Clinical Diabetes Research
Methods and Techniques

Edited by

Michael Roden
Medical Department, Hanusch Teaching Hospital,
Medical University of Vienna
Karl Landsteiner Institute for Endocrinology and Metabolism, Vienna

John Wiley & Sons, Ltd
Clinical diabetes research: methods and techniques/edited by Michael Roden.

Includes bibliographical references.
1. Diabetes. 2. Diabetes—Research. I. Roden, Michael, Dr.
[DNLM: 1. Diabetes Mellitus. 2. Clinical Trials. WK 810 C6413 2007]
RC660.C463 2007
616.4'620072—dc2 2007015950

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library
Typeset in 10/12pt Times by Integra Software Services Pvt. Ltd, Pondicherry, India
Printed and bound in Great Britain by Antony Rowe Ltd., Chippenham, Wilts
This book is printed on acid-free paper responsibly manufactured from sustainable forestry
in which at least two trees are planted for each one used for paper production.
Cover images kindly provided by Dr Julia Szendrödi, Karl-Landsteiner Institute for Endocrinology and Metabolism, at the
MR Center of Excellence, University of Vienna (the magnetic resonance picture) and Agnes Roden, Department of
Internal Medicine III, Medical University of Vienna (the PET scan). The graph displays the suppression of endogenous
glucose production during ingestion of a mixed meal in humans and was produced in the lab of Professor Roden.
Contents

List of Contributors xi

1 Basics of Clinical Metabolic Research 1
   Michael Roden

2 Methods for the Assessment of β-Cell Function In Vivo 7
   Andrea Mari and Giovanni Pacini
   Introduction 7
   Methods for insulin secretion in vivo 7
   β-cell response characteristics 9
   β-cell function tests 12
   Modelling methods 19
   β-cell function and insulin sensitivity 20
   Comparative evaluation of methods 21
   Conclusion 24

3 Assessment of Insulin Sensitivity from Steady-State and Dynamic Tests 27
   Giovanni Pacini and Andrea Mari
   Introduction 27
   Insulin sensitivity from steady-state tests 28
   Insulin sensitivity from dynamic tests 31
   Conclusion 37

4 Glucose Clamp Techniques 43
   Attila Brehm and Michael Roden
   Introduction 43
   Basic principles of the euglycaemic hyperinsulinaemic clamp technique 49
   Methodology 57
   Reproducibility of insulin sensitivity obtained from clamp tests 64
   Safety considerations for hyperinsulinaemic euglycaemic clamp test 65
   Modifications of the euglycaemic hyperinsulinaemic clamp test protocol 65
   Conclusion 67
5 Methods of Assessment of Counterregulation to Hypoglycaemia

Pratik Choudhary, Ming Ming Teh and Stephanie A Amiel

Introduction
Definitions
Pathophysiology
Documentation of hypoglycaemia experience
Investigating the pathogenesis of problematic hypoglycaemia
Hypoglycaemic stimuli for research
Measurement of physiological responses
Conclusion

6 Glucose Kinetics: Measurement of Flux Rates

Jerry Radziuk and Susan Pye

Introduction
Measurement of glucose production and uptake by the liver – tissue balance techniques
Properties of glucose tracers
Measurement of glucose production and uptake by the liver – systemic techniques
Conclusion
Acknowledgements

7 Xenobiotics as Probes of Carbohydrate Metabolism

Bernard Landau

Introduction
Glucuronidation
Glutamination
Ribosylation
Acetylation
Glycination

8 Tracing Hepatic Glucose and Glycogen Fluxes with $^2$H$_2$O

John G Jones

Introduction
Methodology
Theoretical considerations
Quantifying hepatic glucose and glycogen metabolism
Conclusion

9 Lipid Kinetics

John M Miles and Robert H Nelson

Introduction
Tracers for the study of adipose tissue lipolysis
Tracers for the study of triglyceride-rich lipoprotein kinetics
Conclusion
Acknowledgements
## 10 Protein and Amino Acid Kinetics

*Stephen F Previs, Danielle A Gilge and Nadia Rachdaoui*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>169</td>
</tr>
<tr>
<td>Measuring amino acid turnover</td>
<td>170</td>
</tr>
<tr>
<td>Measuring protein turnover</td>
<td>175</td>
</tr>
<tr>
<td>Proteome dynamics</td>
<td>181</td>
</tr>
<tr>
<td>Urea kinetics</td>
<td>182</td>
</tr>
<tr>
<td>Determining the molecular control of protein dynamics</td>
<td>185</td>
</tr>
<tr>
<td>Conclusion</td>
<td>186</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>187</td>
</tr>
</tbody>
</table>

## 11 Assessment of Metabolic Fluxes by *In Vivo* MR Spectroscopy

*Martin Krššák and Michael Roden*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenomena of nuclear magnetic resonance – imaging and spectroscopy</td>
<td>193</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>198</td>
</tr>
<tr>
<td>Liver</td>
<td>207</td>
</tr>
<tr>
<td>Brain</td>
<td>213</td>
</tr>
<tr>
<td>Conclusion</td>
<td>214</td>
</tr>
</tbody>
</table>

