Uncoupling Proteins: 
Regulation by IGF-1 and Neuroprotection 
during Hyperglycemia in Vitro

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Abstract

Diabetic neuropathy is believed to arise due to oxidative stress following hyperglycemic situations. Uncoupling proteins (UCPs) constitute a subgroup of mitochondrial transporter proteins with putative antioxidant properties. By dissipating the proton gradient over the mitochondrial inner membrane, these proteins reduce the mitochondrial inner membrane potential (MMP), and thereby, the mitochondrial production of reactive oxygen species (ROS) is decreased. In this thesis I have examined the regulation of UCP2, UCP3, and UCP4 by the neuroprotective hormone insulin-like growth factor type 1 (IGF-1). I have also investigated the possible involvement of UCP3 in IGF-1-mediated neuroprotection following high glucose treatments. All studies were performed using human neuroblastoma SH-SY5Y cells as an in vitro cell model. The major findings were as follows:

i. Native SH-SY5Y cells expressed UCP2, UCP3, and UCP4.

ii. UCP3 was upregulated by IGF-1 via activation of the IGF-1 receptor. IGF-1 increased UCP3 mRNA and protein levels primarily via activation of the “classical” anti-apoptotic phosphatidylinositol 3 (PI3)-kinase signaling pathway, as shown by incubation with specific inhibitors of the PI3-kinase and mitogen activated protein (MAP) kinase signaling pathways.

iii. UCP2 and UCP4 protein levels were only marginally or not at all regulated by IGF-1. These UCPs are probably not involved in IGF-1-mediated neuroprotection.

iv. High glucose concentrations reduced the UCP3 protein levels in highly differentiated SH-SY5Y cells. Concomitantly, the MMP and the levels of ROS and glutathione increased, whereas the number of neurites per cell was reduced. This supports an antioxidant and neuroprotective role of UCP3.

v. IGF-1 prevented the glucose-induced reduction in UCP3 protein levels. In parallel, the effects on MMP, levels of ROS and glutathione, and number of neurites per cell were abolished or significantly reduced. These data suggest that UCP3 is involved in IGF-1-mediated neuroprotection.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by Roman numerals (I-IV) in the text:


CONTENTS

1. INTRODUCTION ................................................................. 9

1.1 Diabetes mellitus ............................................................ 9
1.1.1 Diabetic neuropathy ...................................................... 9

1.2 Insulin-like growth factor type 1 ...................................... 10
1.2.1 IGF-1 signaling .......................................................... 11
1.2.2 IGF-1, neuronal proliferation, and differentiation .......... 13
1.2.3 IGF-1 and neuronal survival ......................................... 13
1.2.4 IGF-1 and neuroprotection during diabetes .................. 14

1.3 Mitochondria and oxidative stress .................................. 15
1.3.1 Mitochondrial ROS production .................................... 16
1.3.2 Antioxidants; the cellular defense ............................... 18

1.4 Glucose and oxidative stress .......................................... 20
1.4.1 Increased polyol pathway flux ...................................... 21
1.4.2 Formation of advanced glycated end products ............... 22
1.4.3 Protein kinase C hyperactivity ...................................... 24
1.4.4 Mitochondrial dysfunction .......................................... 24

1.5 Uncoupling proteins ...................................................... 26
1.5.1 UCP1 ............................................................................ 27
1.5.2 UCP2 and UCP3 .......................................................... 27
1.5.3 UCP4 and UCP5 .......................................................... 28
1.5.4 Function(s) in the nervous system ................................. 29
1.5.5 UCPs as antioxidants ................................................. 30
1.5.6 UCPs and IGF-1 .......................................................... 32
1.5.7 UCPs and diabetes ...................................................... 32
1.5.8 Hypothesis of thesis .................................................... 33

2. AIMS OF THE STUDY ....................................................... 34

3. METHODOLOGICAL CONSIDERATIONS ....................... 35

3.1 Cell system ................................................................. 35
3.1.1 Native SH-SY5Y cells .................................................. 35
3.1.2 Differentiated SH-SY5Y cells ....................................... 35
3.2 Cell treatments ............................................................ 36
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE(s)</td>
<td>advanced glycated end product(s)</td>
</tr>
<tr>
<td>AR</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>CM-H₂DCFDA®</td>
<td>chloromethyl-dichlorodihydrofluorescein diacetate, acetyl ester</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>DCF</td>
<td>chloromethyl-dichlorodihydrofluorescein (or CM-H₂DCF)</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1 and 2 (or p44/p42)</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transfer chain</td>
</tr>
<tr>
<td>FAD, FADH₂</td>
<td>oxidized and reduced flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide (oxidized glutathione)</td>
</tr>
<tr>
<td>IGF-1, IGF-2</td>
<td>insulin-like growth factor type 1 and 2</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP kinase/ERK kinase</td>
</tr>
<tr>
<td>MKK1, MKK2</td>
<td>MAP kinase kinase 1 and 2</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>MTG</td>
<td>MitoTracker Green FM®</td>
</tr>
<tr>
<td>MTR</td>
<td>MitoTracker Red CMXRos®</td>
</tr>
<tr>
<td>NAD⁺, NADH</td>
<td>oxidized and reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP⁺, NADPH</td>
<td>oxidized and reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>PDK</td>
<td>phosphoinositide dependent kinase</td>
</tr>
<tr>
<td>PI3</td>
<td>phosphatidylinositol 3</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (or Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PI(s)</td>
<td>phosphatidylinositol(s)</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycated end products</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDH</td>
<td>sorbitol dehydrogenase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology/collagen</td>
</tr>
<tr>
<td>SOD2</td>
<td>superoxide dismutase 2</td>
</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>UCP(s)</td>
<td>uncoupling protein(s)</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1 Diabetes mellitus

During the last decades, diabetes mellitus has emerged as one of the major threats to human health. Worldwide, the increase in number of cases has reached epidemic proportions, and no decline is to be expected in the near future. Instead, the number of people with diabetes is forecasted to increase globally by 46% in 10 years, from 151 million in 2000 to 221 million in 2010, and may afflict as many as 300 million in 2025 (Amos et al., 1997; King et al., 1998).

There are two main forms of diabetes. Type 1 diabetes typically appears in early childhood. This form is caused by an auto-immune destruction of pancreatic $\beta$-cells, resulting in absolute insulin deficiency. Patients suffering from Type 1 diabetes are therefore dependent on a life-long administration of exogenous insulin for survival. Type 2 diabetes normally appears later in life and is characterized by insulin resistance (i.e. reduced insulin responsiveness in target tissues) and abnormal insulin secretion. Although type 2 diabetes represents the major form of diabetes, accounting for more than 90% of the cases (Zimmet et al., 2001), the molecular mechanisms underlying the disorder are poorly understood. However, obesity and sedentary life style are known to be important causative factors.

Even though type 1 and type 2 diabetes have different casual backgrounds, the physiological net effect is the same. The complete lack of or the partially reduced insulin signaling leads to disturbed glucose homeostasis, as manifested by chronically elevated blood glucose concentrations (hyperglycemia). Hyperglycemia, in turn, probably induces the multiple secondary complications of diabetes, affecting nerves (neuropathy), retina (retinopathy), kidneys (nephropathy), and the circulatory system (cardio-vascular impairments).

1.1.1 Diabetic neuropathy

Diabetic peripheral neuropathy is the most common form of peripheral neuropathy in developed countries, afflicting more than 50% of the diabetic patients. The most frequent type is distal symmetric polyneuropathy (see Vinik et al., 2000), which primarily involves degeneration of sensory and
motor neurons in nerves projecting the extremities (i.e. feet and hands). This degeneration results in hyperalgesic pain, loss of sensation, and reduced muscle control in the lower limbs, and may in severe cases require amputations. Indeed, diabetic neuropathy is the leading cause of non-traumatic amputations in Western countries (Vinik et al., 2000).

Although diabetic neuropathy is a multifactorial disorder, affecting all components of the nerve (i.e. axons, Schwann cells, extracellular matrix, and neurovasculature), the pathogenesis of the degeneration is poorly understood. However, since the severity of the neuropathy is enhanced when the hyperglycemic situation is uncontrolled, excessive glucose is implicated as a causative factor (The Diabetes Control and Complications Trial Research Group, 1993). It has been suggested that the neuroprotective hormone insulin-like growth factor type 1 (IGF-1) can prevent diabetic neuropathy (see Ishii, 1995). In this thesis, I have examined whether the so called uncoupling proteins (UCPs) are involved in this IGF-1-mediated protection from diabetic neuropathy.

1.2 Insulin-like growth factor type 1

IGF-1 (originally known as somatomedin) is a hormone important for the normal development and cell maintenance in many tissues, including the nervous system. It belongs to the same family of growth factors as insulin and insulin-like growth factor type 2 (IGF-2), and these three proteins also share several functional properties. IGF-1 is a single-chain polypeptide consisting of 70 amino acids with three interchain disulfide bridges, and the molecular weight is 7.6 kDa (Sara and Hall, 1990).

The major source of IGF-1 in humans is the liver. The IGF-1 synthesis is regulated by growth hormone (GH), and it is secreted into the bloodstream to act on target cells in an endocrine manner (Carter et al., 2002). Although the human serum concentration of IGF-1 is nearly 100 nM, its bioavailability is markedly reduced due to binding to carrier molecules, the so called IGF-binding proteins (Sara and Hall, 1990). IGF-1 may reach the central and peripheral nervous system (CNS and PNS) through active transport across the blood brain barrier (Reinhardt and Bondy, 1994; Trejo et al., 2001). However, it is also produced within the CNS and PNS, where it acts locally in autocrine and paracrine manners.
IGF-1 is expressed at high levels in brain during the embryonic development, implicating a role of IGF-1 in neuronal growth and maturation (Feldman et al., 1997). Recent data demonstrate, however, that IGF-1 is important also in adult stages, especially as a neuronal maintaining and survival-promoting factor. Before I discuss these functions further, a short review of the major intracellular IGF-1 signaling pathways is required.

1.2.1 IGF-1 signaling

Most of the effects of IGF-1 are mediated by the IGF-1 receptor, to which IGF-1 has an affinity in the low nanomolar range. IGF-1 may also bind to IGF-2 and insulin receptors, albeit with more than a 10- and 100-fold lower affinity, respectively (Mattsson et al., 1990). The IGF-1 receptor belongs to the large family of receptors with intrinsic tyrosine kinase activity (the RTK family). It is a heterotetramer consisting of two ligand binding α-subunits located on the extracellular side of the plasma membrane, and two membrane spanning β-subunits that exhibit the tyrosine kinase activity (Fig. 1) (see Butler et al., 1998). The subunits are linked by disulfide bridges. The α-subunits of peripherally expressed IGF-1 receptors have a molecular weight of approx. 135 kDa, whereas the weight of α-subunits expressed in adult brain neurons is 115-120 kDa (Burgess et al., 1987). The molecular weight of the β-subunits is approx. 95 kDa (Sara and Hall, 1990).

