific insulin antibody. Thus, the second tube finally contained the same amount of insulin as the first (the amount initially present and that added were the same as that in the first tube), but the amount of labeled insulin present in either was different, as the second tube lacked the added labeled insulin used for the standard RIA procedure. Because the total amount of insulin was the same in both series of tubes, the labeled insulin initially present in plasma bound in the same proportion to the antibody preparation; thus, the second tube could be used as a blank for the first as in a standard RIA procedure. This allowed estimation of the apparent insulin concentration (*i.e.* its binding equivalence to Humulin) regardless of the amount and distribution in molecular species of radioactivity initially present in the plasma. This approach circumvented the problems posed by the presence of radioactive sources (insulin and other) in the samples. The RIA was completed with a series of standards (of both Humulin and rat insulin), blanks, and several tubes for the estimation of nonspecific binding.

As the concentration of insulin in obese rat blood was higher than that in the lean rat blood, the volume of blood extracted allowed for duplicate determinations of all points, whereas lean rats provided just enough plasma for a single determination at each time point.

Calculations

The insulin label present in a given sample of plasma was estimated assuming that labeled insulin was bound by the antibody in the same proportion as unlabeled insulin from the same source. Rat and human insulin bound the antibody differently. However, by using apparent insulin concentrations referred to Humulin standards, the data on insulin radioactivity could be treated as if all insulin present in the rat blood were human insulin. From the RIA data, a plot of insulin bound to the antibody *vs.* the concentration of insulin in the tube was drawn (Fig. 2) using Humulin standards. The data were fitted to an asymmetric sigmoid curve using the FiG-P

FIG. 1. Effect of an oral glucose load on plasma insulin and glucose levels in lean and obese Zucker rats. The data are the mean ± SEM of five different animals per group. The load (0.7 g glucose) was given at time zero.

FIG. 2. Relationship between the human insulin concentration found in the RIA with the proportion of labeled insulin bound to the antibody. The data were fitted to an equation with the formula: $y = Min + (Max - Min)/(1 + [(X/X50)^{-P}])$, where $X50 = 4.525 \times 10^8$, $P = 0.2673$; $Max = 943.017$; $Min = -4.1240$ ($r = 0.992$).

program (Biosoft, Cambridge, UK); the calculated parameters of the curve were used to estimate the percentage of insulin bound to each of the blood samples obtained in the experiment. This percentage also reflected the proportion of insulin radioactivity bound to the antibody; thus, the total amount of insulin radioactivity (r_t) per ml blood at a given time (t) was established for each sample. The r_t values were plotted against time t and fitted to a standard decay graph using the FiG-P program: $r_t = r_0 \times e^{-Kdt}$, from which, K, the decay constant, and r_0, the initial radioactivity per ml blood, were obtained. The half-life ($t_{1/2}$) of insulin was calculated as $t_{1/2} = 1/K$. The ratio of total radioactivity injected (R_0) to r_0 was used to establish the volume (V) of distribution of the injected label (*i.e.* the virtual or practical insulin space): $V = R_0/r_0$. The actual content of human insulin was calculated from total insulin radioactivity and the specific activity of the labeled insulin injected. Human insulin was a maximum of 4–6% of the total blood insulin. Because this proportion was very small, the use of rat insulin standards allowed direct estimation of the rat insulin concentration in the samples. The rat insulin concentration in plasma did not vary during the experiment. The concentration *vs.* time graphs were used to obtain an estimate of the initial (t = 0) insulin concentration (i_0), which was practically identical to the mean of all other time points. As we knew both the virtual distribution volume and concentrations, we could derive the whole mass of circulating insulin (I_0) at time zero: $I_0 = i_0 \times V$. The rate of loss of insulin (rate of degradation, δ) from this circulating pool could be derived from the decay curve and the mass of insulin: $\delta = K \times I_0$. Indeed, because the virtual distribution volume (V) did not change, the insulin mass at a given time (I_t) can be estimated from the plasma concentrations (i_t), and degradation rates for different times (δ_t) may be calculated. The values obtained in all cases were similar, because the changes in insulin concentration during the 2-min analysis were insignificant.

The loss of radioactivity from the labeled insulin pool was studied by establishing the total insulin label values (R_t) at a given time from the radioactivity per ml plasma and the virtual volume of distribution: $R_t = r_t \times V$.