## 12 Positron Emission Tomography in Metabolic Research

*Pirjo Nuutila, Patricia Iozzo and Juhani Knuuti*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>223</td>
</tr>
<tr>
<td>Tracers for metabolic imaging</td>
<td>224</td>
</tr>
<tr>
<td>Principles of modeling</td>
<td>225</td>
</tr>
<tr>
<td>Future perspectives</td>
<td>232</td>
</tr>
</tbody>
</table>

## 13 Assessment of Body Fat Content and Distribution

*Martin Krššák*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>237</td>
</tr>
<tr>
<td>Measurement techniques</td>
<td>237</td>
</tr>
<tr>
<td>Body fat distribution and insulin resistance</td>
<td>247</td>
</tr>
</tbody>
</table>

## 14 Tissue Biopsies in Diabetes Research

*Kurt Højlund, Michael Gaster and Henning Beck-Nielsen*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>265</td>
</tr>
<tr>
<td>Percutaneous needle biopsy of skeletal muscle</td>
<td>266</td>
</tr>
<tr>
<td>Biopsy procedure and sample handling</td>
<td>267</td>
</tr>
<tr>
<td>Discomfort and complications of needle biopsy</td>
<td>269</td>
</tr>
<tr>
<td>Advantages and limitations of needle biopsy</td>
<td>270</td>
</tr>
<tr>
<td>Application of muscle biopsy in diabetes</td>
<td>270</td>
</tr>
<tr>
<td>Morphology of skeletal muscle</td>
<td>273</td>
</tr>
<tr>
<td>Application of muscle morphology in type 2 diabetes</td>
<td>273</td>
</tr>
<tr>
<td>Human myotube cultures</td>
<td>276</td>
</tr>
<tr>
<td>The cell culture principle of human myotubes</td>
<td>277</td>
</tr>
<tr>
<td>Advantages of human myotubes</td>
<td>279</td>
</tr>
</tbody>
</table>
CONTENTS

Limitations of human myotubes 279
Application of myotubes in diabetes research 281
Conclusion 282

15 Assessment of Vascular Function 289
Johannes Pleiner and Michael Wolzt
Basic theoretical concepts 289
The vascular endothelium 289
Strain gauge plethysmography 290
Flow Mediated Dilation (FMD) of the brachial artery 295
Intima media thickness of the carotid artery 299
Assessment of arterial stiffness 303

16 Cardiovascular Autonomic Function Testing 311
Dan Ziegler
Introduction 311
Prognosis 312
Epidemiology 312
Clinical features 313
Diagnostic assessment 316

17 Nerve Function Testing 325
Haris M Rathur and Andrew J M Boulton
Introduction 325
Clinical Assessment 325
Clinical screening devices (not requiring external power source) 328
Quantitative sensory testing 330
Electrophysiology 332
Other methods of assessment 334
Non-invasive assessment 335
Conclusion 336

18 Kidney Function 341
Sally M Marshall
Introduction 341
Glomerular function 341
Tubular function 346
Renal haemodynamics 346
Renal structure 347
Genetic studies 348
Provocation tests 348
Functional magnetic resonance imaging and spectroscopy 349
Conclusion 350
CONTENTS

19 Techniques for the Investigation of the Eye in Diabetes 357
Ayad Al-Bermani and Roy Taylor

Introduction 357
Visual acuity 357
Fluorescein angiography of the fundus 359
Optical coherence tomography (OCT) 362

20 Basics of Molecular Genetics: Lessons from Type 2 Diabetes 367
Leif Groop and Charlotte Ling

Introduction 367
Genetic risk 367
Genetic variability 368
Mapping genetic variability 369
Future directions 375

21 Good Clinical Practice: Friend or Foe? 377
Christian Joukhadar and Markus Müller

Introduction 377
Scientific misconduct 378
Publication bias 378
Institutional review board/independent ethics committee 379
Protocol and informed consent 380
Special populations 381
Serious adverse event reporting 382
Monitoring and inspection 383
Conclusion 384

22 Statistical Considerations in Diabetes Trials 387
Irene M Stratton and Carole A Cull

Introduction 387
Requirements 388
Study design and power calculations 388
Biochemical and clinical measurements 388
Demographics 389
Cross-over studies 390
Long term studies 390
Specialised trial designs 390
Reporting of study protocols 390
Identification and measurement of outcome measures and surrogate outcome measures 391
Adjudicated endpoints 391
Validation of data 391
Other sources of bias and error 391
Analysis 392

Index 395
List of Contributors

Stephanie A. Amiel  BSc, MD, FRCP, RD Lawrence Professor of Diabetic Medicine, Gene and Cell Based Therapy, Diabetes Research Group, Diabetes, Endocrinology and Metabolism, King’s College London School of Medicine, London, UK

Henning Beck-Nielsen  MD, DMSc, Head of the Department of Endocrinology, Odense University Hospital, Professor of Endocrinology, University of Southern Denmark, and Head of the Danish PhD School of Molecular Metabolism, Odense, Denmark

Ayad Al-Bermani  MD, Specialist Registrar in Medical Ophthalmology, Ophthalmology Department, Royal Victoria Infirmary, Newcastle upon Tyne, UK

Andrew J.M. Boulton  MD, FRCP, DSc, Professor of Medicine, Medicine University of Manchester, Manchester, UK

