Measurement of pancreatic islet cell proliferation by heavy water labeling

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1KineMed, Inc., Emeryville; 2Department of Nutritional Sciences and Toxicology, University of California at Berkeley, Berkeley; and 3Division of Endocrinology and Metabolism, Department of Medicine, San Francisco General Hospital, University of California at San Francisco, San Francisco, California

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Chen S, Turner S, Tsang E, Stark J, Turner H, Mahsult A, Keifer K, Goldfinger M, Hellerstein MK. Measurement of pancreatic islet cell proliferation by heavy water labeling. Am J Physiol Endocrinol Metab 293: E1459–E1464, 2007.—We describe a sensitive technique for measuring long-term islet cell proliferation rates in vivo in rats. Pancreatic islets were isolated and the incorporation of deuterium (2H) from heavy water (2H2O) into the deoxyribose moiety of DNA was measured by GC-MS. The results of heavy water labeling, and BrdU staining were compared. The two methods were highly correlated (r = 0.9581, P < 0.001). Based on long-term heavy water labeling, ~50% of islet cells divided in rats between 8 and 15 wk of age. Of interest, long-term BrdU administration suppressed proliferation of islet cells significantly, but not of bone marrow cells. Physiological evidence further supported the validity of the method: older animals (24 wk old) had 60% lower islet cell proliferation rates than younger rats (5 wk old), and partial (50%) pancreatectomy increased proliferation by 20%. In addition, cholecystokinin-8 treatment significantly stimulated proliferation in pancreatectomized rats only. In summary, heavy water labeling is a quantitative approach for measuring islet cell proliferation and testing therapeutic agents.

THE PROLIFERATIVE CAPACITY of pancreatic β-cells is increasingly recognized as a key factor in the pathogenesis of diabetes mellitus (type 1 and type 2) (10, 35). The recent development of agents that may stimulate β-cell proliferation and increase β-cell mass has focused further interest on β-cell regeneration as a therapeutic target in diabetes (2, 13).

Availability of sensitive and efficient techniques for measuring in vivo the proliferation rate of pancreatic β-cells are therefore important for investigations into the pathogenesis and therapy of diabetes. The most widely used technique for measuring proliferation rates of β-cells is through labeling with 5-bromodeoxyuridine (BrdU). This approach has been used for many years and has generated important insights into β-cell dynamics during the progression to diabetes (3, 14, 31).

There are some limitations to the BrdU labeling method, however. Previous reports have demonstrated that BrdU has cytotoxic effects (26, 27) and have reported the impact of chronic BrdU exposure on proliferative capacity of various cells, such as hepatocytes, kidney and immune cells, in mice and rats (21, 32, 36). This approach is also relatively labor intensive, which limits throughput. Genetic analysis (e.g., cell proliferation as a quantitative trait) (4) and drug screening (2, 13, 33) have therefore been constrained.

We recently developed a technique for measuring proliferation rates of cells in vivo, based on heavy water (2H2O) labeling and mass spectrometric analysis (28; Fig. 1, A and B). This approach is particularly well suited for measurement of proliferation of slow-turnover cells, due to the ease of long-term administration of heavy water and the high sensitivity of mass spectrometric analysis (19). Slow-turnover cells for which dynamics have been measured by this approach include vascular smooth muscle cells, adipocytes, T lymphocytes, hippocampal neurons, hepatocytes, endothelial cells, mammary epithelial cells, spermatoocytes, chronic lymphocytic leukemia cells, and others (8, 20, 34). Here, we asked whether the heavy water labeling approach can be used for pancreatic islet cells as a model to study β-cell biology, in that 70~80% of islet cells are β-cells, and the β-cell exhibits relatively slow turnover. We also compare its use to the standard method, BrdU labeling.

METHODS

Animals. Male Wistar rats (Charles River Laboratories) were maintained on an ad libitum diet with commercial chow. Other male rats underwent 50% pancreatectomy (Px) or sham operation at 8 wk old (at Charles River Laboratories) and then were administered cholecystokinin octapeptide (CCK-8; NeoMPS, Strasbourg, France) at 4 μg/kg subcutaneously three times daily or vehicle (15% gelatin) for 14 days beginning the 5th day after surgery (22). Procedures were approved by the Institutional Animal Use Committees.