Binding of a ligand to the α-subunits of the IGF-1 receptor results in a rapid auto-phosphorylation on tyrosine residues of the β-subunits (Butler et al., 1998). The phosphorylated receptor recruits and binds insulin receptor substrates (IRS1-4) and different isoforms of the Src homology/collagen (Shc) protein (p46, p52 or p66), which subsequently become tyrosine phosphorylated. These phosphorylated proteins are then recognized by proteins containing Src homology 2 (SH2)-domains, such as Grb2 and p85, which trigger activation of two major signaling cascades (Fig. 1).

1.2.1.1 MAP kinase pathway

Docking of Grb2 to either IRS or Shc results in the recruitment of Ras, which subsequently becomes activated through a GDP/GTP exchange mediated by the Grb-associated Son of sevenless (Sos). Activated Ras, in turn, initiates a sequence of kinase phosphorylations, known as the mitogen activated protein (MAP) kinase cascade (see Kolch, 2000). First, Ras phosphorylates the small serine kinase Raf, which next phosphorylates MAP kinase kinase 1 (M KK1 or MEK) and MAP kinase kinase 2 (M KK2). Finally, these proteins
phosphorylate the extracellular signal-regulated kinases 1 and 2 (ERK1/2 or p44/p42) on threonine and tyrosine residues at position 202 and 204, respectively. (Kolch, 2000).

Fig. 1. IGF-1 receptor signaling in neurons. Stimulation of the IGF-1 receptor activates two major signaling pathways: the MAP kinase cascade (left) and the PI3-kinase pathway (right). The PI3-kinase pathway is primarily involved in neuronal survival and differentiation, whereas the MAP kinase pathway promotes neuronal proliferation and differentiation. See text for details. IGF-1R, IGF-1 receptor; p70S6K, ribosomal p70S6 kinase. -P indicates protein phosphorylations.

1.2.1.2 PI3-kinase pathway

p85, another protein containing a SH2-domain, constitutes the regulatory subunit of the class 1a phosphatidylinositol 3 (PI3)-kinases. To date, there are 7 p85-isoforms identified in mammals, derived from three separate genes (p85α, p85β and p55γ) (see Anderson and Jackson, 2003). Binding of p85 to activated IRS (Fig. 1) relocates the cytosolic PI3-kinase to the inner surface of the cell membrane. There, the catalytic subunit of PI3-kinase, p110, can phosphorylate phosphatidylinositol inositols (PIs) present in the membrane, predominantly yielding P(3,4,5)IP₃ from P(4,5)IP₂. Phosphorylated PIs function as second messengers, recruiting various cytosolic serine/threonine kinases to the cell membrane. Among them are Akt (or protein kinase B, PKB)
and ribosomal p70S6 kinase, which subsequently become activated by phosphoinositide dependent kinases (PDK) (see Marte and Downward, 1997; Datta et al., 1999; Brunet et al., 2001). In mammals, the PI3-kinase p110 subunit is encoded by at least four genes (Anderson and Jackson, 2003).

1.2.2 IGF-1, neuronal proliferation, and differentiation

IGF-1 induces proliferation of neurons both in vitro and in vivo (Mattsson et al., 1990; Åberg et al., 2000; Kurihara et al., 2000; Östlund et al., 2001; Trejo et al., 2001), a feature probably reflecting the function of IGF-1 in neuronal development and embryogenesis. Although the neuronal proliferation is low in the adult brain, there are neurons with lifelong neurogenesis, such as hippocampal granule neurons. Recently, IGF-1 was shown to modulate the proliferation of these neurons in both hypophysectomized (i.e. GH-depleted) rats and in rats with increased serum IGF-1 levels as induced by treadmill exercise (Åberg et al., 2000; Trejo et al., 2001). These findings implicate a proliferative role of IGF-1 in the nervous system also in adult stages. Kurihara and co-workers (2000) have shown in SH-SY5Y cells that a simultaneous activation of the PI3-kinase and the MAP kinase signaling pathways are necessary for IGF-1-mediated proliferation (Fig. 1).

In parallel with increased proliferation, IGF-1 also induces neuronal differentiation. For instance, IGF-1 stimulates dendritic growth in primary cortical neurons (Niblock et al., 2000). In neuroblastoma cell lines (SH-SY5Y, IMR32) IGF-1-treatment leads to lamellopodia extension, membrane ruffling and neurite outgrowth (Kim et al., 1997; Leventhal et al., 1997; Kim et al., 1998a; Kim et al., 1998b; Kurihara et al., 2000; Kim et al., 2004). The membrane ruffling is dependent on PI3-kinase activation (Kim et al., 1998b; Kim et al., 2004), whereas the neurite formation requires activation of both MAP kinase and PI3-kinase (Fig. 1) (Kim et al., 1998a; Kurihara et al., 2000; Kim et al., 2004).

1.2.3 IGF-1 and neuronal survival

Lately, the importance of IGF-1 in protection of neurons from toxic insults has become increasingly evident. IGF-1 protects neurons from various types of damage, some of which are exemplified in Table 1. The mechanisms for the survival are primarily dependent on PI3-kinase signaling (Fig. 1, Table 1), and involve both changed gene expression and post-translational modifications of cytosolic proteins. For instance, PI3-kinase/Akt-mediated phosphorylation of Forkhead transcription factors inhibits transcription of pro-apoptotic genes,
while phosphorylation of IκB, the negative regulatory factor of nuclear factor κB (NFκB), promotes expression of anti-apoptotic genes (Datta et al., 1999; Brunet et al., 2001). This is illustrated in neuroblastoma cells, where IGF-1 increases the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Singleton et al., 1996; Offen et al., 2001). Furthermore, PI3-kinase/Akt can increase survival directly through inhibition of the pro-apoptotic proteins Bad and caspase 9 (Datta et al., 1999; Brunet et al., 2001).

### Tabel 1. Examples of neuronal insults prevented by IGF-1. Proposed signaling pathways are also indicated.

<table>
<thead>
<tr>
<th>Insult</th>
<th>IGF-1 signaling pathway</th>
<th>Model system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+ and serum deprivation</td>
<td>PI3-kinase/Akt</td>
<td>Rat cerebellar granule neurons</td>
<td>Dudek et al., 1997</td>
</tr>
<tr>
<td>Serum deprivation</td>
<td>PI3-kinase (partially)</td>
<td>Rat cerebral cortical neurons</td>
<td>Yamada et al., 2001</td>
</tr>
<tr>
<td>Serum deprivation</td>
<td>PI3-kinase/Akt/FKHRL1</td>
<td>Rat hippocampal neurons, PC12 cells</td>
<td>Zheng et al., 2002</td>
</tr>
<tr>
<td>K+ deprivation</td>
<td>PI3-kinase</td>
<td>Rat cerebellar granule neurons</td>
<td>Miller et al., 1997</td>
</tr>
<tr>
<td>NGF withdrawal</td>
<td>PI3-kinase</td>
<td>Rat DRG neurons</td>
<td>Russell et al., 1998</td>
</tr>
<tr>
<td>Amyloid β-peptide</td>
<td>PI3-kinase/JNK/ERK1/2</td>
<td>SH-SY5Y cells</td>
<td>Wei et al., 2002</td>
</tr>
<tr>
<td>Dopamine treatment</td>
<td>Not determined</td>
<td>Rat cerebellar granule neurons</td>
<td>Offen et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SK-N-SH cells</td>
<td></td>
</tr>
<tr>
<td>Hyperosmotic stress</td>
<td>Not determined</td>
<td>SH-SY5Y cells</td>
<td>van Golen and Feldman, 2000</td>
</tr>
<tr>
<td>Hyperosmotic stress</td>
<td>Akt</td>
<td>SH-SY5Y cells</td>
<td>Stoothoff and Johnson, 2001</td>
</tr>
<tr>
<td>H2O2</td>
<td>PI3-kinase/NFκB</td>
<td>Rat cerebellar granule neurons</td>
<td>Heck et al., 1999</td>
</tr>
</tbody>
</table>

FKHRL1, Forkhead transcription factor; NGF, nerve growth factor; PC12, rat pheochromocytoma cell line; SH-SY5Y and SK-N-SH, human neuroblastoma cell lines

### 1.2.4 IGF-1 and neuroprotection during diabetes

Since IGF-1 is important for neuronal maintenance and survival, disturbed IGF-1 signaling might be detrimental to neurons, leading to reduced neurotrophic support, axonal degeneration, and neuronal cell loss.
Intriguingly, both the levels of IGF-1 and the IGF-1 receptor are reduced in states of diabetes. For instance, in streptozotocin (STZ)-induced diabetic rats (a type 1 diabetes animal model), the IGF-1 mRNA levels are decreased in liver, sciatic nerve, spinal cord, and superial cervical ganglia (Ishii et al., 1994; Wuarin et al., 1994; Bitar et al., 1997; Zhuang et al., 1997). Neurons from superial cervical ganglia also exhibit decreased IGF-1 mRNA receptor levels, as well as number of specific IGF-1 binding sites (Bitar et al., 1997). Furthermore, the levels of IGF-1 and IGF-1 receptors are reduced in blood samples of type 2 diabetic patients (Migdalis et al., 1995). Importantly, changed IGF-1 activity in diabetic rats and humans correlates with increased nerve dysfunction (Ishii et al., 1994; Migdalis et al., 1995), and in diabetic rats, administration of exogenous IGF-1 reversed the established neuronal damages associated with autonomic neuropathy (Schmidt et al., 1999). These data clearly indicate that IGF-1 prevents diabetes-induced neuronal damages, and reduced IGF-1 activity may hence significantly contribute to the development of diabetic neuropathy (see Ishii, 1995).

