How is the insulin content regulated in nondiabetic hyperinsulinaemic, insulin resistant states? Study of spontaneously hypertensive rats

Jack L. Leahy[1], **Chuan Chen**[1], **Christopher J. Rhodes**[2]

[1] Division of Endocrinology, Diabetes and Metabolism, University of Vermont College of Medicine, Burlington, USA
[2] Departments of Internal Medicine and Pharmacology, University of Texas Southwestern Medical Center, Dallas, USA

The successful adaption to insulin resistant states occurs through a beta-cell hyperfunction [1]. The traditional explanation invokes the well-known feedback loop with glycaemia: it is generally presumed that a minor degree of hyperglycaemia elicits the increased insulin secretion. We previously studied insulin resistant hyperinsulinaemic spontaneously hypertensive rats (SHR), and found their basal and post stimulation glycaemia levels were identical to normally insulin sensitive Wistar Kyoto rats (WK) [2]. The mechanism of the hyperinsulinaemia was a subnormal glucose set-point (ED_{50}) for insulin secretion. The same observation has been made in pregnant rats, another insulin resistant state [3, 4]. Thus, the compensation to insulin resistance appears to entail an altered beta-cell glucose sensing system, with the result being normoglycaemia. A similar situation may occur in humans since a hypersecretion of insulin has been observed in obese subjects in tandem with glycaemia values that exactly matched nonobese control subjects over a 24 h period [5].

The current report has addressed what happens to the beta-cell insulin stores in SHR. We have noted a close parallel relationship between the pancreas insulin content and insulin secretory responses in nondiabetic and diabetic rats [6, 7], suggesting the insulin stores exert a regulatory influence over insulin secretion. In that case, an adaptive mechanism must exist in SHR that counterbalances the insulin hypersecretion. Confirming that idea, Iwase reported the pancreas insulin content of SHR rats was twice that of age-matched WK rats with no difference in the pancreas beta-cell mass [8]. However, current knowledge provides little insight into the mechanism. Insulin content is viewed as a balance of synthesis, secretion and lysosomal degradation (so-called crinophagy). All three are glucose-responsive. Hyperglycaemia induces a near-instantaneous increase of insulin secretion and post-transcriptional proinsulin synthesis [9] followed by adaptive changes of proinsulin gene transcription and intracellular degradation after several hours [10, 11]. The adaptive mechanism in SHR is unclear since glycaemia is unchanged. Proinsulin synthesis is increased in pregnant rats [12] and we expected to find increased transcriptional or translational production or both of proinsulin as another manifestation of the enhanced beta-cell glucose sensitivity. Instead, as described in this report, proinsulin synthesis was unchanged in SHR. Also, intracellular granule degradation was not obviously changed within the limits of current technology for its measurement. Thus, our results raise the possibility that regulatory factors exist for the insulin content beyond the traditional triad, and are operative in insulin resistant states.

Increased islet insulin content in SHR. We investigated normoglycaemic 12 week-old SHR which our previous study showed had twice normal plasma insulin levels compared with age-matched WK rats [2]. An additional finding was the insulin secretion at 7.8 mmol/l glucose (glycaemia level of a normal rat) from SHR isolated islets was twice that of WK islets when the DNA contents were identical, confirming the hyperinsulinaemia stemmed from hyperfunctioning beta cells [2]. We now have found that SHR islets have a greater insulin content than WK islets (80 ± 8 ng/islet SHR vs 59 ± 5 ng/islet WK, $n = 7$, $p < 0.05$) which confirms the report of Iwase in whole pancreas [8] that a mechanism exists in SHR that counterbalances (even surpasses) the insulin hypersecretion and preserves the insulin content.