Heavy water labeling protocol. A priming intraperitoneal bolus (0.35 ml/g body wt) of 99% 2H2O in 0.9% NaCl was given to reach a body water enrichment of roughly 5% (using an estimated 60% body weight as water) and then received 8% 2H2O in drinking water for the study duration. Animals were killed after different time points of 2H2O intake. The 2H2O enrichments in body water achieved stable steady-state values after 3 days of labeling in rodents on this protocol (28).

Pancreatic islet purification. The islet isolation technique has been described in detail previously (9). Briefly, 30 ml of cold Hanks’ buffer with collagenase solution was infused into the pancreatic duct. The inflated pancreas was digested in a water bath at 37°C. Islets were picked out by hand. DNA was extracted from all islets in each animal for measurement of 3H incorporation to avoid sampling bias.

Fresh live islets isolated by this technique, including islet cells were immunostained with anti-insulin antibodies (see Supplementary Fig. 1 online).

Bone marrow cell isolation. Bone marrow cells were flushed out with a needle and syringe containing PBS from the femur, as described previously (28).

Enrichment and incorporation of 2H2O in cell DNA. The incorporation of 2H from 2H2O into the deoxyribose (dR) moiety of...
purine deoxyribonucleotides in genomic DNA was measured as described previously (28). In brief, DNA was extracted from islets by using Qiagen kits and was hydrolyzed to deoxyribonucleosides. DNA was heated with magnesium chloride and zinc sulfate, followed by incubation for 2 h in a 37°C water bath with DNase, nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase. The dR moiety was derivatized to pentane tetraacetate, as described (28). Pentane tetraacetate was analyzed by positive chemical ionization GC-MS with a model 5973 mass spectrometer and model 6890 gas chromatograph (Agilent, Palo Alto, CA). Selected ion monitoring was performed with mass-to-charge ratios (m/z) of 245 and 246, representing the M + 0 and M + 1 ions, respectively. The excess fractional M + 1 enrichment (EM1) of dR was calculated as

\[
EM1 = \frac{\text{abundance m/z 246}_{\text{sample}}}{\text{abundance m/z 245}_{\text{sample}}} - \frac{\text{abundance m/z 246}_{\text{STD}}}{\text{abundance m/z 245}_{\text{STD}}}
\]

where sample and STD represent the analyzed sample and unenriched standards, respectively. Standards of natural abundance (unlabeled) pentane tetraacetate were analyzed concurrently with samples. Abundance matching of samples to standards (Table 1) and other corrections were as described in detail elsewhere (28).

**Calculation of fraction of newly divided islet cells.** The fraction of newly divided islet cells was calculated from the ratio of 2H incorporation into DNA from islets to 2H incorporation into bone marrow cells (20, 28):

\[
\frac{\text{dR enrichment of islet cells (EM1)}}{\text{dR enrichment of bone marrow cells (EM1)}} \times 100 = \text{fraction newly divided cells (%)}
\]

where EM1 is molar excess of 2H in dR from DNA (28). Because bone marrow cells are nearly fully turned over in rats after 4–5 days of 2H2O labeling, their labeling values serve as an internal reference (representing 100% newly divided cells) for calculating the fraction of any comparison cell that have proliferated in the same animal (Table 1).

**Comparison of 2H2O labeling alone to the double-labeling, 2H2O-BrdU approach.** To examine the effect of BrdU on islet cell proliferation, rats were labeled continuously for periods of 7, 14, and 28 days with 2H2O alone or double-labeled with 2H2O-BrdU in parallel studies. The 2H2O labeling protocol was performed as described above. BrdU (1 mg/ml), was added to 2H2O in drinking water to create a 2H2O-BrdU solution. To ensure that BrdU was bioactive, the 2H2O-BrdU solution was protected from light exposure and was changed every 3 days with freshly prepared solution, as described previously (31). Islet cell proliferation was measured by mass spectrometry, as described above. Additionally, a portion of pancreatic segments in the double-labeled 2H2O-BrdU animals was fixed in 4% paraformalin for immunohistochemistry to explore the correlation between the 2H2O labeling and BrdU staining approaches.