Statistical comparison between groups was established with standard analysis of variance programs and Student's *t* test.

Results

Figure 3 shows the lack of change in plasma insulin levels of the four groups studied during the 2-min analysis of insulin turnover. Zucker obese (fa/fa) rats showed higher insulin levels than lean controls. The infusion of noradrenaline slightly decreased obese rat insulin levels, but in lean animals it did not affect insulin levels.

Figure 4 presents the decay curves for plasma insulin radioactivity *vs.* time. Lean and obese rats showed a similar pattern over time. Noradrenaline treatment, however, resulted in higher radioactivity settings, mainly for lean rats and less for obese, compared with controls. These decay curves were used for the calculation of insulin space, turnover rates and cleavage, shown in Table 1. Insulin levels were higher in obese than in the lean rats. Noradrenaline infusion resulted in no significant change in the insulin pool size and a decrease in virtual insulin space. Obese rats had a higher insulin mass than lean controls. The half-life of insulin was in the same range for all groups and was unaffected by noradrenaline treatment. Insulin degradation rates were higher in obese than in lean rats and were practically unchanged by noradrenaline treatment. These results were maintained even when the data were expressed per unit of animal weight.

Figure 5 depicts the fall in total insulin radioactivity in the plasma pool calculated from the data in Fig. 4 and Table 1. The decay curves thus calculated were fully in line with the

FIG. 3. Plasma insulin concentrations in Zucker lean and obese rats after the injection of labeled insulin. ○ and ●, Lean rats; □ and ■, obese rats; ● and ■, noradrenaline (NA)-treated rats; ○ and □, untreated controls. The data are the mean ± SE of five or six different rats per group. Statistical differences between groups were determined by two-way analysis of variance. There were significant differences between lean and obese groups ($P = 0.000$). The effects of noradrenaline treatment were significant ($P = 0.039$) for obese, but not for lean, rats. The effects of time were not significant ($P > 0.05$) in any case.

zero time values, corresponding to the initial (injected) labeled insulin mass. Lean rats showed a higher loss of absolute insulin radioactivity than the obese; under noradrenaline infusion, the differences between lean and obese rats were minimal in absolute terms.

Discussion

The method presented is conceptually very simple, but requires careful development, especially at the critical point of evaluation of insulin radioactivity in the samples. This is dependent on precise measurements of radioactivity and the construction of a well defined RIA standard curve, from which an equation could be derived. Another critical point is the need for high insulin specific activity; this is essential for the precise RIA estimation of circulating insulin, but it is equally preferable for injection into the rat bloodstream, because the higher the specific activity, the lower the disturbance. In our case, the insulin injected was about 7% of the whole circulating insulin pool in lean controls and 0.6–1% of that in obese rats.

A critical point in the investigation of insulin cleavage is the assignment of radioactivity measurements to intact (*i.e.* fully functional) insulin, without interference by free iodine or labeled fragments freed by the cleavage of insulin. The method presented prevents this interference, as only the label bound to insulin is measured; free iodine and labeled peptides are removed during the RIA procedure. Only labeled, complete, insulin is bound to the antibody, and thus only this

FIG. 4. Insulin radioactivity decay with time in the plasma of Zucker lean and obese rats injected with labeled insulin. Data represent the mean (±SE) radioactivity in 1 ml plasma as a percentage of the total radioactivity injected, found at given times after injection. The curves were fit to exponential decay curves with r values of 0.989 and 0.978 for lean and obese controls, respectively, and 0.994 and 0.979 for lean and obese rats during noradrenaline infusion.
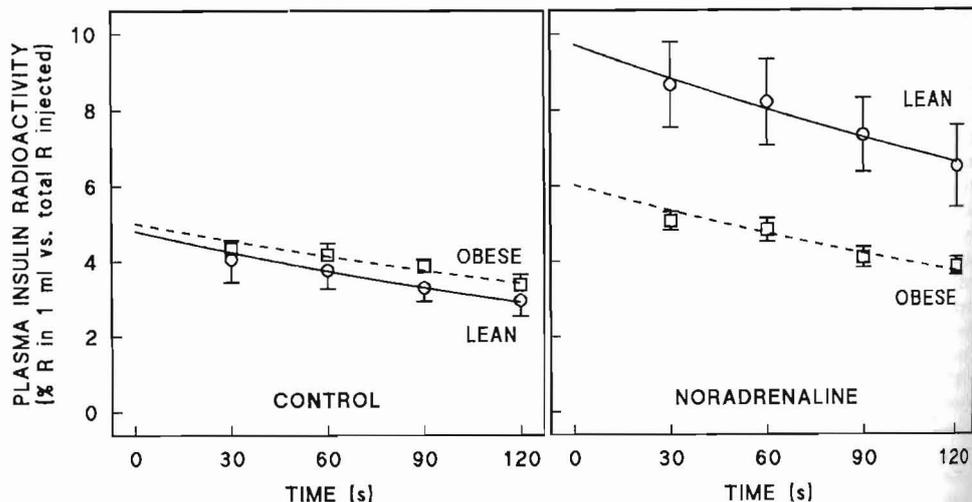
radioactive molecular species is taken into account; this is true for both the insulin injected into the rat and that used in the RIA.