The molecular mechanisms by which IGF-1 protects neurons during diabetes are poorly understood. A possible pathway is IGF-1-mediated inhibition of the c-Jun N-terminal kinase (JNK). This kinase, together with p38, constitutes a subset of MAP kinases involved in cell death signaling and is primarily activated by stressful stimuli, such as osmotic and oxidative stress (see Harper and LoGrasso, 2001). In cultured adult rat dorsal root ganglia (DRG) neurons and SH-SY5Y cells, it has been demonstrated that high glucose concentrations induce phosphorylation of both p38 and JNK (Cheng and Feldman, 1998; Purves et al., 2001). Activation of these proteins was also observed in DRG neurons of STZ-induced diabetic rats and in sural nerves of diabetic patients (Purves et al., 2001). IGF-1 is known to prevent phosphorylation of JNK induced by glucose and other stimuli (Cheng and Feldman, 1998; Okubo et al., 1998; Wei et al., 2002), and may thus inhibit JNK signaling. IGF-1 has also been shown to protect SH-SY5Y cells and embryonic rat DRG neurons from both caspase 3 activation and apoptosis following treatment with high glucose concentrations in vitro (Russell et al., 1999; Tanii et al., 2000).

1.3 Mitochondria and oxidative stress

Reactive oxygen species (ROS) are highly reactive molecules formed during normal cell activity in reactions where molecular oxygen (O2) serves as the
electron acceptor. In this process free radicals, i.e. species with an unpaired electron such as superoxide anion ($O_2^-$), or non-radicals such as hydrogen peroxide ($H_2O_2$) are generated (Table 2).

Excessive levels of ROS are highly detrimental to cells. ROS induce cell damage either directly through infliction with cellular macromolecules (proteins, lipids, and nucleic acids), or indirectly via activation of intracellular stress signaling pathways that induce expression of genes related to cell damage (see Evans et al., 2002). During evolution cells have developed a network of compensatory antioxidant mechanisms, in order to neutralize and eliminate the ROS. However, in some situations the ROS production and the antioxidant defense may be seriously imbalanced. This results in a state known as oxidative stress, which eventually can lead to cell damage, apoptosis, or necrosis, depending on the severity.

<table>
<thead>
<tr>
<th>Table 2. Examples of reactive oxygen species.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td>Free radicals</td>
</tr>
<tr>
<td>Superoxide anion</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>Peroxyl radical</td>
</tr>
<tr>
<td>Hydroperoxyl radical</td>
</tr>
<tr>
<td>Non-radicals</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Hydrochlorous acid</td>
</tr>
</tbody>
</table>

### 1.3.1 Mitochondrial ROS production

A major cellular source of ROS is the mitochondria (Loschen et al., 1971; Boveris et al., 1972; Loschen et al., 1974; Turrens and Boveris, 1980). These organelles consist of two phospholipid membranes, where the outer membrane faces the cytosol and the inner, highly folded membrane, surrounds the mitochondrial interior; the matrix. The space between the two membranes is termed the intermembrane space.

Mitochondria are often described as cellular “power-plants” because of their ability to conserve energy released from oxidation of glucose and lipids in the form of ATP. This is accomplished by the stepwise process known as the electron transfer chain (ETC), involving four enzyme complexes and ATP synthase. Both the ETC complexes and ATP synthase are situated in the mitochondrial inner membrane.
1.3.1.1 Electron transfer chain

The ETC is initiated by the donation of electrons from NADH and FADH$_2$ to complex I (NADH-dehydrogenase) and complex II (succinate dehydrogenase, Fig. 2) (see Nicholls and Budd, 2000). NADH and FADH$_2$ are generated during the oxidation of glucose and lipids in the series of reactions that constitute the glycolysis, Krebs cycle, and β-oxidation. The electrons are shuttled from complex I and II to complex III (ubiquinone-cytochrome C oxidoreductase) by ubiquinone, and to complex IV (cytochrome oxidase) by cytochrome C. In the final step of the ETC, complex IV transfers four electrons to O$_2$, which together with four protons yields two water molecules (Fig. 2).

The transport of electrons through complex I, III and IV is coupled to the transfer of protons from the matrix side of the mitochondrial inner membrane to the intermembrane space. This leads to the generation of a mitochondrial membrane potential (MMP) in the form of an electrochemical proton gradient, which is used as a driving force by ATP synthase to produce ATP (oxidative phosphorylation, Fig. 2).
1.3.1.2 Generation of ROS from the ETC

It has been known for decades that the ETC is intimately correlated with generation of ROS (Loschen et al., 1971; Boveris et al., 1972; Loschen et al., 1974; Turrens and Boveris, 1980), and that the rate of ROS production is dependent on the metabolic state of the mitochondria (Loschen et al., 1971). The ATP synthesis is controlled by the availability of ADP (and Pi), which, in turn, is dependent on the cell activity. When the ATP consumption is low, i.e. when the cell is at rest (state 4 respiration), re-entry of protons to the matrix through ATP synthase is reduced due to the lack of ADP. Continued activity of ETC leads to further increased MMP (hyperpolarization), which progresses until a critical value of the MMP is reached, and the proton transfer of the complexes is inhibited. This inhibition prolongs the half life of the reduced ETC electron carriers and enhances the risk that surrounding O₂ becomes reduced to O₂⁻, particularly at the interface between ubiquinol and complex III (Fig. 2) (see Cadenas and Davies, 2000; Nicholls and Budd, 2000). Of the total daily oxygen consumption, as much as 1-2% goes to the generation of O₂⁻ by the ETC (Cadenas and Davies, 2000). Thus, mitochondria may contribute substantially to the total cellular ROS production.

1.3.2 Antioxidants; the cellular defense

The ROS production is balanced by the cellular antioxidant defense systems. Halliwell (1995) defined an antioxidant as “any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate”. Hence, the group of antioxidants is highly miscellaneous, embracing both enzymes and non-enzymatic substances, and their strategies to protect the cell from oxidative stress are diverse. Here, I will present the antioxidant mechanisms that are particularly important for elimination of O₂⁻ produced by the ETC. These are schematically outlined in Fig. 3.

1.3.2.1 Detoxification of ETC-generated ROS

In normal situations, O₂⁻ generated by the ETC is immediately converted to H₂O₂ by superoxide dismutase 2 (SOD2) present in the mitochondria (Fig. 3) (Loschen et al., 1974; see Cadenas and Davies, 2000; Evans et al., 2002). SOD2 is relatively abundant in the mitochondrial matrix, and is hence localized at high levels at the site of the ROS-production.
The produced mitochondrial \( \text{H}_2\text{O}_2 \) is in close proximity to reduced pools of copper and iron (\( \text{Cu}^+ \) and \( \text{Fe}^{2+} \)) present in the mitochondrial inner membrane. Thus, if \( \text{H}_2\text{O}_2 \) is not quickly eliminated, there is a significant risk that these transition metal ions catalyze the conversion of \( \text{H}_2\text{O}_2 \) to the highly reactive hydroxyl radical, \( \cdot\text{OH} \), through Fenton chemistry (Fig. 3) (Cadenas and Davies, 2000; Evans et al., 2002). The \( \cdot\text{OH} \), which easily diffuses from the site of production, attacks most molecules present in the cell (Halliwell, 1995), and can hence induce significant cellular damage. To avoid this scenario, \( \text{H}_2\text{O}_2 \) is quickly converted to \( \text{H}_2\text{O} \) by glutathione peroxidase present in the matrix, in a reaction where reduced glutathione (GSH) is used as electron donor (Fig. 3). Alternatively, \( \text{H}_2\text{O}_2 \) diffuses into the cytosol where it is detoxified by catalase in peroxisomes (Evans et al., 2002). This reaction yields \( \text{H}_2\text{O} \) and \( \text{O}_2 \) (Fig. 3).

1.3.2.2 Glutathione

Glutathione (L-\( \gamma \)-glutamyl-L-cysteinglycine) is the main non-protein substance involved in the cellular antioxidant defense. It is a tripeptide, consisting of cystein, glutamic acid, and glycine, and the thiol (-SH) group of the cystein residue constitutes its reactive group (see Dringen and Hirrlinger, 2003; Pastore et al., 2003). Glutathione is produced in cells by the subsequent actions of \( \gamma \)-glutamylcystein syntethase and glutathione syntethase, and it may approach concentrations up to 10 mM intracellularly. Since mitochondria lack the enzymes for glutathione synthesis, glutathione is actively transported into the mitochondrial matrix, where it deals with ETC-generated ROS. Within the
cytosol and organelles, free glutathione is present mainly as GSH (Pastore et al., 2003), and there is also a protein-bound glutathione fraction.

Although glutathione may scavenge free radicals directly, the main mechanism by which glutathione function as an antioxidant is as a cofactor for enzymes involved in the detoxification of ROS. For instance, during elimination of peroxides by GSH peroxidase, GSH is used as an electron donor, and becomes in this reaction oxidized to glutathione disulfide (GSSG, Fig. 4). GSSG is re-converted to GSH by GSSG reductase, a process that is dependent on NADPH as cofactor. Together, these reactions are referred to as the glutathione redox cycle (Fig. 4) (Dringen and Hirrlinger, 2003; Pastore et al., 2003).

Besides being an antioxidant, glutathione also has other cellular functions (Pastore et al., 2003). For instance, it is an important intracellular reducing agent involved in the maintenance of thiol groups of other molecules. Glutathione also conjugates with various xenobiotics through a reaction catalyzed by glutathione S-transferase. This detoxifies the xenobiotics, and allows them to be excreted in the urine (phase II toxicokinetic reactions).

![Glutathione redox cycle](image)

**Fig. 4.** Glutathione redox cycle. When peroxides (R-O-O-H) are detoxified by GSH peroxidase, GSH is oxidized to GSSG. The re-convertion of GSH by GSSG reductase is dependent on NADPH as a cofactor.

### 1.4 Glucose and oxidative stress

As pointed out in Section 1.1.1, a causative factor of diabetic neuropathy is probably uncontrolled hyperglycemia (The Diabetes Control and Complications Trial Research Group, 1993). There are multiple hypotheses about how glucose induces cell damage, which are highly interrelated and synergistic. Lately, oxidative stress has emerged as a unifying mechanism of these pathways (see Greene et al., 1999; Brownlee, 2001; Evans et al., 2002).
Below, I will describe some of the best characterized pathways, \textit{i.e.} the polyol pathway, formation of advanced glycated end products (AGEs), protein kinase C (PKC) hyperactivity, and mitochondrial dysfunction, and their relation to oxidative stress.

### 1.4.1 Increased polyol pathway flux

In the first step of the polyol pathway, glucose is reduced by the enzyme aldose reductase (AR) to sorbitol. During this reaction NADPH is oxidized to NADP$^+$ (Fig. 5). Since AR is acting only on the straight-chained conformation of the glucose molecule and not on the predominant “chair” conformation, the affinity of AR for glucose \textit{in vivo} is low ($K_m = 100-400$ mM, Oates, 2002). Thus, in non-diabetic individuals with low blood glucose concentrations, reduction of glucose by AR is a scare event, whereas in hyperglycemic patients substantial levels of sorbitol may be formed. The endogenous substrate for AR is unknown.