![Fig. 1.](http://ajpendo.physiology.org/) A: labeling pathway for measuring DNA synthesis and thus cell proliferation from 2H2O label incorporation. B: sites of 2H incorporation from 2H2O into C-H bonds of deoxyribose (dR) in replicating DNA. GNG, gluconeogenesis/glycolysis; PPP, pentose-phosphatase pathway; ribonucleotide reductase; DNPS, de novo purine/pyrimidine synthesis pathway; DNNS, de novo nucleotide synthesis pathway. C: time course of islet cell proliferation in rats (n = 5/time point). Animals were labeled from 8 wk of age until 15 wk of age. D: comparison of proliferation rates of smaller and larger islets (n = 4/group). Islets of different sizes were isolated from the same animal after 2 wk of labeling. E: comparison of proliferation rates of islet cells and exocrine tissue cells (n = 4–6/group). Tissues were harvested from the same animal after labeling for 2 wk.
**RESULTS**

**Basal rates of islet cell proliferation in rats.** The time course of islet cell proliferation is shown (Fig. 1C). Labeling with \(^2\text{H}_2\text{O}\) was begun at 8 wk of age and continued for up to 7 wk duration. Islet cell proliferation approached an apparent plateau value of 55% new cells during the 7-wk period in these growing rats. Because some smaller islets might be newer or represent recent buds from ducts (5), we determined whether smaller islets had higher growth rates than larger islets. The fraction of newly divided cells in smaller islets (>150 \(\mu\)m in diameter) was compared with larger islets (>150 \(\mu\)m in diameter) isolated from the same animal after a 14-day labeling period. There was no difference between the two groups (smaller islets 17.3 ± 2.9% vs. larger islets 17.1 ± 3.3%; Fig. 1D) (6).

The most serious practical problem in dynamic studies of slow-turnover tissues is contamination by small amounts of exocrine tissue would therefore be unlikely to have affected results substantially, even if present. Correlation between fraction of newly divided islet cells by \(^2\text{H}_2\text{O}\) labeling and percentage by BrdU immunohistochemistry. To validate the reliability of the \(^2\text{H}_2\text{O}\) labeling approach, we compared the fraction of newly divided cells by \(^2\text{H}_2\text{O}\) labeling and the percentage of BrdU-insulin-stained cells from the same rats, which had undergone a double-labeling \(^2\text{H}_2\text{O}-\text{BrdU}\) protocol. The fraction of newly divided islet cells by \(^2\text{H}_2\text{O}\) labeling was highly correlated with the percentage of BrdU/insulin staining (\(r = 0.9581, P < 0.001\); Fig. 2A) with no significant differences (9.3 ± 1.3% vs. 8.9 ± 0.8%, 7 days, NS; 18.5 ± 1.6% vs. 20.3 ± 2.0%, 14 days, NS, respectively; Fig. 2B).

**Suppressive effects of long-term BrdU administration on islet cell proliferation.** We considered the possibility that BrdU administration might alter proliferation rates of islet cell during continuous administration (21, 32, 36). The results from \(^2\text{H}_2\text{O}\) labeling alone were compared with double labeling with \(^2\text{H}_2\text{O}-\text{BrdU}\) (Fig. 3A). The fraction of newly divided islet cells was significantly higher with \(^2\text{H}_2\text{O}\) labeling alone than with double labeling with \(^2\text{H}_2\text{O}-\text{BrdU}\) (12.7 ± 1.2% vs. 9.3 ± 1.3%, 7 days, \(P < 0.01, 22.2 ± 3.1\%\) vs. 18.5 ± 1.6%, 14 days, \(P < 0.05, 31.2 ± 1.8\%\) vs. 26.3 ± 4.2%, 28 days, \(P < 0.05\); respectively). Thus, long-term BrdU administration suppressed islet cell proliferation by 16–25%. BrdU did not affect the turnover of bone marrow cells (Fig. 3B). Long-term BrdU administration appeared to have little effect on body weight, food intake, glucose, or organ weights (Table 2), consistent with reports by others (31).