The main difficulty that may arise from a study based on calculated constants taken from calculated values and used to derive the final results is a cumulative effect of residuals in calculations that may lead to widening errors. This study has been designed to minimize this effect. The adequacy of decay curve fitting is apparent in Fig. 3, which shows the loss of radioactive insulin per ml plasma. The data for total insulin radioactivity remaining in the rat were calculated by applying the data derived from these equations and insulin levels. Figure 4 shows the tight fit of these calculated data to decay curve equations. The lack of dispersion of data suggests an acceptable degree of precision in the derivation of the virtual volume of diffusion and decay rates shown in Table 1.

The compartments occupied by the circulating insulin pool are probably not uniform with respect to insulin concentration (22, 24), as this is affected by the varying ability of tissues to extract and inactivate insulin (12, 13). It is difficult to establish the mean representative values of circulating insulin in a given subject, because insulin levels may differ substantially in different blood vessels and under distinct

conditions and times. For this reason, only blood from the same source was analyzed. The carotid cannula allows the extraction of arterial blood just as it leaves the heart. The virtual volume of distribution of insulin derived from data of arterial blood may not be real, because we do not know whether its insulin levels are representative. The insulin pool size (I_t) is more reliable, because it may be derived directly from decay curves and insulin levels.

Virtual insulin space was higher in lean than in obese rats under standard conditions; in any case, this value was higher than the total rat plasma volume, but much smaller than the sum of insulin compartments (24). Obese rats have a lower extracellular space than lean because of large fat deposits. Insulin pool size was much higher in obese than in lean rats, both in absolute terms and in relation to body weight; in the latter case, the differences were somewhat diminished because of the dilution effect of the large fat mass in obese rats.

Hyperinsulinemia is associated with obesity (32), as it is related to higher fat deposition and insulin resistance (33). The apparent changes observed in lean rats as a consequence of catecholamine infusion may be due in part to the shrinkage of insulin virtual space, largely the space occupied by the blood, *i.e.* blood vessels, because of vasoconstriction (34, 35). The significant changes in plasma insulin radioactivity decay

TABLE 1. Comparison of injected labeled insulin turnover in anesthetized lean and obese Zucker rats: effect of noradrenaline infusion

Parameter	Lean control	Lean noradrenaline	Obese control	Obese noradrenaline
Rat wt (g)	339 ± 9	311 ± 21	450 ± 44[a]	461 ± 13[a]
Mean plasma insulin concentration, i (pM)	231 ± 23	297 ± 65	2228 ± 416[a]	1787 ± 460[a]
Insulin pool size				
I_0 (pmol)	5.56 ± 0.49	4.21 ± 0.98	49.7 ± 14.2[a]	31.3 ± 10.3[a]
I_0 (pmol/kg BW)	15.7 ± 1.6	12.7 ± 2.3	107 ± 28[a]	71 ± 25[a]
Virtual vol of insulin distribution				
V (ml)	21.5 ± 2.4	11.4 ± 1.8[b]	19.7 ± 0.9	16.5 ± 1.4
V (% of BW)	6.3 ± 0.6	3.6 ± 0.4[b]	4.5 ± 0.4[a]	3.6 ± 0.3
Insulin half-life, $t_{1/2}$ (sec)	226 ± 15	269 ± 23	314 ± 40	254 ± 28
Insulin degradation rate				
δ (fkat)	27.6 ± 2.9	16.2 ± 3.9	198 ± 54[a]	110 ± 22[a]
δ (fkat/kg BW)	77.6 ± 8.3	45.1 ± 8.2	425 ± 105[a]	245 ± 57[a]

The data are the mean ± SE of five or six animals in each group. See *Materials and Methods* for the calculations and derived magnitudes. Statistical significance of the differences between groups was determined by Student's *t* test.

