A ROLE FOR GLYCOGEN SYNTHASE KINASE 3 BETA IN THE REGULATION OF GLUCAGON GENE TRANSCRIPTION BY INSULIN
CONTENTS

1. INTRODUCTION
   1.1 Glucagon and diabetes mellitus 1
   1.2 The insulin signalling pathways 2
      1.2.1 The PI(3)K pathway 2
      1.2.2 The MAP kinase pathway 3
      1.2.3 Phosphatases in insulin signalling 4
   1.3 Glucagon and pancreatic islets 4
   1.4 The glucagon promoter 5
      1.4.1 Regulation by insulin 5
      1.4.2 Cis-control elements on the glucagon promoter 6
   1.5 Glycogen synthase kinase 3 (GSK3) 8
      1.5.1 Characteristic features of GSK3 8
      1.5.2 Structure of GSK3 and regulation of its enzymatic activity 8
      1.5.3 GSK3 consensus site for phosphorylation 9
      1.5.4 PKB mediated inhibition of GSK3 10
   1.6 GSK3 substrates 10
   1.7 The Wnt pathway 12
   1.8 GSK3 inhibitors 13
   1.9 Aim of the study 14

2. MATERIALS and METHODS
   2.1 MATERIALS 15
      2.1.1 Instruments 15
      2.1.2 Consumables 16
      2.1.3 Antibiotics 16
      2.1.4 GSK3 inhibitors 17
      2.1.5 General Chemicals 17
      2.1.6 Kits 19
      2.1.7 Bacterial culture materials 19
      2.1.8 Eukaryotic cell line 19
      2.1.9 Eukaryotic cell culture materials 19
      2.1.10 General buffers and media 20
2.1 Reporter gene plasmids and expression plasmids 21
2.1.12 Antibodies, proteins, peptides, molecular weight standards and enzymes 22

2.2 METHODS 23
2.2.1 Standard methods of molecular cloning 23
2.2.1.1 Preparation of competent Escherichia coli bacteria 23
2.2.1.2 Transformation of competent bacteria 23
2.2.1.3 Mini preparation of plasmid DNA 23
2.2.1.4 Maxi preparation of plasmid DNA 24
2.2.1.5 Measurement of DNA concentration 25
2.2.1.6 Restriction enzyme analysis of DNA 25
2.2.1.7 Agarose gel electrophoresis 25
2.2.1.8 Purification of DNA from agarose gel 26
2.2.1.9 Blunt-end cloning 26
2.2.1.10 Dephosphorylation of 5' protruding DNA ends 27
2.2.1.11 Ligation 27
2.2.1.12 DNA Sequencing 27
   Sequencing reaction 27
   Sequencing polyacrylamide gel electrophoresis 28
2.2.2 GST-recombinant protein expression and purification in bacteria 29
2.2.3 Affinity purification of GSK3 from InR1G9 cells 30
2.2.4 SDS-polyacrylamide gel electrophoresis 31
2.2.5 Western Blot Analysis 33
2.2.6 Phosphorylation of GST-fusion proteins by recombinant GSK3β in vitro 35
2.2.7 GSK3 activity measured by ex vivo assay 36
2.2.8 Eukaryotic cell culture methods 37
2.2.8.1 Cell culture 37
2.2.8.2 DEAE-Dextran transfection 37
2.2.8.3 Insulin or GSK3 inhibitors treatment 38
2.2.8.4 Cell extract preparation 38
2.2.8.5 Luciferase reporter gene assay 39
2.2.8.6 GFP reporter gene assay 40
2.2.9 Immunocytofluorescence 40
2.2.10 Nuclear Extracts from Tissue Culture Cells 41
2.2.11 Two-dimensional gel electrophoresis 42
2.2.12 MTT test 42
2.2.13 Software 43