The second step of the polyol pathway is oxidation of sorbitol to fructose by the enzyme sorbitol dehydrogenase (SDH, Fig. 5). In this reaction, the cofactor NAD$^+$ is reduced to NADH.

![Fig. 5. The polyol pathway. Glucose is reduced to sorbitol by AR with NADPH as cofactor. Next, sorbitol is oxidized to fructose by SDH, during which NADH is formed. Increased concentrations of sorbitol and fructose may induce osmotic stress. Depletion of NADPH inhibits re-reduction of GSSG to GSH. Raised levels of NADH may serve as substrate for ETC in the mitochondria or be oxidized by NADH oxidase, both of which result in ROS generation.](image)

### 1.4.1.1 Polyol pathway and oxidative stress

The proposed mechanisms for how increased polyol pathway activity is detrimental to neurons are numerous (see Oates, 2002). Originally, increased intracellular concentrations of the reaction products, \textit{i.e.} sorbitol and fructose, were believed to induce osmotic stress. Lately, however, the changed balance of the cofactors has received great attention. Hyperactivity of AR depletes the
cell from NADPH (Cheng and Gonzalez, 1986; Lee and Chung, 1999), which is necessary for the reformation of GSH from GSSG by GSSG reductase (Fig. 5, cf. 1.3.2). Thereby the cellular antioxidant defense is reduced. Furthermore, raised levels of NADH following SDH activity may induce oxidative stress through oxidation of NADH by NADH oxidase (Kim et al., 2002), or when NADH is used as substrate for the ETC in the mitochondria (cf. 1.3.1, Oates, 2002).

1.4.2 Formation of advanced glycated end products

AGEs is the collective term for the stable adducts formed from non-enzymatic reactions between glucose or its metabolites and proteins, nucleotides, or lipids. The classical and most studied glycation process involves the formation of Amadori products (see Kikuchi et al., 2003). When glucose reacts with amino groups, Schiff’s bases are formed, which subsequently may undergo Amadori rearrangements, yielding relatively stable fructosamine adducts (the Amadori product, see Fig. 6). After a complex cascade of reactions, these fructosamine adducts are irreversibly converted to stable AGEs (Kikuchi et al., 2003).

Recently, the importance of highly reactive α-oxoaldehydes, e.g. glyoxal, methylglyoxal, and 3-deoxyglucosone, in AGE formation has become increasingly evident (see Brownlee, 2001; Thornalley, 2002). These AGE precursors, which react with amino groups of intracellular or extracellular proteins to form AGEs, are initially formed from glucose through several biochemical mechanisms (Fig. 6). Glucose may undergo autooxidation to form glyoxal (Wells-Knecht et al., 1995), and thereby directly contribute to the AGE precursor pool. In addition, glucose enhances AGE precursor formation through increased glycolysis, leading to accumulation of glyceraldehyde-3-phosphate that is fragmented to methylglyoxal (Brownlee, 2001). Both the Schiff’s base and the fructosamine can also generate AGE precursors (Thornalley et al., 1999), and additionally, lipid peroxidation may lead to formation of glyoxal (Fu et al., 1996) (Fig. 6). Since the α-oxoaldehydes are formed intracellularly, it seems likely that elevated glucose concentrations inside cells are the primary trigger for formation of both intracellular and extracellular AGEs (Brownlee, 2001; Thornalley, 2002).

AGEs are detrimental to the nerve by three general mechanisms. First, modifications of intracellular proteins may result in altered protein functions. For instance, glycation of tubulin and actin may prevent their polymerization
to form microtubules and microfilament. Second, modification of extracellular proteins may lead to abnormal interaction with components of the extracellular matrix (Brownlee, 2001). Finally, extracellular AGEs may interact with the receptor for AGE (RAGE) (Neeper et al., 1992).

Fig. 6. Formation of AGEs and its relation to oxidative stress. The classical pathway for AGE formation includes generation of an Amadori product (fructosamine adduct), a reaction that occurs intra- and extracellularly. AGEs are also formed from intracellular AGE precursors (reactive α-oxoaldehydes) that are generated from autoxidation of glucose, degradation of Amadori products and glycolytic intermediates, and from lipid peroxidation. AGE formation leads to generation of ROS directly or through interaction with RAGE. ROS, in turn, can enhance AGE formation via increased accumulation of glycolytic intermediates, lipidperoxidation, and inhibition of AGE precursor detoxification systems. R-NH₂, amino group

1.4.2.1 AGE and oxidative stress
AGE formation and oxidative stress are highly interrelated. Beside a direct production of ROS through autooxidation of glucose (Greene et al., 1999), the formation of AGEs is enhanced by oxidative stress (Fig. 6). For instance, ROS inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Du et al., 2003), which leads to accumulation of glyceraldehyde-3-phosphate and increased methylglyoxal production. Furthermore, glucose-induced ROS production by the ETC may promote lipid peroxidation, thus resulting in increased glyoxal formation (Fig. 6) (Fu et al., 1996). ROS also
deplete cells of GSH and NADPH, which constitute important cofactors for the AGE precursor detoxification systems (e.g. the glyoxalase system) (Thornalley, 2002). Extracellular AGEs may in turn enhance oxidative stress through activation of RAGE via an unknown mechanism. However, it was shown recently that RAGE activates NADPH oxidase (Wautier et al., 2001), an enzyme known to substantially contribute to cellular ROS production in many cell types (Inoguchi et al., 2000; Lee et al., 2003).

1.4.3 Protein kinase C hyperactivity
The classical isoforms of the PKC family are activated by 1,2-diacylglycerol (DAG) and Ca\(^{2+}\). In states of hyperglycemia, glucose may generate increased \textit{de novo} synthesis of DAG (see Eichberg, 2002), with a subsequent activation of PKC. In addition, glucose-induced oxidative stress may increase PKC activity. PKC can in turn enhance the oxidative stress through activation of NADPH oxidase (Inoguchi et al., 2000), thus forming a viscous circle.

PKC hyperactivity is highly linked to diabetes-induced endothelial dysfunction, leading to microvascular insufficiencies. This is particularly important for the development of diabetic retinopathy and nephropathy (Tomlinson, 1999). However, in diabetic nerves, the DAG levels are rather decreased, and there are no consistencies in the changes of PKC activity (Eichberg, 2002), thus contradicting the involvement of PKC in diabetic neuropathy. Despite this, PKC inhibitors corrected reduced Na,K-ATPase activity and nerve conduction velocity in nerves of STZ-induced diabetic rats (Cameron et al., 1999; Cameron and Cotter, 2002), implicating that increased PKC activity indeed is important for diabetic neuropathy. PKC may play a significant role in the development of the glucose-induced neurovascular dysfunction, leading to reduced nerve blood flow and nerve ischemia. Whether this effect is important for the onset of diabetic neuropathy or just accelerates the neurodegeneration, is, however, uncertain (Tomlinson, 1999; Eichberg, 2002).

1.4.4 Mitochondrial dysfunction
Lately, the mitochondrion has occupied a central position in the research of diabetic neuropathy. Hyperglycemia affects numerous of factors that are directly related to mitochondrial function (Table 3). For instance, in DRG neurons isolated from STZ-induced diabetic rats, as well as in neurons treated with high glucose concentrations \textit{in vitro}, the mitochondria are depolarized (Srinivasan et al., 2000; Russell et al., 2002; Vincent et al., 2002; Huang et al.,
2003; Vincent et al., 2004). Furthermore, hyperglycemia leads to decreased Bcl-2 expression (Srinivasan et al., 2000), increased intracellular concentrations of Ca\(^{2+}\) (Huang et al., 2003), and translocation of cytochrome C into the cytoplasm (Srinivasan et al., 2000; Russell et al., 2002), all features of mitochondrial dysfunction.

Table 3. Mitochondrial-related effects of hyperglycemia or treatments with high glucose concentrations in neurons.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Model system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial depolarization</td>
<td>DRG neurons from STZ-rats*</td>
<td>Srinivasan et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Russell et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Vincent et al., 2002</td>
</tr>
<tr>
<td></td>
<td>DRG neurons from STZ-rats†</td>
<td>Huang et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Vincent et al., 2004</td>
</tr>
<tr>
<td>↓ Cytochrome oxidase activity</td>
<td>DRG sections from STZ-rats¶</td>
<td>Schmeichel et al., 2003</td>
</tr>
<tr>
<td>↑ Mitochondrial ROS production</td>
<td>Primary rat DRG neurons</td>
<td>Russell et al., 2002</td>
</tr>
<tr>
<td></td>
<td>SH-SY5Y cells</td>
<td>Vincent et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Vincent et al., 2004</td>
</tr>
<tr>
<td>↑ Intracellular Ca(^{2+}) concentrations</td>
<td>DRG neurons from STZ-rats†</td>
<td>Huang et al., 2003</td>
</tr>
<tr>
<td>↓ Bcl-2 expression</td>
<td>DRG neurons from STZ-rats*</td>
<td>Srinivasan et al., 2000</td>
</tr>
<tr>
<td>Cytochrome C translocation</td>
<td>DRG neurons from STZ-rats*</td>
<td>Srinivasan et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Russell et al., 2002</td>
</tr>
<tr>
<td>Mitochondrial swelling</td>
<td>DRG neurons from STZ-rats*</td>
<td>Russell et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Primary rat DRG neurons</td>
<td>Russell et al., 2002</td>
</tr>
</tbody>
</table>

SH-SY5Y, human neuroblastoma cell line; STZ-rats, STZ-induced diabetic rats. *4-6 weeks, †8 weeks or ¶12 months of diabetes.