Attila Brehm  MD, Assistant, First Medical Department, Hanusch Hospital (Academic Teaching Hospital, Medical University of Vienna), Vienna, Austria

Pratik Choudhary  MB, BS, Gene and Cell Based Therapy, Diabetes Research Group, Diabetes, Endocrinology and Metabolism, King’s College London School of Medicine, London, UK

Carole Cull  PhD, University Research Lecturer, Senior Medical Statistician, Diabetes Trials Unit, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Headington, Oxford, UK

Michael Gaster  MD, PhD, Specialist in General Medicine. Associated Professor at the Molecular Endocrinology Unit, Odense University Hospital, Odense, Denmark

Danielle A. Gilge  PhD, Candidate, Nutrition, Case Western Reserve University, Cleveland, Ohio, USA

Leif Groop  MD, PhD, Professor, Head, Lund University Diabetes Centre, Department of Clinical Sciences, Diabetes and Endocrinology, Lund University, Malmoe, Sweden

Kurt Højlund  MD, PhD, Senior Registrar, Post Doc, Department of Endocrinology, Odense University, Odense, Denmark

Patricia Iozzo  PhD, Turku PET Centre, University of Turku, Turku, Finland and PET Centre, Institute of Clinical Physiology, CNR National Research Council, Pisa, Italy

Christian Joukhadar  MD, Lecturer, Department of Clinical Pharmacology, Medical University Vienna, Vienna General Hospital, Vienna, Austria
John Griffith Jones  DSc, Senior Researcher and Invited Assistant Professor, Intermediary Metabolism, Center for Neurosciences and Cell Biology, Department of Biochemistry, University of Coimbra, Coimbra, Portugal

Juhani Knuuti, Professor, Turku PET Centre, University of Turku, Turku, Finland

Martin Krššák PhD, Departments of Radiodiagnostics and Internal Medicine III, Medical University Vienna, Vienna, Austria

Bernard Landau MD, PhD, Division of Clinical and Molecular Endocrinology, Case Western Reserve University, Cleveland, Ohio, USA

Charlotte Ling MSc, PhD, Associate Professor, Lund University Diabetes Centre, Department of Clinical Sciences, Diabetes and Endocrinology, Lund University, Malmö, Sweden

Andrea Mari PhD Senior Research Scientist, Institute of Biomedical Engineering, National Research Council, Padova, Italy

Sally M. Marshall BSc, MD, FRCP, Professor of Diabetes, Diabetes Research Group, School of Clinical Sciences, Faculty of Medicine, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

John M. Miles MD, Professor of Medicine, Endocrine Research Unit, Division of Endocrinology, Diabetes, Metabolism and Nutrition, Mayo Clinic, Rochester, Minnesota, USA

Markus Müller MD, Professor, Head, Department of Clinical Pharmacology, Medical University of Vienna, Vienna General Hospital, Vienna, Austria

Robert H. Nelson MD, Instructor, Endocrine Research Unit, Division of Endocrinology, Diabetes, Metabolism and Nutrition, Mayo Clinic, Rochester, Minnesota, USA

Pirjo Nuutila MD, Professor, Turku PET Centre and Department of Medicine, University of Turku, Finland

Giovanni Pacini DSC, Head, Metabolic Unit, Institute of Biomedical Engineering, Italian National Research Council, Padova, Italy

Johannes Pleiner MD, Lecturer in Clinical Pharmacology, Cardiovascular Pharmacology, Department of Clinical Pharmacology, Medical University Vienna, Vienna, Austria

Stephen F. Previs PhD, Assistant Professor, Nutrition, Case Western Reserve University, Cleveland, Ohio, USA

Susan Pye MSc, Research Associate, Diabetes and Metabolism Research Unit, Ottawa Hospital and Ottawa Health Research Unit, Ottawa, Canada

Nadia Rachdaoui PhD, Instructor, Division of Clinical and Molecular Endocrinology, Department of Medicine, Case Western Reserve University, Cleveland, Ohio, USA

Haris Rathur MRCP, Specialist Registrar, Diabetes and Endocrinology, Medicine, Manchester Royal Infirmary, Manchester, UK
Jerry Radziuk PhD, MD, CM, Professor, Departments of Medicine and of Physiology, Director, Diabetes and Metabolism Research Unit, University of Ottawa, Ottawa Hospital and Ottawa Health Research Unit, Ottawa, Canada

Michael Roden, MD, Head/Director, Medical Department, Hanusch Teaching Hospital, Medical University of Vienna and Karl Landsteiner Institute for Endocrinology and Metabolism, Vienna, Austria

Irene Stratton MSc, University Research Lecturer, Senior Medical Statistician, Diabetes Trials Unit, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, UK

Roy Taylor MD, Professor of Medicine and Metabolism, Honorary Consultant Physician, School of Clinical Medical Sciences, Newcastle University and Diabetes Centre, Newcastle upon Tyne, UK

Ming Ming Teh MB, BS, Clinical Research Fellow, Gene and Cell Based Therapy, Diabetes Research Group, Diabetes, Endocrinology and Metabolism, King’s College London School of Medicine, London, UK

Michael Wolzt MD, Consultant Physician, Associate Professor, Medical University of Vienna, Division of Cardiovascular Medicine, Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria

Dan Ziegler MD, Professor of Internal Medicine, Consultant Physician, Institute of Clinical Diabetes Research, German Diabetes Center, Leibniz Center at the Heinrich Heine University, Düsseldorf, Germany
1

Basics of Clinical Metabolic Research

Michael Roden

Metabolic diseases, particularly obesity, dyslipidaemia and type 2 diabetes mellitus (T2DM), as well as conditions of increased risk for these diseases such as factors of the (dys)metabolic syndrome have become dramatically more prevalent over the last decade. Both in the industrialised world and, even more so, in developing regions and countries – which feature rapidly increasing economies and are adopting the so-called Western lifestyle – these diseases, particularly overweightness and obesity, are a growing health problem (Kopelman 2000). It is assumed that T2DM could be the largest epidemic in human history (Zimmet 2005) as more than 190 million people worldwide are diabetic and more than 300 million suffer from impaired glucose tolerance, the immediate pre-diabetic state. Recent calculations suggest that in the year 2025 more than 300 million people will have overt diabetes, mainly T2DM, with excessive growth in developing countries (King et al. 1998).

Diabetes mellitus is already the leading cause of blindness among working-age adults, of end-stage renal disease and of non-traumatic loss of limb (Ullbrecht et al. 2004; Williamson et al. 2004). The global mortality attributable to diabetes in the year 2000 was estimated to be 2.9 million deaths, which was 5.2 % of all death. Thus diabetes is the fifth leading cause of death globally. About 2–3 % of the population in low-economy countries and up to 8 % in the United States and Canada die because of diabetes (Roglic et al. 2005). The costs caused by diabetes are enormous. Currently diabetes care accounts for 2–7 % of the total national health care budgets in Western countries, amounting to $132 billion in the United States in 2002 (ADA 2003). These data underline the importance of understanding the cellular mechanisms of the causes and complications of type 2 diabetes mellitus in order to offer better targeted and more effective treatment or even prevention of the disease.

Over the last decades, we have learned a lot from in vitro experiments in isolated tissues and cell cultures, but especially from in vivo studies in mouse models of modified insulin secretion or action (Nandi et al. 2004). Nevertheless, the phenotypes resulting from the various tissue-specific knockout or overexpression mouse models of diabetes or metabolic diseases do not generally resemble the phenotypes of corresponding diseases in humans. In addition to species differences and technical limitations of metabolic studies in small animals,
this observation can likely be explained by gene-environment interactions influencing the human phenotypes of metabolic disorders. This makes detailed studies in humans under \textit{in vivo} conditions mandatory.

Starting from simple endocrine and metabolic stimulation or suppression tests such as the insulin and glucagon administration or glucose tolerance tests, a series of more sophisticated tests has been developed over the last three decades. This development is mirrored by the near-exponential rise in original papers and reviews on the topics ‘diabetes’ and ‘metabolic diseases’ in peer-reviewed journals since 1970 (Figure 1.1). Among those publications, some key methodological papers speeded up the development of clinical metabolic research. These key papers include the description and validation of standardised insulin sensitivity and secretion tests such as the glucose clamp (Tobin et al. 1979) and minimal modeling of glucose and insulin concentrations during the intravenous glucose tolerance test (Bergman et al. 1981). Later on, tissue-specific metabolism became accessible by applying \textit{in vivo} multinuclear magnetic resonance spectroscopy (MRS) (Shulman et al. 1990; Krssák et al. 1999) or positron emission tomography (PET) (Nuutila et al. 1993). Novel applications of stable isotopes as labels of molecules, e.g. $^2\text{H}_2\text{O}$, later allowed quantification of complex metabolic fluxes such as rates of gluconeogenesis from different sources and glycogenolysis from one single blood sample (Landau et al. 1995) (NOTE: I would like to express my grief for my long-term mentor and friend, Professor Landau, an outstanding researcher and scientist in the field of metabolism, who passed away during the printing of this book). Combining the different techniques, e.g. MRS and $^2\text{H}_2\text{O}$ (Kunert et al. 2003), further stimulated research on human metabolism and detailed metabolic phenotyping of various populations is now widely used.

![Figure 1.1](image-url)  
**Figure 1.1** Number of original papers (dark columns) and reviews (light columns) in five-year intervals from 1970 to 2005 based on MEDLINE searching for the terms ‘diabetes’ or ‘metabolic diseases’.
Despite the rising interest in clinical research, many basic questions regarding metabolism in humans are unanswered or have not been addressed in sufficient detail. A major issue is the role of aging in intermediary metabolism, which was studied in detail in vivo in humans only recently. Petersen et al. (2004) assessed glucose fluxes and ectopic lipid deposition as well as mitochondrial oxidation and phosphorylation under in vivo conditions and found $\sim 40\%$ lower flux rates through the tricarboxylic acid cycle and adenosine-trisphosphate synthesis, possibly explaining the reduced insulin sensitivity and elevated ectopic lipid content in elderly humans. Nevertheless, it remains unclear whether aging is generally associated with impaired insulin sensitivity; specific genetic or even acquired abnormalities could be responsible for reduced mitochondrial function not only during aging but also in other metabolic disorders (Stark & Roden 2007). Likewise, sex and ethnicity can variably affect metabolic characteristics including body fat and its distribution, lipid metabolism and insulin action, which further complicates interpretation of clinical metabolic studies (Woods et al. 2003; Carulli et al. 2005).