Corresponding author: Dr. J. Leahy, University of Vermont College of Medicine, Given C331, Burlington, VT 05405, USA
Abbreviations: SHR, Spontaneously hypertensive rats; WK, Kyoto rats; IRI, insulin immunoreactivity.
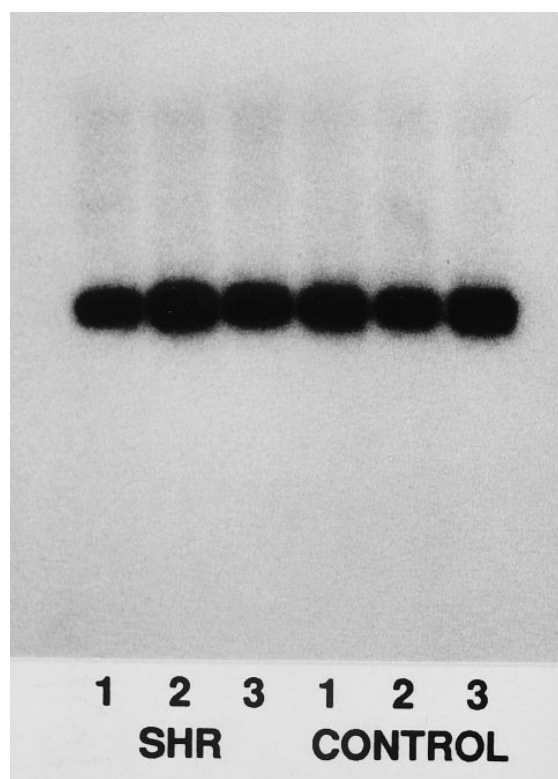
Fig. 1. Proinsulin mRNA in isolated islets of SHR and WK rats. Lanes were loaded with 2.5 μg total islet RNA from 3 separate SHR and 3 WK rats followed by electrophoresis on a 1 % agarose/6 % formaldehyde gel and transfer onto a nylon membrane. After prehybridization with denatured salmon sperm DNA, membranes were hybridized overnight at 42 °C with a [32P]-CTP radiolabelled riboprobe for rat proinsulin I (gift from L. G. Moss, New England Medical Center, Boston, Mass., USA), washed with high stringency conditions, and visualized by autoradiography
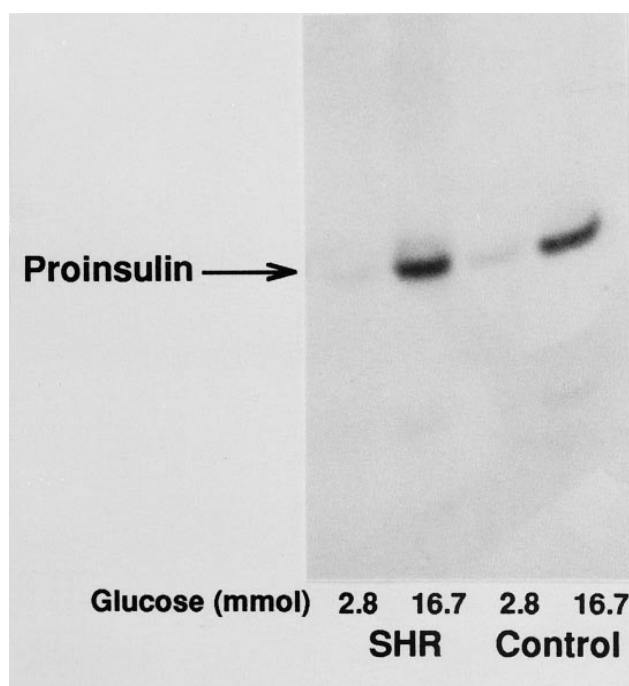

Fig. 2. Proinsulin biosynthesis by [35S]methionine incorporation in freshly isolated islets of SHR and WK rats. Islets (150) were incubated 40 min at 37 °C in 100 μl modified KRB, 20 mmol/l N-2-hydroxyethyl piperazine-N′-2ethanesulphonic acid (HEPES), 0.1 % bovine serum albumin (BSA), 2.8 or 16.7 mmol/l glucose, then radiolabelled 30 min in 100 μl incubation media with the same glucose concentration and 0.25 mCi [35S]methionine. They underwent lysis, immunoprecipitation with anti-insulin serum, alkaline-urea polyacrylamide gel electrophoresis, and fluorography. A pair of bands is shown for each animal: the 2.8 mmol/l glucose sample is on the left, and 16.7 mmol/l glucose on the right

In vitro proinsulin synthesis in SHR. We expected to find increased proinsulin synthesis in SHR because of that finding having been made in pregnant rats [12]. Proinsulin gene expression was measured in freshly isolated SHR and WK islets using a previously described Northern blot method [13]. Figure 1 shows there was no difference between three SHR and three WK rats. An additional two pairs were studied: the total group showed no difference between SHR and WK islets (SHR 109 ± 14 % of WK, *n* = 5).