Recently, a mechanism for hyperglycemia-induced damage of mitochondria was suggested (Nishikawa et al., 2000). Excessive glycolysis following hyperglycemia leads to increased generation of NADH and FADH\(_2\). These cofactors are used as electron donors in the ETC, resulting in increased ETC-activity and raised MMP. Thereby the mitochondrial production of O\(_2^•\)- increases (cf. 1.3.1), which, in turn, induces oxidative stress (Nishikawa et al., 2000; Du et al., 2001; Russell et al., 2002; Vincent et al., 2002; Vincent et al., 2004). Indeed, high glucose treatments increase the mitochondrial ROS production in both primary rat DRG neuron and in neuroblastoma SH-SY5Y cells (Table 3) (Russell et al., 2002; Vincent et al., 2002; Vincent et al., 2004).
In rat DRG neurons, Schwann cells, and SH-SY5Y cells, inhibition of the glucose-induced increase in MMP by ETC-inhibitors prevents the subsequent mitochondrial ROS production, MMP depolarization, cytochrome C translocation, as well as mitochondrial swelling (Russell et al., 2002; Vincent et al., 2002). Furthermore, in bovine aortic endothelial and human mesangial cells, normalization of elevated MMP corrected the glucose-induced activation of the polyol pathway, formation of AGEs, PKC hyperactivity, as well as other events implicated in diabetic complications (Du et al., 2000; Nishikawa et al., 2000; Du et al., 2001; Du et al., 2003; Kiritoshi et al., 2003). These data clearly indicate that regulation of MMP represents an attractive target for prevention of glucose-induced oxidative stress, cellular damage and progression of diabetic neuropathy.

### 1.5 Uncoupling proteins

Uncoupling proteins (UCPs) belong to a family of anion transporter molecules situated in the inner membrane of mitochondria. As indicated by the term “uncoupling”, these proteins have the ability to uncouple oxygen consumption from oxidative phosphorylation, i.e. ATP synthesis, through induction of transport of protons from the mitochondrial intermembrane space into the matrix (see Fig. 9). During this process, the proton gradient becomes relaxed, the MMP decreased, and stored energy may be released as heat. The exact mechanism for how the UCPs dissipate the proton gradient remains unknown.

![Fig 7. Schematic model of topology of UCPs in the mitochondrial inner membrane. The UCPs are believed to consist of 6 transmembrane α-helices connected with hydrophilic loops. Both the amino (N)- and the carboxy (C)-terminals face the intermembrane space. (Adapted from Rousset et al., 2004).](image)

To date, there are five UCPs identified in mammals, numbered from UCP1 to UCP5. All these proteins are approx. 300 amino acids long with a molecular
weight of about 35 kDa (Table 4). The three-dimensional structures of the UCPs are unknown, but based on their amino acid sequence they are believed to be composed of six transmembrane α-helices connected with hydrophilic loops, with the amino- and carboxy-terminals facing the intermembrane space (Fig. 7) (see Dalgaard and Pedersen, 2001; Rousset et al., 2004). The UCPs are suggested to form homodimers within the mitochondrial inner membrane (Dalgaard and Pedersen, 2001).

1.5.1 UCP1

UCP1, originally known as thermogenin, is the best characterized UCP, and was also the first to be cloned nearly 20 years ago (Jacobsson et al., 1985). The protein is exclusively expressed in brown adipose tissue (BAT, Table 4), where it is involved in heat production during non-shivering thermogenesis. This primarily occurs in rodents that are exposed to cold or arousing from hibernation and in infants at birth. The thermogenesis is induced by stimulation of the β-adrenergic signaling system (see Cannon and Nedergaard, 2004). UCP1 is activated by free fatty acids (FFA) and inhibited by purine di- or tri-phosphate nucleotides, with the highest sensitivity to GDP (Dalgaard and Pedersen, 2001; Cannon and Nedergaard, 2004; Rousset et al., 2004).

<table>
<thead>
<tr>
<th>Protein</th>
<th>No of amino acids</th>
<th>MW (kDa)</th>
<th>Tissue or cell type of distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1</td>
<td>307</td>
<td>33</td>
<td>BAT</td>
</tr>
<tr>
<td>UCP2</td>
<td>309</td>
<td>33</td>
<td>spleen, lung, stomach, intestines, WAT, macrophages, pancreatic β-cells, brain, spinal cord</td>
</tr>
<tr>
<td>UCP3</td>
<td>312</td>
<td>34</td>
<td>skeletal muscle, heart, BAT, DRG neurons, spinal cord</td>
</tr>
<tr>
<td>UCP4</td>
<td>323</td>
<td>36</td>
<td>brain</td>
</tr>
<tr>
<td>UCP5</td>
<td>325</td>
<td>36</td>
<td>brain, heart, kidney</td>
</tr>
</tbody>
</table>

MW, molecular weight; no, number

1.5.2 UCP2 and UCP3

In 1997, two novel homologous isoforms of UCP1 were identified, consecutively named UCP2 and UCP3 (Boss et al., 1997; Fleury et al., 1997; Gimeno et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997). UCP2 is expressed in numerous tissues, including spleen, lung, stomach, intestine, and
white adipose tissue (WAT) (Pecqueur et al., 2001; Zhang et al., 2001), see Table 4. In addition, UCP2 protein has been recognized in structures of the nervous system, for instance in the hypothalamus, in the spinal cord, and in cortical neurons (Horvath et al., 1999; Horvath et al., 2002; Mattiasson et al., 2003).

In contrast to the ubiquitous UCP2, UCP3 is generally considered skeletal muscle specific, although it is also expressed at low levels in heart and BAT (Table 4) (Boss et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997; Gong et al., 2000; Vidal-Puig et al., 2000; Cadenas et al., 2002). Most recently, UCP3 was also detected in the nervous system: the UCP3 mRNA and protein was identified in mouse spinal cord and rat DRG neurons, respectively (Vincent et al., 2002; Dupuis et al., 2003; Vincent et al., 2004).

UCP2 and UCP3 were originally believed to regulate energy expenditure and body weight (Boss et al., 1997; Fleury et al., 1997; Gimeno et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997). Indeed, mice overexpressing UCP3 are leaner than their wildtype littermates and have reduced adipose tissue mass (Clapham et al., 2000; Cadenas et al., 2002). However, knockout mice lacking UCP2 or UCP3 are neither obese nor have decreased metabolic rate or changed energy expenditure (Arsenijevic et al., 2000; Gong et al., 2000; Vidal-Puig et al., 2000; Cline et al., 2001; Cadenas et al., 2002). These findings indicate that UCP2 and UCP3 are not directly involved in the control of energy metabolism, but that the effect on body mass rather are indirect effects of so-far unidentified protein functions. Lately, evidence implicates UCP2 in regulation of pancreatic insulin secretion and in macrophage function (Arsenijevic et al., 2000; Zhang et al., 2001), whereas the physiological role of UCP3 remains elusive. Recent data suggest, however, that UCPs may work as antioxidants.

1.5.3 UCP4 and UCP5

Only a few years after the identification of UCP2 and UCP3, two other mammalian UCPs were cloned, i.e. UCP4 (Mao et al., 1999) and UCP5 (or brain mitochondrial carrier protein 1) (Sanchis et al., 1998). These proteins are more distantly related to the “original” group of UCPs, exhibiting an amino acid identity of 35-40% with UCP3 (Fig. 8). UCP4 is exclusively expressed in the brain (Mao et al., 1999), while UCP5 also is expressed in kidney and heart (Table 4) (Sanchis et al., 1998).
1.5.4 Function(s) in the nervous system

Of the five cloned mammalian UCPs, four are expressed within CNS and PNS. This indicates that presence of UCPs in the nervous tissue is important for the normal neuronal function. As summarized in Table 5, UCPs have been implicated in various types of neuronal activities, ranging from regulation of synaptic neurotransmission (Horvath et al., 1999; Horvath et al., 2002; Yamada et al., 2003) to control of brain temperature (Horvath et al., 1999; Yu et al., 2000).

Lately, mounting evidence suggests, however, that UCPs are involved in the protection of neurons (Table 5). For instance, in rat brain, mRNA expression of UCP2 and UCP5 is increased following aging (Kondou et al., 2000; Mizuno et al., 2000), and in rat cortical neurons, ischemia-treatment raised both the mRNA and protein levels of UCP2 (Mattiasson et al., 2003). Furthermore, in mice and PC12 cells that overexpress UCP2, cells are protected from damage and cell death induced by ischemia, brain injury, seizures, and H$_2$O$_2$-treatment (Table 5) (Bechmann et al., 2002; Diano et al., 2003; Mattiasson et al., 2003). Sullivan and coworkers (2003) also showed that in low-fat-fed rat pups, UCP-activity in brain is reduced, which in turn enhanced the neuronal vulnerability to kainic acid-induced seizures. All of the neuroprotective effects are believed to be related to the antioxidant properties of the UCPs.
Tabel 5. Suggested functions of UCPs in the nervous system

<table>
<thead>
<tr>
<th>Suggested function</th>
<th>Protein</th>
<th>Model system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotransmission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine secretion</td>
<td>UCP2</td>
<td>UCP2-transfected PC12h cells</td>
<td>Yamada et al., 2003</td>
</tr>
<tr>
<td>Pain or temperature sensation</td>
<td>UCP2</td>
<td>Mouse/primate spinal cord</td>
<td>Horvath et al., 2002</td>
</tr>
<tr>
<td>Thermal synapses</td>
<td>UCP2</td>
<td>Rat hypothalamus</td>
<td>Horvath et al., 1999</td>
</tr>
<tr>
<td>Thermal effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adaptation to cold</td>
<td>UCP4</td>
<td>Mouse brain</td>
<td>Yu et al., 2000</td>
</tr>
<tr>
<td></td>
<td>UCP5</td>
<td>Mouse brain</td>
<td>Yu et al., 2000</td>
</tr>
<tr>
<td>Heating the brain</td>
<td>UCP2</td>
<td>Rat brain</td>
<td>Horvath et al., 1999</td>
</tr>
<tr>
<td>Neuroprotection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging</td>
<td>UCP2</td>
<td>Rat brain</td>
<td>Mizuno et al., 2000</td>
</tr>
<tr>
<td></td>
<td>UCP5</td>
<td>Rat brain</td>
<td>Kondou et al., 2000</td>
</tr>
<tr>
<td>Ischemia</td>
<td>UCP2</td>
<td>UCP2tg-mouse brain</td>
<td>Mattiasson et al., 2003</td>
</tr>
<tr>
<td></td>
<td>UCP2</td>
<td>Primary rat cortical neurons</td>
<td>Mattiasson et al., 2003</td>
</tr>
<tr>
<td>Brain impact or lesions</td>
<td>UCP2</td>
<td>UCP2tg-mouse cortex</td>
<td>Bechmann et al., 2002</td>
</tr>
<tr>
<td></td>
<td>UCP2</td>
<td>UCP2tg-mouse cortex</td>
<td>Mattiasson et al., 2003</td>
</tr>
<tr>
<td>Epileptic seizures</td>
<td>UCP2</td>
<td>UCP2tg-mouse hippocampus</td>
<td>Diano et al., 2003</td>
</tr>
<tr>
<td>Kainic acid-induced seizures</td>
<td>UCP2</td>
<td>Rat limbic neurons</td>
<td>Sullivan et al., 2003</td>
</tr>
<tr>
<td>H2O2-induced oxidative stress</td>
<td>UCP2</td>
<td>UCP2-transfected PC12 cells</td>
<td>Diano et al., 2003</td>
</tr>
<tr>
<td>Glucose-induced oxidative stress</td>
<td>UCP3</td>
<td>UCP3-transfected embryonic rat DRG neurons</td>
<td>Vincent et al., 2004</td>
</tr>
</tbody>
</table>

PC12, rat pheochromocytoma cell line; PC12h, PC12 subclone; UCP2tg-mouse, mouse transgenic for UCP2

1.5.5 UCPs as antioxidants

In cells, most of the produced O2− is generated by the mitochondrial ETC (cf. 1.3.1), particularly in states when respiration is low and MMP high (state 4 respiration). UCPs would in this sense function as antioxidants by inducing mild uncoupling, that is increasing the proton transport from the mitochondrial intermembrane space into the matrix, and thus lowering the MMP below the critical level for the O2− production (Fig. 9) (Nègre-Salvayre et al., 1997).