3. RESULTS
3.1 Effect of GSK3β overexpression on glucagon gene transcription 44
3.2 Expression and regulation by insulin of endogenous GSK3 activity in InR1G9 cells 45
3. 2. 1 Expression of GSK3 in InRlG9 cells 45
3. 2. 2 Regulation of GSK3 enzymatic activity by insulin in InRlG9 cells 46
3. 3 GSK3 inhibitors and their effect on glucagon gene transcription 48
3. 3. 1 Effect of various GSK3 inhibitors on glucagon gene transcription 48
3. 3. 2 Effect of SB-216763 on glucagon gene transcription in a dose dependent manner 48
3. 3. 3 Effect of GSK3 inhibitors on the transcriptional activity of a CMV promoter 50
3. 3. 4 The GSK3 inhibitors were tested for potential cytotoxic effects via the MTT test 51
3. 3. 5 GSK3 inhibitors abolished the effect of GSK3βwt overexpression on glucagon gene transcription 52
3. 3. 6 Inhibition of GSK3 activity in InRlG9 cells as revealed by ex vivo assay 53
3. 3. 7 GSK3 inhibitors stabilized β-catenin protein levels 54
3. 4 Deletion and internal mutation analysis of the glucagon promoter after treatment of InRlG9 cells with the GSK3 inhibitor, SB-216763 57
3. 4. 1 Effect of SB-216763 on the transcriptional activity of 5'-deleted fragments of the glucagon promoter 57
3. 4. 2 Effect of SB-216763 on the transcriptional activity of 3'-deleted fragments of the glucagon promoter 57
3. 4. 3 Effect of SB-216763 on a glucagon reporter gene that the Pax6 binding sites have been mutated 57
3. 5 Effect of SB-216763 on Pax6 mediated transcriptional activity 60
3. 6 Effect of SB-216763 on CBP mediated transcriptional activity 60
3. 7 Effect of GSK3βwt overexpression on the activity of Gal4-CBP 64
3. 7. 1 Effect of GSK3βwt overexpression on Gal4-CBP activity in the context of the glucagon promoter 64
3. 7. 2 Effect of GSK3βwt overexpression on the transcriptional activity conferred by N-terminal and C-terminal part of CBP 64
3. 7. 3 Mapping the effect of GSK3βwt overexpression within the carboxy-terminal part of CBP 64
3. 8 GSK3β mediated phosphorylation of Pax6 and CBP as revealed by in vitro assay 68
3. 8. 1 Phosphorylation of Pax6 transactivation domain (TAD) by GSK3 in vitro 68
3. 8. 2 Phosphorylation of a C-terminal part of CBP consisting of amino acids 2040-2305 by GSK3β in vitro 68
3. 9 Two-dimensional gel electrophoresis of nuclear proteins from InRlG9 cells 70
### 4. DISCUSSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 GSK3 expression in InR1G9 cells and inhibition of enzymatic activity by insulin-triggered phosphorylation</td>
<td>72</td>
</tr>
<tr>
<td>4.2 Regulation of glucagon gene transcription by GSK3β</td>
<td>73</td>
</tr>
<tr>
<td>4.3 GSK3 responsive element in the glucagon promoter</td>
<td>75</td>
</tr>
<tr>
<td>4.4 GSK3 regulates transcriptional activity and phosphorylation of Pax6 <em>in vitro</em></td>
<td>76</td>
</tr>
<tr>
<td>4.5 GSK3 regulates transcriptional activity and phosphorylation of CBP <em>in vitro</em></td>
<td>77</td>
</tr>
<tr>
<td>4.6 Final concept</td>
<td>78</td>
</tr>
<tr>
<td>4.7 Perspectives</td>
<td>80</td>
</tr>
<tr>
<td>4.7.1 GSK3 inhibitors as novel antidiabetic drugs?</td>
<td>80</td>
</tr>
<tr>
<td>4.7.2 Studying insulin resistance in InR1G9 cells</td>
<td>80</td>
</tr>
</tbody>
</table>

### REFERENCES

81