Applications and validation of the heavy water labeling approach. Two physiological experiments were performed that provided validation of the heavy water labeling approach. First, the effects of age on the proliferation dynamics of islet cells was determined (Fig. 4A). In younger rats (5 wk old at the beginning of the labeling period), islet cell proliferation rates were 20.8, 42.4, and 55.7% for 7, 14, and 28 days, respectively. In older rats (24 wk old at the beginning of the labeling period), islet cell proliferation rates only were 4.7, 11.3, and 21.3% for 7, 14, and 28 days, respectively. Thus, older rats had more than a 60% reduction in the fraction of newly divided islet cells compared with younger animals.

Next, we tested the effects of partial Px on islet cell proliferation (7). After 50% Px in rats, islet cell proliferation in the residual pancreas was ~20% elevated compared with sham-operated, age-matched rats (Fig. 4B). This increase in proliferating fraction was observed after both 14 days and 28 days of \(^2\text{H}_2\text{O}\) labeling (15.8 ± 1.8% vs. 12.8 ± 1.7% for Px vs.

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**Table 1. Sample calculation for islet cell fractional proliferation**

<table>
<thead>
<tr>
<th></th>
<th>M0 (abundance)</th>
<th>M1 (abundance)</th>
<th>%EM1</th>
<th>EM1, %</th>
<th>Average EM1, %</th>
<th>f, %</th>
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<tr>
<td>Standards</td>
<td>12,337,343</td>
<td>2,618,069</td>
<td>17.50</td>
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<td></td>
<td></td>
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<td>Bone marrow</td>
<td>43,513,178</td>
<td>9,384,745</td>
<td>17.74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41,729,268</td>
<td>21,964,295</td>
<td>34.48</td>
<td>16.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>42,652,958</td>
<td>22,515,849</td>
<td>34.55</td>
<td>16.81</td>
<td>16.77</td>
<td></td>
</tr>
<tr>
<td>(islets)</td>
<td>13,752,563</td>
<td>4,009,560</td>
<td>22.57</td>
<td>5.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14,072,648</td>
<td>4,089,390</td>
<td>22.52</td>
<td>4.90</td>
<td>5.15</td>
<td>30.7</td>
</tr>
</tbody>
</table>

1. EM1(islet)/EM1 (bone marrow). %EM1 (corrected) was calculated from abundance-matched standard curves. Standards refers to natural abundance (unlabeled) DNA. Sample refers to islets from a rat labeled with \(^2\text{H}_2\text{O}\). Bone marrow cells were isolated from the same rat labeled with \(^2\text{H}_2\text{O}\).
sham-operated, 14 days, $P < 0.05$, respectively. We also tested the effects of CCK-8, a peptide agent with known insulin-secretory agonist effects (22, 29). CCK-8 was given to sham-operated and Px rats (the study design, Fig. 4C). No effect was observed in the sham-operated controls. Px plus CCK-8 significantly increased islet cell proliferation further, although modestly (by 13%), compared with Px alone (Fig. 4D). All groups remained euglycemic. No differences in plasma insulin concentrations were measurable among groups (not shown).

**DISCUSSION**

Our objective here was to develop an alternative technique for measuring the proliferative dynamics of $\beta$-cells in pancreatic islets. The goal was to have a method that would complement the BrdU labeling approach and perhaps has advantages, such as higher throughput (1, 2, 34).

Several technical points deserve comment. The results shown herein highlight the sensitivity and quantitative reproducibility of heavy water labeling and GC-MS analysis. The results of heavy water labeling correlated well with BrdU administration (Fig. 2). Heavy water can be administered without toxicity for several weeks, allowing integrated proliferation rates to be measured over long time periods. Indeed, we (23) had shown previously that $^{2}$H$_2$O and BrdU gave similar results for colonocyte proliferation, although with superior...
The availability of the heavy water labeling method also allowed us to directly assess the impact of BrdU administration on proliferation rates of various cell types, including pancreatic islet cells (Fig. 3A), through double-labeling, 2H2O-BrdU experiments. We observed that long-term BrdU administration significantly reduced the proliferation of islet cells. These findings are consistent with previous reports of cells other than β-cells (21, 32, 36), although others have reported no adverse effects of BrdU on β-cell proliferation (31). Interestingly, BrdU administration did not affect the turnover of bone marrow cells, which may reflect the fact that the bone marrow is a rapid-turnover tissue (30). In any case, the possibility of an interaction between BrdU administration and cell type or drug candidates is of some concern (16, 21, 26).