The idea of UCPs as antioxidants was first formulated by Nègre-Salvayre and coworkers in 1997, who demonstrated that the UCP-inhibitor GDP increased the mitochondrial H2O2 generation in mitochondrial fractions from liver, thymus, and spleen. Since then, the hypothesis has been tested and supported by several groups. For instance, in knockout mice lacking UCP2 or UCP3, both
the ROS production and markers of oxidative damage are higher than in their wildtype littermates (Arsenijevic et al., 2000; Vidal-Puig et al., 2000; Brand et al., 2002). Furthermore, in several cell types, including rat embryonic DRG neurons and neuroblastoma GT1-1 cells, overexpression of UCP1, UCP3, and UCP5 reduced the mitochondrial ROS production (Nishikawa et al., 2000; Kim-Han et al., 2001; Vincent et al., 2004). Intriguingly, $\text{O}_2^{\bullet-}$ present in the mitochondrial matrix was recently shown to activate UCP1, UCP2 and UCP3 (Echtay et al., 2002a; Echtay et al., 2002b; Krauss et al., 2003; Talbot et al., 2004), implicating the existence of a negative feedback response to overproduction of ROS from the ETC.

![Fig. 9. Mechanism for antioxidant activity of UCPs. Mitochondrial $\text{O}_2^{\bullet-}$ production from the ETC is enhanced when the re-entry of protons (H$^+$) to the matrix through ATP synthase is low and the MMP high. UCPs transport protons across the mitochondrial inner membrane into the matrix by an unknown mechanism. Thereby, the MMP is decreased below the critical value for $\text{O}_2^{\bullet-}$ production.](image)

According to these findings, the physiological function of the novel UCPs (UCP2-UCP5) is to participate in the cellular defense from oxidative stress. Of this follows that reduced UCP activity in neurons, particularly in states of enhanced oxidative stress, would lead to increased neuronal damage and cell loss. However, this may be prevented by upregulation of UCP expression. Identification of factors that can affect UCP expression is therefore of great interest.
1.5.6 UCPs and IGF-1

One attractive candidate for mediating regulation of UCP expression in neurons is IGF-1. This idea is based on the observation that UCP1 mRNA is induced by IGF-1 in brown adipocytes (Guerra et al., 1994; Lorenzo et al., 1996; Porras et al., 1998; Teruel et al., 1998). The signal is mediated via the PI3-kinase dependent pathway (Teruel et al., 1998), although the MAP kinase pathway may (Teruel et al., 1998) or may not (Porras et al., 1998) also be involved. In addition, GH-administration increased the levels UCP3 mRNA in skeletal muscle and adipose tissue of GH-deficient patients, and in parallel the concentration of serum IGF-1 was enhanced (Pedersen et al., 1999), thus further supporting a UCP-regulatory role of IGF-1. As already noted, IGF-1 is an important neurotrophic factor that protects neurons from numerous types of insults, including hyperglycemia and diabetes (cf. 1.2.4). If IGF-1 also regulates expression of UCPs in neurons, it is possible some of the neuroprotective effects exerted by IGF-1 involve UCPs.

1.5.7 UCPs and diabetes

As described in Section 1.4, a suggested mechanism for development of diabetic neuropathy is increased ETC-flux and mitochondrial dysfunction following elevated intracellular glucose concentrations. In this context, UCPs are highly interesting. There is a possibility that UCPs expressed in peripheral neurons would correct or slow down the progression of diabetic neuropathy via mild control of MMP. Indeed, overexpression of UCP1 and UCP3 in bovine aortic endothelial cells as well as in rat DRG neurons, prevented glucose-induced mitochondrial ROS production and many of the subsequent cellular and mitochondrial effects (Du et al., 2000; Nishikawa et al., 2000; Du et al., 2001; Vincent et al., 2002; Du et al., 2003; Vincent et al., 2004).

Intriguingly, the UCP expression is affected in states of diabetes. In muscles of patients with type 2 diabetes, the UCP3 expression is reduced (Krook et al., 1998; Schrauwen et al., 2001), and in some forms of type 2 diabetes, mutations in the UCP3 gene have been identified (Argyropoulos et al., 1998; Otabe et al., 1999). Furthermore, high glucose treatments in vitro decreased the UCP3 mRNA expression in rat skeletal muscle (Pedersen et al., 2001), and similar results were obtained recently in rat DRG neurons, when the UCP3 protein level was examined (Vincent et al., 2004). The latter study also demonstrated reduced UCP3 protein expression in DRG neurons of STZ-induced diabetic rats (Vincent et al., 2004).
1.5.8 Hypothesis of thesis
These exciting data open for the possibility that defective UCP(3) expression in peripheral neurons during states of diabetes would enhance the mitochondrial ROS production and thereby contribute to the development of diabetic neuropathy. This would be prevented by a possible IGF-1-mediated regulation of the UCP expression. In the present study, this hypothesis was tested using human neuroblastoma SH-SY5Y cells as an in vitro cell model.
2. AIMS OF THE STUDY

i. To examine the expression of UCP2, UCP3 and UCP4 in SH-SY5Y cells (Papers I and III)

ii. To explore the regulation of UCP2, UCP3 and UCP4 expression by IGF-1 (Papers I, II and III)

iii. To investigate the role of UCP3 in IGF-1-mediated neuroprotection from glucose-induced oxidative stress (Paper IV)
3. METHODOLOGICAL CONSIDERATIONS

In this part, theoretical aspects of the methods used in the thesis are briefly discussed. For detailed descriptions, the reader is referred to the original papers.

3.1 Cell system

Throughout this study I have used the human neuroblastoma SH-SY5Y cell line, either in its native or differentiated form.

3.1.1 Native SH-SY5Y cells (Paper I, II and III)

The human neuroblastoma SH-SY5Y cell line is a subclone of the SK-N-SH cell line, which was established from a bone marrow aspirate of a metastatic tumor of a 4-year old girl in 1971 (Biedler et al., 1973; Biedler et al., 1978). The cells show several neuronal characteristics in their native form. Morphologically, they are small and dense with triangular-shaped cell bodies that possess short and delicate processes (Fig. 10A). The cells express acetylcholine receptors, as well as enzymes involved in adrenergic neurotransmitter biosynthesis (Biedler et al., 1978; Adem et al., 1987), and they have a low resting membrane potential (Tosetti et al., 1998). SH-SY5Y cells also exhibit intact IGF-1- and insulin- signaling systems, with both functional receptors and downstream signaling molecules (Mattsson et al., 1990; Kim et al., 1997; Kim et al., 1998a; Kim et al., 1998b; Kurihara et al., 2000).

3.1.2 Differentiated SH-SY5Y cells (Paper IV)

The native SH-SY5Y cells can be further differentiated into a mature neuronal phenotype by incubation with retinoic acid (RA) (Pålman et al., 1984). During this process they cease to proliferate, while developing a fine network of neurites with synaptic contacts (Fig. 10B). Furthermore, the number of muscarinic acetylcholine receptors increases, which is accompanied by enhanced membrane excitability (Adem et al., 1987; Tosetti et al., 1998).

In order to obtain highly differentiated neuronal cells, native SH-SY5Y cells were cultivated with 1 µM RA in serum-free defined N2-medium (Bottenstein and Sato, 1979) for 6 days. Unlike their native counterparts, these cells are sensitive to high glucose concentrations. For instance, high glucose-treatment clearly affects the cell morphology, yielding cells with both shorter and fewer
neurite processes (Fig. 10C). Hence, in Paper IV, these differentiated cells were used as a cell model for “diabetic” neurons.

Fig. 10. Human neuroblastoma SH-SY5Y cells. Native cells (A), highly differentiated cells obtained by incubation with 1 µM RA for 6 days (B), and highly differentiated cells incubated with 60 mM glucose for 3 days (C). Note the shorter and reduced number of neurites in (C) as compared to (B). Cells originally magnified 200x.

3.2 Cell treatments

3.2.1 UCP expression and IGF-1 regulation (Papers I, II and III)

In Papers I, II and III, expression of UCPs in SH-SY5Y cells and their regulation by IGF-1 were examined. Native cells were starved for growth factors for 18 h in insulin-free N2 medium (N2 medium with all the supplements except insulin), after which treatments with human recombinant IGF-1 or insulin started. The treatments lasted for up to 96 h.

In order to discriminate between signaling from the IGF-1 receptor and the insulin receptor, a truncated variant of IGF-1 was administered to the cells. This molecule lacks the last carboxy-terminus amino acid residue of IGF-1 and is only 20% as potent at IR binding as the authentic IGF-1. Its affinity to the IGF-1R remains, however, unaffected (Eriksson et al., 1996).

I also investigated the contribution of the PI3-kinase and MAP kinase signaling pathways in IGF-1-mediated UCP expression. Cells were treated with 10 nM IGF-1, together with LY294002 and PD98059 (Fig. 11). LY294002 is a specific PI3-kinase inhibitor that competitively binds to the ATP-binding site of the 110 kDa catalytic subunit (Vlahos et al., 1994), whereas PD98059 specifically interacts with the inactive form of MEK (or MKK1) in a
noncompetitive manner (Alessi et al., 1995). Thereby the access of upstream activating enzymes (e.g. Raf) is blocked. PD98059 also works as a weak inhibitor of MKK2.