Proinsulin synthesis was quantified by [35S]methionine incorporation as described previously [13, 14]. Glucose concentrations of 2.8 and 16.7 mmol/l were used to investigate whether the glucose setpoint was lowered in SHR, akin to insulin secretion. As shown in Figure 2, there was no difference in the band intensities between SHR and WK islets at either glucose concentration. In five pairs of animals, the results were 61 ± 26 % at 2.8 mmol/l glucose and 90 ± 30 % at 16.7 mmol/l glucose for SHR relative to WK (*p* = NS for both values).

Neither of the tested glucose concentrations was physiologic. We repeated the experiment using 7.8 mmol/l glucose during the incubation and labelling periods. Also, a "chase" period at 2.8 mmol/l glucose was included after the labelling period to study insulin degradation. In SHR and WK, the band intensity of the newly-formed proinsulin at 1 h (now insulin) was greater than at 0 h, reflecting additional incorporation of label from intracellular [35S]methionine. Thus, the 1 h measure was felt to best reflect proinsulin synthesis. Using this measure, a decrease was noted in SHR at 55 ± 10 % of WK (*n* = 6, *p* < 0.006). Viewed together, the Northern and radiolabel incorporation results showed no increase in proinsulin biosynthesis in SHR islets, and possibly a decrease depending on the in vitro condition studied.

In vivo proinsulin synthesis in SHR. We were concerned that a potentiating factor for proinsulin synthesis was active in SHR that was not apparent during the in vitro testing. To address this issue, proinsulin synthesis was assessed in vivo based on the reports of Permutt, Giddings, and Logothetopoulis [15–17]

Table 1. In vivo proinsulin biosynthesis in SHR and WK rats

	WK (n = 3)	SHR (n = 3)	P
Weight (g)	190 ± 8	198 ± 9	NS
Administered radiolabel (mCi)	2.7 ± 0.1	2.8 ± 0.1	NS
Serum specific activity (cpm/μl)	25561 ± 1510	25244 ± 542	NS
Proinsulin activity (cpm/ng IRI)	1.6 ± 0.2	1.7 ± 0.1	NS
Proportion proinsulin (%)	7.1 ± 1.1	4.1 ± 0.4	0.06

Data are expressed as means ± SEM. Statistical significance was assessed by the Student's unpaired t-test

but with HPLC replacing column chromatography to separate the insulins and proinsulins. Three pairs of nonanesthetized SHR and WK rats were given 1.4 mCi/100 g [^{35}S]methionine through a jugular catheter. After 30 min, the pancreas was excised, homogenized in acid ethanol, and processed for insulin/proinsulin precipitation as described previously [18]. Serum isotope enrichment was determined by liquid scintillation counting. The extracts underwent reverse-phase HPLC using Altex Ultrasphere ODS 420 columns (Beckman, San Ramon, Calif., USA; 4.6 mm i.d., 10 cm precolumn and 25 cm main column). Buffer A: 50 mmol/l phosphoric acid, 20 mmol/l triethylamine, 50 mmol/l sodium perchlorate, pH 3.0. Buffer B: 90% acetonitrile, 10% H$_2$O. The programme was a 90 min gradient from 67.5%:32.5% buffer A:B to 60%:40% A:B. One ml fractions (flow rate 1 ml/min) were collected for insulin RIA and liquid scintillation counting. The insulins eluted at 15–20 min and the proinsulins at 40–45 min. Earlier (30–35 min) and later (48–52 min) RIA peaks were presumed to be conversion intermediates although definitive characterization was not performed. Virtually no [^{35}S] cpm above the baseline existed in any peak other than proinsulin as expected from the 30 min time period of the protocol. The calculations made from the HPLC profiles were insulin immunoreactivity (IRI) in the insulin and proinsulin peaks from the RIA results; % proinsulin = IRI proinsulin ÷ IRI (proinsulin + insulin); proinsulin activity = cpm in the proinsulin peak minus background ÷ IRI proinsulin.

The body weights, administered radiolabel, and serum isotopic enrichment were equal in the SHR and WK rats (Table 1). No difference in proinsulin synthesis was observed between SHR and WK as reflected in similar [^{35}S] activities of the proinsulin peak.