These aspects of human metabolism culminate in the recent discussions on definitions of metabolic disorders summarised by the term ‘(dys)metabolic syndrome’ or ‘syndrome X’, which was introduced by Reaven about 20 years ago (Reaven 1988) and increasingly studied over the next decades (Figure 1.2). The World Health Organisation (WHO), the European Group for Insulin Resistance (EGIR), the Adult Treatment Panel (ATP III) and the International Diabetes Foundation (IDF) re-defined the metabolic syndrome, and more definitions are coming up due to different combinations and cut-off points of continuous variables, such as fasting plasma glucose, triglycerides or variable indices of body fat content (Table 1.1). The current discussion focuses on the relative importance of the compounds of the metabolic syndrome and the issue of whether it is a syndrome at all (Kahn et al. 2005). This controversy mostly results from different interpretations and understandings of this term, which can be used 1) to explain a series of factors by one underlying causal factor, be it insulin

![Number of publications](image)

**Figure 1.2** Number of original papers (dark columns) and reviews (light columns) in five-year intervals from 1970 to 2005 based on MEDLINE searching for the term 'metabolic syndrome'.
Table 1.1  Definitions of the metabolic syndrome by the World Health Organisation (WHO), the European Group for Insulin resistance (EGIR), the Adult Treatment Panel (ATP III) and the International Diabetes Foundation (IDF). Key: DM – Diabetes mellitus; IGT – impaired glucose tolerance; IFG – impaired fasting glucose

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main criteria</strong></td>
<td><strong>Main criterion</strong></td>
<td><strong>Main criterion</strong></td>
<td><strong>Main criterion</strong></td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>Insulin resistance</td>
<td>Abdominal obesity</td>
<td>Abdominal obesity</td>
</tr>
<tr>
<td>OR</td>
<td></td>
<td></td>
<td>PLUS</td>
</tr>
<tr>
<td>DM / IGT / IFG</td>
<td></td>
<td></td>
<td>Hyper-triglyceridaemia</td>
</tr>
<tr>
<td><strong>Other components</strong></td>
<td><strong>Other components</strong></td>
<td><strong>Abdominal obesity</strong></td>
<td><strong>Abdominal obesity</strong></td>
</tr>
<tr>
<td>Hypertension</td>
<td>Hyperglycaemia</td>
<td>High triglycerides</td>
<td>Low HDL-C</td>
</tr>
<tr>
<td>≥ 140/90 mmHg</td>
<td></td>
<td>Low HDL-C</td>
<td></td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>Hypertension</td>
<td>≥ 130/85 mmHg High</td>
<td></td>
</tr>
<tr>
<td>Central obesity</td>
<td>Dyslipidaemia</td>
<td>fasting glucose/DM</td>
<td></td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>Central obesity</td>
<td>(two or more)</td>
<td>(two or more)</td>
</tr>
<tr>
<td>(two or more)</td>
<td>(two or more)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

resistance or obesity; 2) to provide an easy cardiovascular risk cluster in addition to the known traditional risk makers such as cigarette smoking or LDL-cholesterol; or 3) to create a novel clinical syndrome or disease requiring specific treatment. We are currently lacking detailed knowledge of the interaction between numerous polygenic and environmental mediators, variation between populations and ethnic groups and time-dependent changes in these continuous variables. Above all, the physiological basis for the several metabolic disorders remains largely unknown at present.

In addition to its relevance for understanding human pathophysiology, clinical metabolic research is becoming important as a tool in clinical trials on non-pharmacological treatment and even more on drug treatment for today’s major epidemiological threats. Of note, searching the MEDLINE detects 580 clinical trials in the 1970s, 1,464 in the 1980s, 5,236 in the 1990s – and already 7,568 between 2000 and 2006.

The sections of this book cover the relevant aspects of current clinical research in humans and are designed to address the researcher’s need for theoretical and practical knowledge in this field.

References

Methods for the Assessment of \( \beta \)-Cell Function \textit{In Vivo}

Andrea Mari and Giovanni Pacini

Introduction

The \( \beta \) cell plays a key role in the maintenance of glucose homeostasis and \( \beta \)-cell dysfunction is a characteristic feature of many states of glucose intolerance. Thus, the assessment of \( \beta \)-cell function is of fundamental importance in the study of metabolic disorders, particularly type 2 diabetes, and in the evaluation of drugs to treat \( \beta \)-cell dysfunction. At the present time, \( \beta \)-cell function continues to be very actively studied, as strategies for preventing the decline of \( \beta \)-cell function and design of drugs that may achieve this effect are a promise for the alleviation of the burden of diabetes.

The scope of this chapter is to provide a comparative and critical account of the most important methods for the assessment of \( \beta \)-cell function \textit{in vivo}. We illustrate the basic technical aspects of the methodologies, referring the reader to the original publications for the details of test protocols. We also try to relate the characteristics of the \textit{in vivo} tests to the \( \beta \)-cell molecular processes, knowledge of which has been greatly increased in the recent years. We hope that this attempt, though imperfect, will improve critical understanding of the methods. Other useful reviews of \( \beta \)-cell function assessment can be found in several journals (Hovorka & Jones 1994; Kahn 2003; Pacini & Mari 2003; Ferrannini & Mari 2004; Mari 2006) and textbooks (Porte et al. 2003; DeFronzo et al. 2004; LeRoith et al. 2004).