Fig. 11. Chemical structures of the PI3 kinase inhibitor LY294002 (A) and the MEK inhibitor PD98059 (B).

3.2.2 Protection by IGF-1 during high glucose treatment (Paper IV)
Highly differentiated and growth factor starved SH-SY5Y cells were exposed to 8, 17, 30, or 60 mM glucose in insulin-free N2 medium, together with 10 nM human recombinant IGF-1. To cells treated without IGF-1, 10 nM human recombinant insulin was added as a survival factor. This insulin concentration neither affects the IGF-1 receptor nor the UCP3 expression (Mattsson et al., 1990; Paper I). The osmolarity of the high glucose media was 305 mOsm/l for 17 mM glucose, 319 mOsm/l for 30 mM glucose, and 346 mOsm/l for 60 mM glucose. Treatments lasted for 72 h.

3.3 mRNA expression

3.3.1 Northern blot analysis (Paper II)
In Paper II, changes in UCP3 mRNA expression following IGF-1 treatment was detected by Northern blot analysis. Total RNA was separated in a denaturing agarose gel and blotted onto a nylon membrane. Once immobilized on the membrane, the UCP3 mRNA was detected by hybridization with a UCP3 specific probe, i.e. a cDNA sequence that was complementary to a part of the UCP3 mRNA sequence and radioactively labeled with [32P]-dCTP. After exposure to a phosphor imager screen, the UCP3 mRNA signal was quantified by the use of a computer program. In order to compensate for sample variability caused by differences in RNA
extraction and handling, the mRNA of the house keeping gene β-actin was used as internal standard.

A major benefit of Northern blot analysis is that it quantitatively detects changes in mRNA expression. The method is, however, comprised with severe limitations, especially when the samples under examination are of low quantity or when the target RNA is rare. Therefore, reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were also conducted.

### 3.3.2 RT-PCR analysis (Papers I and II)

RT-PCR is an extremely sensitive method that detects small, but physiologically relevant, changes in expression of a particular mRNA. It consists of two reactions, which in this thesis were performed separately. In the first reaction, extracted total RNA was transcribed to cDNA using reverse transcriptase and random hexamer primers. Thereafter, the UCP3 cDNA was amplified by PCR with specific primers for human UCP3. Although RT-PCR constitutes a powerful and simple tool for detecting changes in mRNA expression, the method is accompanied with problems. As the PCR greatly amplifies the target cDNA, errors are also amplified, which can lead to large variability between samples and obscure accurate quantification. Therefore, a careful valuation of the experimental design and choice of quantification method is crucial when performing a RT-PCR. In Paper II, UCP3 mRNA levels were quantified using an approach of kinetic RT-PCR for endpoint analysis (Freeman et al., 1999). This technique is based on the mathematical theory behind PCR:

The amount of PCR-product yielded in a PCR is expressed by the equation

\[ N = N_0 \times (1+E)^n \]  

(1)

or

\[ \log(N) = \log(1+E)n + \log(N_0) \]  

(2)

where \( N \) is the PCR-product (amount measured after \( n \) cycles), \( N_0 \) the amount of target cDNA, \( n \) the number of repeated cycles, and \( E \) the efficiency of the reaction (proportion of target cDNA copied in a cycle). This gives, that when the amount of PCR-product from a series of cycles is plotted on a logarithmic scale against cycle number, the \( y \)-intercept of the created line is equal to the logarithm of the initial target cDNA amount (\( N_0 \)), as stated by Equation (2).
Hence, in Paper II, 10-µl samples were removed from the PCR reaction after cycle 22, 25, 28 and 31 and analyzed on agarose gel (Fig. 12A). The logarithm of the density of the bands in the gel, as quantified by a computer program, was plotted against cycle number, and the y-intercept, corresponding to the amount of UCP3 cDNA at start of the PCR (N₀), was calculated by linear regression (Fig. 12B).

![Fig. 12. Kinetic RT-PCR for endpoint analysis.](image)

Quantification according to this approach does not, however, take into consideration sample variability caused by differences in RNA isolation and efficiency in the RT-reaction. In order to adjust for this, UCP3 mRNA levels were normalized to the amount of ribosomal 18S RNA in samples. The 18S cDNA was amplified by the use of QuantumRNA™ 18S Internal Standards, a system developed by Ambion®. This technique is based on the addition of 18S competimers to the PCR-mixture along with 18S primers. The competimers, which are identical to the 18S primers but with altered 3′-ends that block extension by DNA polymerase, compete with the primers for 18S cDNA hybridization. Whenever a competimer binds to a 18S cDNA molecule, that particular molecule cannot act as template in the PCR reaction. Consequently, the availability of the 18S cDNA in the PCR-reaction is reduced, leading to an amplification of 18S in the same linear range as the cDNA under study.
3.4 Protein expression

3.4.1 Western blot analysis (Papers I, II, III and IV)
Western blot analyses were performed in order to investigate the protein expression of the UCP proteins, IGF-1 receptors, insulin receptors, and the phosphorylation state of the IGF-1 receptor, Akt, and the MAP kinases ERK1 and ERK2. Cells were lysed in a hypotonic buffer containing 1% Triton X-100 and protease inhibitors for 10 min. Thereafter, the extracted proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Unequal protein loading and uneven protein transfer were controlled for by staining the membranes with Ponceau S; a red-colored dye that reversibly binds to positively charged amino acid groups of proteins. The individual proteins under study were subsequently detected by immunoblotting with appropriate antibodies.

In Western blot analyses, the use of primary and secondary antibodies results in a several-fold amplification of the detection signal that significantly enhances the sensitivity of the method. However, this also means that the final performance and interpretation of the results are highly dependent on the specificity of the antibodies. Data about the primary antibodies used in this thesis for UCP detection are presented in Table 6.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope mapping</th>
<th>Supplier</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP2 (C-20)</td>
<td>C-terminus of human UCP2</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>III</td>
</tr>
<tr>
<td>UCP31-A</td>
<td>Between 2nd and 3rd TM domains of human UCP3</td>
<td>Alpha Diagnostics International, Inc.</td>
<td>I, II</td>
</tr>
<tr>
<td>UCP32-A (AB3046)</td>
<td>Near C-terminus of human UCP3</td>
<td>Chemicon International, Inc.</td>
<td>IV</td>
</tr>
<tr>
<td>UCP4 (G15)</td>
<td>Near N-terminus of human UCP4</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>III</td>
</tr>
</tbody>
</table>

C, carboxy; N, amino; TM, transmembrane

3.4.2 Immunofluorescence (Paper I)
In addition to Western blot analysis, UCP3 protein was detected by immunofluorescence. By this method it is possible to both detect and determine the intracellular localization of a protein. After cell fixation with
paraformaldehyde and permeabilization with 0.1% Triton X-100, UCP3 was visualized by incubation with the UCP31-A antibody (Table 6) and an Alexa 488-conjugated IgG secondary antibody. Mitochondria were detected by incubation with MitoTracker Red CMXRos® (MTR, Fig. 13A) prior to cell fixation. Images of the UCP3 and mitochondria signals were created by fluorescence microscopy.

### 3.5 Neuroprotection during high glucose treatment

In paper IV, cellular changes induced by high glucose treatments in highly differentiated SH-SY5Y cells were assayed. Along with examination of the effects on cell neurites and mitochondrial membrane potential, two assays were established to determine states of oxidative stress. Whereas cellular levels of H₂O₂ were represented a direct measure of ROS production, changes in total glutathione represented an indirect estimate of oxidative stress.

#### 3.5.1 Neurite degeneration (Paper IV)

The number of neurite processes with a length exceeding the diameter of the cell bodies and the number of cells were manually scored according to Nordin-Andersson and co-workers (1998). Cells with non-neuronal phenotype, *i.e.* epithelial-like cells, and dead cells were not counted.

![Molecular structures of the fluorescent probes MTR (A), MTG (B) and CM-H₂DCFDA (C).](image)

#### 3.5.2 Mitochondrial membrane potential (Paper IV)

Cells in a 24-well plate were incubated with MTR (Fig. 13A), a cell-permeant fluorescent stain that selectively concentrates in active mitochondria in a MMP-dependent manner. After consecutive washings, the MTR-fluorescence
was measured in a plate reader at 578/599 nm (ex/em). The estimated MMP was related to the mitochondrial mass, as measured in a sister cell plate treated with MitoTracker Green FM® (MTG, Fig. 13B). Unlike MTR, this dye preferentially accumulates in the mitochondria regardless of the MMP. The MTG-fluorescence was read at 490/516 nm (ex/em).

3.5.3 Reactive oxygen species (Paper IV)
Intracellular levels of ROS were assessed by incubating a 24-well plate of cells with 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA®, Fig. 13C) that passively diffuses across the plasma membrane. Inside the cell, the acetate groups are cleaved by esterases, while the chloromethyl group forms adducts with surrounding thiols. As a result, the yielded CM-H₂DCF (or DCF) becomes fluorescent and is trapped inside the cell, where it can react with H₂O₂.

The DCF-fluorescence was monitored at 488/520 nm (ex/em) and related to MTG-fluorescence as described in Section 3.5.2. The mitochondrial mass (MTG-fluorescence) was proportional to the amount of cells in wells.

![Fig. 14. Principle for measuring total cellular glutathione content. Glutathione (GSH), along with all the mercaptans in the sample, forms adducts with the chromogenic reagent R1. In alkaline conditions, the adducts obtained with glutathione specifically transform into chromophoric thiones, with a maximal absorbance at 400 nm.](image)

3.5.4 Intracellular glutathione content (Paper IV)
Total glutathione content in cells was determined by the Glutathione Assay Kit provided by Calbiochem®. This method is based on two subsequent reactions that are outlined in Fig. 14.

After lysing the cells in 5% metaphosphoric acid, the chromogenic reagent R1 (Fig. 14) was added to the lysate. This initiated the first reaction, in which all the mercaptans in the sample formed adducts with the reagent. Thereafter, the pH was elevated by addition of 30% NaOH. In the resulting alkaline
environment, only adducts created with glutathione were transformed into a chromophoric thione. This thione was monitored by light absorbance at 400 nm, and the glutathione content, as estimated from a glutathione standard curve, was related to the protein content in samples.
4. RESULTS AND DISCUSSION

In this section, the major findings of the Papers included in this thesis are discussed.