Proinsulin synthesis in SHR: conclusions. Proinsulin synthesis was not increased in SHR based on rigorous in vitro and in vivo assessment. A concern could be that our methods were not sensitive enough to identify a small increase in synthesis. However, it must be emphasized that SHR secrete insulin at twice the normal rate [2], so that a doubling of synthesis (more so, since the insulin stores are increased in SHR islets) would be needed to prevent depletion of the insulin stores which should easily have been observed. Another issue is the large amount of radioactivity needed for the in vivo protocol caused us to use younger animals (8 week-old WK and 10 week-old SHR) than for the rest of the study although this was felt not to be significant since insulin resistance or hyperinsulinaemia or both have been found in 10 week-old SHR [19, 20]. Thus, our results argue against proinsulin synthesis being the site of compensation in SHR in terms of how the beta-cell insulin stores are maintained. Of great interest, this conclusion disagrees with pregnancy [12], another insulin resistant state, which may reflect the unique factors which mediate the beta-cell compensation in pregnancy: oestrogen, prolactin and placental hormones [4, 21]. Thus, our findings raise the intriguing idea of multiple mechanisms to conserve insulin stores based on the inherent beta-cell compensatory mediators.

Intracellular insulin degradation in SHR islets. We also investigated intracellular degradation of insulin granules in SHR islets. The first direct evidence for this process in beta-cells came from Halban in the early 1980s. Using "pulse chase" radiolabelling of rat islets, they noted a 41% decrease in the cellular content of labelled insulin after 24 h. They found 16% in the media, i.e. had been secreted. The remaining 25% was unaccounted for [10]. Further evidence was obtained with mannoheptulose, an inhibitor of glucose phosphorylation that blocked insulin synthesis and secretion. Recoverable insulin again fell in islets cultured with mannoheptulose, proving the existence of a third regulatory factor for the insulin stores [22]. Insight was provided by the morphological studies of Schnell which correlated these findings to the presence of secondary lysosomes that contained granule material [23]. A number of factors are known to influence degradation. Most relevant for the current study, it varies inversely with the functional activity of islets, i.e. lowered when insulin secretory function is increased or islet insulin content is reduced or both [22–25]. The glucose concentration also is a regulatory factor independent of its effect on insulin secretion [26]. We thus expected to find the degradation rate was lowered in SHR islets because of the beta-cell hyperfunction.

Insulin degradation was investigated in isolated islets using [^{35}S]methionine incorporation at 7.8 mmol/l glucose followed by a "chase" period at 2.8 mmol/l glucose to minimize secretion of the newly synthesized material. Chase periods up to 12 h showed a stable band intensity for the labelled insulin in both

groups, consistent with minimal degradation, and with no obvious difference between the SHR and WK islets. However, the correctness of this finding for the in vivo state is clearly open to question, since assessment of the degradation rate entailed studying islets ex vivo for multiple hours at a subphysiologic glucose concentration which artifactually lowered the degradation rate to virtually zero [26]. As such, confirmation of our results awaits development of more physiologically relevant measures for intracellular degradation.

Lowered proinsulin to insulin ratio in SHR. A well recognized finding in diabetic states is a raised serum ratio of proinsulin to insulin [27]. We previously investigated diabetic rats, and found pancreas extracts also had a raised proinsulin to insulin ratio [18, 28] which we suggested highlighted the importance of the stored material for determining what is secreted. Table 1 shows a lowered pancreas % proinsulin in SHR that nearly achieved statistical significance ($p = 0.06$). That result supports our prior conclusion, since a lowered serum proinsulin to insulin ratio has been reported in nondiabetic insulin resistant states such as obesity [29, 30]. Thus, our results focus attention on disordered storage rather than secretory mechanisms per se mediating the altered serum proinsulin to insulin ratio in different metabolic states.

Conclusions. Proinsulin synthesis was unchanged in SHR based on rigorous in vitro and in vivo assessment. Also, intracellular granule degradation was not obviously changed within the limits of current technology. We thus lack an explanation as to how the insulin stores are preserved in these hyperinsulinaemic rats. The current understanding of beta-cell physiology is based on the dogma that a coordinated balance of insulin production, secretion and degradation underlies the regulation of stored insulin. The current results suggest that additional regulatory factors may be operative in insulin resistant states, with the intracellular trafficking, targeting, and storage of granules being potential regulatory sites that affect granule life span.

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