Methods for insulin secretion \textit{in vivo}

The assessment of \( \beta \)-cell function requires the determination of the insulin secretory response to a given stimulus and, if the stimulus is not standardised, normalisation of the response to the stimulus. Thus, the evaluation of insulin secretion is a prerequisite for the assessment of \( \beta \)-cell function. The insulin secretory response can be simply determined from insulin concentration, or else from C-peptide levels using more complex methodologies.
Insulin concentration

Simple measurement of plasma insulin is a classic approach still employed in many studies. However, peripheral insulin concentration reflects pancreatic insulin secretion only partially. Insulin in fact undergoes a first pass hepatic removal of about 50%, i.e. about half the insulin secretion never reaches the periphery. Most importantly, insulin clearance, of which hepatic extraction is a major determinant, may vary in different metabolic conditions (Duckworth et al. 1998). Differences in peripheral insulin may not only reflect differences in insulin secretion but also differences in insulin clearance (Ferrannini et al. 1997; Camastra et al. 2005). Nevertheless, the observed insulin concentration profile almost parallels that of insulin secretion, as insulin kinetics is fast. Rapid insulin release, such as that seen in first phase secretion, is clearly reflected in insulin concentration.

C-peptide methods

To avoid the problem of non-constant insulin clearance, an alternative approach based on the measurement of C-peptide was developed almost 30 years ago (Eaton et al. 1980). C-peptide

![Diagram of deconvolution process](image)

**Figure 2.1** Illustration of deconvolution. The mathematical representation of the relationship between insulin secretion (ISR) and C-peptide concentration is based on convolution. The determinant of this relationship is the C-peptide concentration response to a C-peptide bolus injection, which quantitatively describes C-peptide kinetics. Convolution is the operation with which C-peptide concentration is calculated from ISR. If C-peptide kinetics is known (either by direct assessment or using the allometric formula of Van Cauter et al. (1992)), it is possible to reverse the convolution operator and calculate ISR from C-peptide concentration. This operation is called deconvolution and is illustrated in the graphs on the top (data from Figure 2.4). In this example, ISR is represented as a piecewise constant function over one-minute intervals (top left). For a given ISR time-course, the C-peptide kinetic model allows calculation (by convolution) of the corresponding C-peptide concentration. Thus, the ISR values can be determined by fitting the calculated C-peptide values to the measured ones. This is done using a modified least-squares method that ensures a smooth ISR profile. The graphs on top show measured (dots) and fitted (solid line) C-peptide values obtained with this procedure (right graph) and the calculated ISR (left graph).
is co-secreted with insulin in equimolar amounts, undergoes negligible hepatic extraction and has linear and relatively constant kinetics. Thus, C-peptide concentration reflects more precisely true pancreatic insulin secretion, although its time-course, compared to that of insulin concentration, is somewhat blunted and delayed with respect to insulin secretion. For this reason, a mathematical operation, called deconvolution, is used to reconstruct insulin secretion from C-peptide concentration. Deconvolution, illustrated in Figure 2.1, is the mathematical operation with which C-peptide (i.e. insulin) secretion is calculated from C-peptide concentration (see Hovorka & Jones 1994 for details).

To perform deconvolution, C-peptide kinetics must be known. In the original approach (Eaton et al. 1980), followed in several successive studies, C-peptide kinetics was determined by a bolus injection of biosynthetic C-peptide in each individual. In a later study (Van Cauter et al. 1992), the difficulty of the assessment of the individual C-peptide kinetics was circumvented by developing a method by which approximate C-peptide kinetic parameters could be derived from anthropometric measurements. This approach has made wide application of the C-peptide deconvolution methodology possible, and is currently one of the most common methods for insulin secretion.

C-peptide deconvolution remains unfortunately a rather specialised approach, as it requires specific software and technical expertise (Hovorka & Jones 1994). A publicly available (though rather old) program for deconvolution (Hovorka et al. 1996) can be found at http://www.soi.city.ac.uk/~sg331/software.html (web search keywords: isec,site:city.ac.uk).