[a] $P < 0.05$ *vs.* corresponding lean group.

[b] $P < 0.05$ *vs.* corresponding control group.
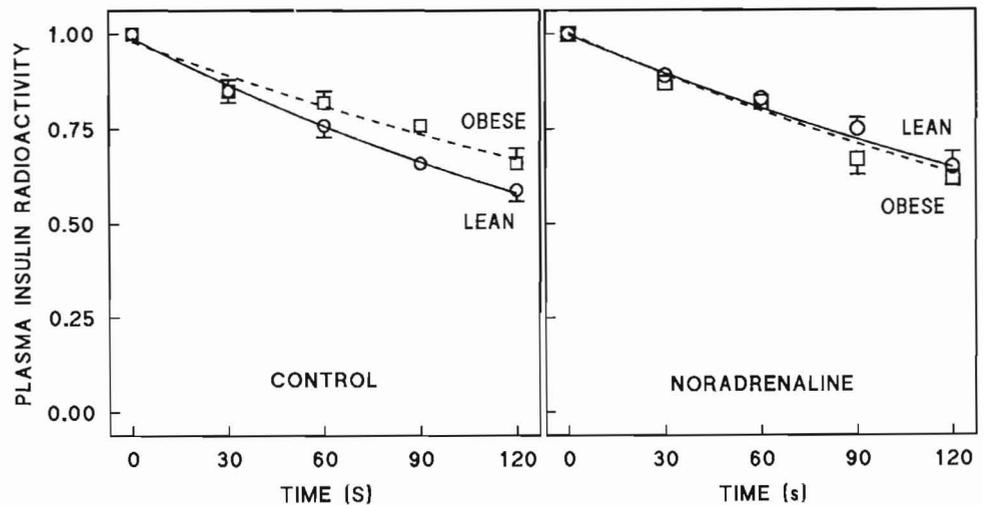
FIG. 5. Total radioactivity present in the plasma insulin pool of Zucker lean and obese rats after the injection of labeled insulin. The data are the mean (±SE) of the quotients between radioactivity found (that in 1 ml plasma multiplied by the virtual volume of distribution) and that injected for the time given. Time zero shows the unity, *i.e.* the injected radioactivity only. The curves were fit to exponential decay curves, with r values of 0.998 and 0.977 for lean and obese controls, respectively, and 0.994 and 0.989 for lean and obese rats during noradrenaline infusion.

may be the consequence of a lower distribution space; the size of the insulin pool changed less than the insulin levels. However, in obese rats the data presented show a lack of response of insulin pool dynamics to noradrenaline infusion; the effects of noradrenaline were less apparent than those in lean rats, changing the insulin space and degradation rate only fractionally. This lack of effect may be related to higher basal adrenergic stimulation in obese rats related to hypertension (36) and counteractive adaptation to hyperinsulinemia (37, 38).

The obese rats used in this experiment showed a marked resistance to glucose, with hyperinsulinemia and basal normoglycemia, as described for Zucker fa/fa rats (37).

Insulin half-life was similar in lean and obese rats; all values were in the 4-min range. The similarities between groups in this respect were, however, only apparent, as the estimated rates of insulin inactivation were much higher in obese than in lean rats (~7-fold under the conditions tested). Noradrenaline injection had no significant effect on these rates in either group of rats.

There is a direct relationship in humans between fat accumulation and built-in capability to inactivate insulin (39), which is partly confirmed by the finding that obese rats cleave insulin much faster than lean controls. The maintenance of higher insulin levels with similar half-lives for insulin molecules implies a higher insulin turnover in the obese animals. The increased degradation of insulin with maintenance of high circulating levels and pool size implies increased secretion (synthesis) by the pancreas. It may be postulated that the constant release of large amounts of insulin (in the range of 210 μg/day·kg in obese rats compared with ~38 μg/day·kg in lean animals) may be instrumental in the development of late-onset diabetes often associated with obesity.

Acknowledgment

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