There are also methods for evaluating cell division rates that do not involve metabolic labeling [e.g., staining for Ki67 or proliferating cell nuclear antigen (17, 37)]. These methods reflect the fraction of cells that are “in cycle” but do not reveal the rate at which cell division is actually occurring in a population. However, if a G1/S-phase block is present, for example, cells will accumulate that are positive for these cell cycle antigens even though the true rate of mitosis and passage through S-phase of the cell cycle is low.

The heavy water labeling approach described herein has disadvantages as well as advantages compared with BrdU staining. Because pancreatic islet cells, not purified β-cells, are isolated by our protocol, the proliferation rate of all islet cells is assumed to represent that of β-cells and it is assumed that there are no highly proliferative contaminating cells present. These assumptions are reasonable, in that 70–80% of islet cells are β-cells, exocrine tissue is not present (and has a low proliferation rate), and proliferating glucagon-, somatostatin-, or pancreatic polypeptide-containing cells in islets are rare in adulthood (11, 18). The correlation between BrdU and 2H2O results that we observed (Fig. 2A) also supports this conclusion. Nevertheless, BrdU labeling directly visualizes β-cell proliferation and would be advantageous in any circumstance where other cells in the islet might confound proliferative results. In the future, isolation of β-cells by cell sorting could resolve concerns about non β-cells in islets measured by the heavy water labeling approach.

The heavy water labeling approach detects all input sources into new β-cells, including neogenesis and proliferation, although it is unable to distinguish among these sources. The possibility of quantitative artifacts arising from smaller, new islets is unlikely, however, based on our finding that smaller and larger islets exhibited the same fractional proliferation rates (6). Thus, as long as the neogenic islets can be isolated physically as an islet after a 2- to 4-wk labeling period, measured proliferation results should not be biased. The proliferative capacity to include neogenic islets in the mass balance equation therefore allows true net cell growth to be calculated (2).

This approach is compatible with relatively high efficiency for an in vivo technique (5). GC-MS measurements can be reliably performed from a small number of islets, if necessary, which

### Table 2. Physiological characteristics of rats labeled with 2H2O alone and with 2H2O + BrdU for 28 days

<table>
<thead>
<tr>
<th></th>
<th>2H2O alone</th>
<th>2H2O + BrdU</th>
</tr>
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<tbody>
<tr>
<td>Body Weight (g)</td>
<td>431±12</td>
<td>414±22</td>
</tr>
<tr>
<td>Food Intake (g/d)</td>
<td>26±2</td>
<td>27±1</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>111±4</td>
<td>113±3</td>
</tr>
<tr>
<td>Pancreas Weight (g)</td>
<td>1.28±0.1</td>
<td>1.15±0.1</td>
</tr>
<tr>
<td>Spleen Weight (g)</td>
<td>1.18±0.1</td>
<td>1.05±0.1</td>
</tr>
<tr>
<td>Kidney Weight (g)</td>
<td>3.35±0.2</td>
<td>3.67±0.3</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>18.6±0.5</td>
<td>19.0±1.9</td>
</tr>
</tbody>
</table>

Data are means ± SE, weights in g; n = 6/group. brdU, 5-bromodeoxyuridine.
would allow a larger number of animals to be studied. Preparation of DNA and GC-MS analyses can be batched and performed at high efficiency (e.g., >50 samples per day by a technician).

Type 2 diabetes is characterized by a deficit in β-cell mass and by an increasing incidence with age. We confirmed previous findings (12, 25) of an age-dependent decline in β-cell proliferation rates (Fig. 4A). Our results with CCK in the partial pancreatectomy model represent a pilot application of a drug testing strategy. Use of heavy water labeling has also recently been used to identify strain effects on islet cell proliferation in mice (unpublished observations).

In summary, heavy water labeling represents a convenient, reliable, nontoxic, and highly efficient alternative to BrdU labeling for measurement of proliferation rates of pancreatic islet cells and thus β-cells. Applications to the pathophysiology and treatment of diabetes may prove useful.

DISCLOSURES
S. Chen holds stock in KineMed Inc. M. K. Hellerstein is on the Board of Directors of, serves as Chair of the Scientific Advisory Board of, and holds stock in KineMed Inc.

REFERENCES