**β-cell response characteristics**

**in vivo β-cell response**

Figure 2.2 summarises the most relevant characteristics of a normal β-cell response: 1) when glucose is raised gradually, insulin secretion is progressively stimulated (Figure 2.2A) and a dose-response relationship is observed between glucose concentration and insulin secretion (Figure 2.2B). 2) When glucose concentration is briskly increased and maintained at a suprabasal level, insulin secretion shows a biphasic pattern, with an initial burst (first phase insulin secretion) followed by a gradually increasing secretion that approaches a nearly constant level after about 1–2 h (second phase insulin secretion) (Figure 2.2C). The amplitude of both first and second phase response is a function of the glucose increment; the amplitude of the second phase depends on the β-cell dose-response. A biphasic response is also observed with the intravenous glucose tolerance test (IVGTT), in which a strong first phase secretion peak is followed by a slower and more blunted secretion rise. 3) Prolonged exposure to hyperglycaemia produces an increase of the insulin response (both first and second phase). This phenomenon, called potentiation, is clearly visible by repeating the same stimulus after a short rest period (Figure 2.2C). 4) The β-cell response to an oral glucose stimulus, such as an oral glucose tolerance test (OGTT), is considerably higher than that obtained with an intravenous glucose infusion at matched glucose levels (Figure 2.2D). This augmentation of the secretory response is mainly attributed to gut-secreted hormones called incretins, and in particular to glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). 5) The β-cell responds also to various non-glucose stimuli (e.g. some aminoacids, sulphonylureas and glucagon). An aminoacid frequently used in β-cell function testing is arginine, which is a powerful secretagogue. Injection of arginine produces a strong
first phase insulin response, which is potentiated by hyperglycaemia (Figure 2.2E). 6) In the presence of insulin resistance, β-cell function is increased to cope with the increased insulin demand necessary to set resistant glucose uptake and production processes to adequate levels. This phenomenon is exemplified in Figure 2.2F for the acute insulin response (AIR),

**Figure 2.2** Characteristics of the β-cell response: A) insulin secretion (ISR) obtained with a gradual increase in glucose concentration; B) the corresponding dose-response relating glucose concentration to insulin secretion. Redrawn from Byrne et al. (1995); C) response to a repeated square wave of hyperglycaemia (mean glucose levels shown in the bars). Both glucose stimulations produce the typical biphasic secretory pattern. Potentiation of the secretory response is evident in the second stimulus. Redrawn from Cerasi (1981); D) Enhancement of the secretory response observed when glucose is administered orally. The insulin curves are obtained with an OGTT (closed circles and solid line) and with an intravenous glucose infusion reproducing the same glucose levels of the OGTT (open circles and dashed line). Redrawn from Nauck et al. (1986); E) response to arginine injection at different glucose levels. The four smaller peaks are relative to a basal glucose concentration of ~5 mM – glucose levels for the higher peaks are shown in the bars above the peaks. The response to arginine is potentiated by hyperglycaemia. Redrawn from Ward et al. (1984); F) inverse relationship between the minimal model parameter of insulin sensitivity (S_i) and the acute insulin response to an IVGTT (AIR). The solid line represents a hyperbolic interpolation in a group of 93 subjects (the dashed lines represent the dispersion, as percentiles of the disposition index). As a subject becomes insulin resistant (from point S to R), his secretion increases to maintain glucose tolerance normal. Redrawn from Kahn et al. (1993).
A parameter of first phase secretion of the IVGTT (see below). Fasting insulin secretion exhibits a similar relationship with insulin sensitivity.

While the β-cell characteristics illustrated in Figure 2.2 are well known, the relative importance of the various response modes to glucose regulation and the pathophysiology of type 2 diabetes is still debated. The response modes are often but not always correlated (Ferrannini & Mari 2004). Therefore, although the use of a single β-cell function index may be a practical necessity, complete assessment of β-cell function requires multiple indices.

**Cellular processes underlying the β-cell response**

Some background on the relevant cellular processes is useful to shed light on the physiological meaning of the β-cell response characteristics observed in the various tests, although our understanding of the molecular aspects of insulin secretion is still largely incomplete. The molecular aspects of β-cell secretion are discussed in depth in several excellent reviews (Henquin 2000; Rorsman et al. 2000; Bratanova-Tochkova et al. 2002; Henquin et al. 2002; Straub & Sharp 2002).

Figure 2.3 is a simplified representation of the β-cell secretory machinery. Two pathways, denoted as triggering and amplifying (Henquin 2000), are shown. In the triggering pathway, glucose activates exocytosis by increasing cytosolic calcium concentration through a chain of events that ends with the opening of the calcium channels and an influx of extracellular calcium (see the legend to Figure 2.3). Through the amplifying pathway, glucose modulates insulin secretion independently of changes in calcium concentration. Both these pathways are important determinants of the β-cell response.

In the β cell, insulin is stored in granules. Only granules in a specific status (usually denoted as immediately releasable) can undergo exocytosis by activation of the triggering pathway. The translocation of granules from one status to another is an additional determinant of the phasic insulin response. According to some viewpoints, an immediately releasable pool of granules is responsible for first phase insulin release (Daniel et al. 1999), while the second phase involves the translocation of new granules to the plasma membrane (see the reviews cited above).
CH02 METHODS FOR THE ASSESSMENT OF β-CELL FUNCTION IN VIVO

Figure 2.3  Simplified representation of the β cell. In the triggering pathway, glucose metabolism, which depends on glucose influx through the glucose transporters, modulates the ATP/ADP ratio, which increases (block arrow) when glucose concentration and metabolism increase. The increase in the ATP/ADP ratio closes the ATP-sensitive potassium channels; the closure of these channels produces a membrane depolarisation that opens the voltage-dependent calcium channels. Opening of the calcium channels increases the calcium influx and the cytosolic calcium concentration, which triggers exocytosis of the insulin granules that are ready for release. The amplifying pathway, which encompasses complex and incompletely understood phenomena depicted here very schematically, is responsible for increases in insulin secretion independent of changes in cytosolic calcium concentration. This pathway is also activated by glucose metabolism. The incretin hormones (GLP-1 in the figure) are thought to interact with the amplifying pathway to augment insulin secretion. Another key phenomenon in insulin release is the translocation of granules from pools inside the cell to the plasma membrane, as only granules in a particular state on the plasma membrane can be released by calcium-mediated exocytosis.

β-cell function tests

Intravenous vs. oral tests

An important distinction should be made between intravenous and oral β-cell function tests. In fact, ingestion of glucose stimulates the entero-insular axis, i.e. a complex hormonal and neural response that markedly potentiates insulin secretion (Figure 2.2D) (Unger & Eisentraut 1969; Fehmann et al. 1995; Creutzfeldt 2001). The magnitude of the potentiation response depends not only on the degree of neural activation and secretion of gut incretin hormones but also on intrinsic β-cell function, as incretin hormones bind to specific β-cell receptors and activate signalling for secretion (Figure 2.3). Thus, oral tests give a more comprehensive assessment of β-cell function, but they cannot distinguish the intrinsic β-cell defects from those of the entero-insular axis (e.g. a defective GLP-1 production or impaired neural stimulation). In addition, with oral tests the secretory stimulus (e.g. glucose concentration)
cannot be standardised, and thus assessment of β-cell function requires appropriate methods for normalisation of insulin secretion to the stimulus.

**Intravenous glucose tolerance test (IVGTT)**

The IVGTT is the typical test for first phase insulin secretion, although a second phase is also present. For first phase assessment, a 10-min IVGTT is sufficient. However, the IVGTT is often also used to evaluate insulin sensitivity with the minimal model and possibly second phase secretion. Here the test format for the minimal model, which is the most widely used, will be described. More details can be found in Chapter 3.

The IVGTT minimal model protocol (Figure 2.4) is as follows: 1) a standardised glucose bolus (0.3 g/kg body weight) is injected after a baseline control period of about 20–30 min; 2) glucose, insulin and often C-peptide concentrations are measured at frequent intervals (12–30 samples) for 3–4 h. Frequent samples (at 1–2 min intervals) are collected in the initial 8–10 min for first phase assessment; 3) the typical first phase secretion index is the *acute insulin response* (AIR), i.e. the average incremental insulin concentration obtained in the first 5–10 min of the test; 4) second phase insulin secretion is calculated using empirical indices, usually computed from the areas under the insulin and glucose concentration curves, or by modeling (Toffolo et al. 1980; Toffolo et al. 1995; Toffolo et al. 1999), using both insulin and C-peptide; 5) In the insulin-modified IVGTT (shown in Figure 2.4), used to improve the minimal model insulin sensitivity estimate, a standardised insulin dose (0.03–0.05 U/kg) is administered 20 min after the glucose bolus. Exogenous insulin obviously masks the endogenous insulin response. However, if C-peptide concentration is measured, the second phase can be still determined (Toffolo et al. 1999) (with the proviso that exogenous insulin may interfere with endogenous secretion).

The IVGTT, together with the hyperglycaemic clamp, is the typical test for first phase insulin secretion, and AIR is the most widely used index. Assessment of second phase secretion is on the other hand made difficult by the necessity of accounting for glucose levels, which may vary considerably. Compared to AIR, the empirical and the model-based indices of second phase secretion have received limited attention. First phase insulin release depends on the amplitude of the glucose increment after the bolus. However, as the glucose dose is standardised, it is reputed that AIR does not require normalisation to the glucose peak. On the other hand, in normal subjects AIR is dependent on insulin sensitivity (Figure 2.2F). Thus, AIR per se may not be a good index of β-cell function, i.e. comparison of AIR in populations with different insulin sensitivity may lead to inappropriate conclusions. This important problem is discussed in a later section.

One drawback of the IVGTT is that first phase insulin secretion, though important, is only one of the modes of response of the β cell, and is thus insufficient to characterise β-cell function satisfactorily. As known since long time (Seltzer et al. 1967) and re-emphasized recently (Ferrannini & Mari 2004), diabetic subjects may totally lack first phase secretion but still respond to an OGTT.

First phase secretion quite likely represents the discharge of a pool of immediately releasable insulin granules through the activation of the triggering pathway (Daniel et al. 1999; Rorsman et al. 2000; Straub & Sharp 2002). As the magnitude of this pool depends on a complex equilibrium between exocytosis and refilling from a precursor pool, in which several cellular processes are involved, an observed defect of first phase insulin release may result from quite different causes. Therefore, while there is ample evidence that
first phase secretion is a sensitive marker of β-cell function, impairment of this function may not be a primary β-cell defect (Mari 2006).

**Hyperglycaemic glucose clamp**

The hyperglycaemic glucose clamp (DeFronzo et al. 1979) assesses both first and second phase secretion. The protocol is as follows (see Figure 2.5): 1) after an initial baseline control period, an intravenous priming dose of glucose, followed by a variable glucose infusion, is administered to sharply raise glucose concentration to the desired hyperglycaemic value; 2) to keep glucose concentration constant in the successive period, glucose infusion rate is frequently adjusted based on quick bedside measurement of glucose concentration, similarly to the euglycemic clamp (see Chapter 4). An approximate equilibrium is reached after about