4.1 UCP expression in SH-SY5Y cells (Papers I, II and III)

In Papers I and III, demonstrated by Western blot analyses that native human neuroblastoma SH-SY5Y cells expressed UCP2, UCP3, and UCP4. A closer examination of the UCP3 expression (Papers I and II) revealed that both the UCP3 mRNA and protein were expressed in the cells. The basal level of the UCP3 transcript seemed, however, relatively low, although fully detectable with Northern blot and RT-PCR analyses. Investigations of the subcellular distribution of UCP3 using immunofluorescence technique manifested that the UCP3 protein was localized in the mitochondria. This finding supports that UCP3 is expressed and working as a true UCP in the SH-SY5Y cells.

This was the first time UCP3 expression was reported in cells of neuronal origin. Unlike UCP2 and UCP4, which previously had been identified in the nervous system (Horvath et al., 1999; Mao et al., 1999; Horvath et al., 2002; Mattiasson et al., 2003), UCP3 was believed to be expressed predominantly in skeletal muscles (Gong et al., 2000; Vidal-Puig et al., 2000; Cadenas et al., 2002) and not in neurons. However, this conclusion was based on Northern blot examinations of whole brain homogenates, a method that could leave mRNA scarcely expressed or present at high levels only in small regions of the brain, undetected. Furthermore, UCP3 might be expressed in the PNS or in the spinal cord, a possibility that had not yet been examined.

Intriguingly, the latter hypothesis was recently proved to be true: the presence of UCP3 mRNA was reported in mouse lumbar spinal cord, and both UCP3 mRNA and protein were detected in rat DRG neurons (Dupuis et al., 2003; Vincent et al., 2002; Vincent et al., 2004). Thus, it was concluded that expression of UCP3 in SH-SY5Y cells (Papers I and II) was not an artifact resulting from the tumorogenic phenotype of the cells, but rather a manifestation of their neuronal properties. Thereby, the SH-SY5Y cells constitute a relevant cell model system for studies on UCP regulation and functions in the nervous system.
4.2 Regulation of UCP expression by IGF-1

Despite intensive research since the identification of the “new” mammalian UCPs (UCP2-UCP5), their physiological function remains elusive. However, increasing amount of data indicate that the UCPs take part in the cellular defense from oxidative stress (Nègre-Salvayre et al., 1997; Arsenijevic et al., 2000; Nishikawa et al., 2000; Vidal-Puig et al., 2000; Brand et al., 2002; Echtay et al., 2002a). This function would be particularly important in the nervous system, because of its relatively low antioxidant capacity.

IGF-1 is a neurotrophic factor that protects neurons from oxidative stress and apoptosis induced by various types of injury. Although the PI3-kinase signaling cascade has been assigned as the major survival-promoting pathway, little is known about the exact mechanism behind the protection. However, IGF-1 is known to regulate the BAT-specific UCP1 via the PI3-kinase signaling pathway (Teruel et al., 1998). If this also accounts for neuronal UCPs, there is a possibility that these proteins participate in IGF-1-mediated neuroprotection. In Papers I, II and III, I therefore examined the regulation of UCP2, UCP3, and UCP4 by IGF-1.

Before I deal with the results of these Papers, I will briefly discuss the functionality of the IGF-1 signaling system in the SH-SY5Y cells used for these studies.

4.2.1 IGF-1 signaling system in SH-SY5Y cells (Papers I and II)

As expected, the SH-SY5Y cells responded to both IGF-1 and insulin with increased cell proliferation and formation of neurite processes (not shown). A closer inspection of the IGF-1 signaling system demonstrated that the SH-SY5Y cells expressed both IGF-1 and insulin receptors (Paper I), which was in agreement with several previous reports (Mattsson et al., 1990; Kim et al., 1997; Kim et al., 1998a; Kim et al., 1998b; Kurihara et al., 2000). After treatment with IGF-1 or insulin, the receptors were desensitized, as shown by reduced receptor protein expression. These findings indicate that the receptors were stimulated by the ligands.

Activation of the IGF-1 receptor by IGF-1 was further confirmed in Western blot experiments where an antibody specific for phosphorylated tyrosine residues was used (Paper I). Incubation with 10 nM IGF-1 induced a distinct tyrosine phosphorylation of a protein with a molecular weight estimated to 95 kDa. This matched the size of the β-subunit of the IGF-1 receptor (Fig. 15A).
The results clearly indicated IGF-1 receptor stimulation. The insulin receptor was probably not activated, since the expression of this receptor was unaffected by IGF-1-treatment (Paper I). Insulin, on the other hand, significantly downregulated the IGF-1 receptor at 860 nM, a concentration at which insulin is known to bind to the IGF-1 receptor in SH-SY5Y cells (Mattsson et al., 1990).

As shown in Paper II, the SH-SY5Y cells also expressed functional intracellular signaling systems. When the cells were treated with 10 nM IGF-1, the phosphorylation of the downstream targets of MEK and PI3-kinase, i.e. ERK1/2 and Akt, increased (Fig. 15B), indicating that both of these pathways were activated. The PI3-kinase inhibitor LY294002 specifically blocked the IGF-1-induced activation of Akt, whereas the MEK inhibitor PD98059 prevented ERK1/2 activation. (Fig. 15B). Unexpectedly, the MEK inhibitor also seemed to enhance the IGF-1-mediated phosphorylation of Akt (Fig. 15B), suggesting a cross-talk between the two signaling pathways.

Taken together, these data clearly demonstrated that the SH-SY5Y cells used in this thesis exhibited fully functional IGF-1 receptors and intracellular signaling systems.

4.2.2 Regulation of UCP expression by IGF-1 (Papers I, II and III)

Next, I investigated the regulation of UCP2, UCP3, and UCP4 by IGF-1.

4.2.2.1 UCP3 expression

As shown in Papers I and II, IGF-1 significantly upregulated the expression of UCP3. Incubation with 10 nM IGF-1 increased the UCP3 mRNA approx. 2.5-
fold after 5 h, as judged from Northern blot and RT-PCR analyses (Paper II). This was followed by increased UCP3 protein expression that reached a 2-3-fold maximum after 72 h, and remained sustained for at least another 24 h (Paper I). The raise in UCP3 mRNA imply that IGF-1 did not regulate UCP3 translation or protein degradation, but rather affected transcription of the \textit{ucp3} gene or UCP3 mRNA turnover.

Since IGF-1 exhibits cross-reactivity with the insulin receptor, I wanted to assure that the UCP3-induction was mediated by the IGF-1 receptor. The cells were stimulated with a truncated form of IGF-1, a molecule which displays the same affinity to the IGF-1 receptor as the authentic IGF-1, but binds to a markedly lesser extent to the insulin receptor (Eriksson et al., 1996). Thereby, it was possible to distinguish between IGF-1 and insulin receptor signaling. In addition, cells were incubated with 860 nM insulin, a concentration at which insulin is known to activate the IGF-1 receptor (Mattsson et al., 1990). After 96 h of treatment with these substances, the UCP3 protein expression increased to the same level as after IGF-1-incubation (Paper I). Low insulin concentrations were, however, ineffective. These data show that IGF-1 induced UCP3 expression via stimulation of the IGF-1 receptor.

Downstream of the IGF-1 receptor, the PI3-kinase pathway was found to be crucial for enhancing the UCP3 expression (Paper II). The inhibitor LY294002 concentration-dependently prevented the IGF-1-induced increase in UCP3 mRNA and protein, with a complete restoration to control levels at 1 µM. Nevertheless, it seemed as if the ability of PI3-kinase to fully transmit the UCP3 inducing signal also was dependent on activation of the MAP kinase pathway: after incubation with the effective concentration of the MEK inhibitor PD98059 (10 µM), the expression of UCP3 mRNA and protein decreased. The protein expression was, however, only partially, and not statistically significantly, reduced as compared to cells treated with mere IGF-1. This can be explained by a possible interaction between the MAP kinase pathway and an unknown modulator downstream of PI3-kinase, as proposed in Fig 16. Intriguingly, it was recently demonstrated that ribosomal p70S6 kinase, which is a downstream target of the PI3-kinase pathway, is also activated by ERK1/2 (Lehman and Gomez-Cambronero, 2002). This kinase therefore constitutes an attractive candidate for this modulator.

Like UCP3, UCP1 is also regulated by IGF-1 via the PI3-kinase signaling cascade in primary brown adipocytes (Teruel et al., 1998). However, in this case, the contribution of the MAP kinase pathway is elusive, since PD98059
was found to either abolish or potentiate the UCP1 expression (Porras et al., 1998; Teruel et al., 1998). Furthermore, UCP1 is regulated by insulin (Guerra et al., 1994; Porras et al., 1998; Teruel et al., 1998; Valverde et al., 2003), which was not the case for UCP3 in SH-SY5Y cells (Paper I). These discrepancies probably reflect that the UCPs are differently regulated in different tissues.

Fig. 16. Suggested signaling pathway for IGF-1-mediated UCP3 expression. Activation of the PI3-kinase pathway is crucial for inducing UCP3 expression. However, a full submission of the UCP3-inducing signal is dependent on activation of MAP kinase. Supposedly, the MAP kinase pathways is interfering with an unknown positive modulator downstream of PI3-kinase (gray arrow). IGF-1R, IGF-1 receptor; MAPK, MAP kinase pathway; PI3-K, PI3-kinase pathway.

4.2.2.2 UCP2 and UCP4 expression

IGF-1 also increased the protein levels of UCP2, however, not to the same extent as for UCP3 (Paper III). The UCP2 level increased approx. 1.8-fold after 96 h of treatment with 10 nM IGF-1, indicating that the closely related UCP2 and UCP3 to some extent are co-regulated by IGF-1. However, in contrast to UCP3, the UCP2 protein expression was only moderately decreased by the PI3-kinase inhibitor LY294002, suggesting that downstream the IGF-1 receptor, the two proteins are regulated by separate signaling pathways. Furthermore, UCP4 was only marginally affected by IGF-1. Consequently, I concluded that IGF-1 is not as an important regulatory factor for UCP2 and UCP4 as it is for UCP3, and that the UCPs in the nervous system are differently regulated.
4.2.2.3 Significance of IGF-1-mediated UCP expression

In Paper I and II, I demonstrated that UCP3 is regulated by IGF-1 via the neuroprotective PI3-kinase pathway. This finding supports the theory that UCP3 takes part in the IGF-1-mediated protection from oxidative stress and apoptosis in neurons. The fact that UCP2 and UCP4 (Paper III) were not regulated by IGF-1 to a great extent, indicates that they are probably not contributing to the IGF-1-mediated neuroprotection. Instead, these UCPs may be regulated by other cell-surviving signals, allowing the neuronal defense system to handle various types of insult.

4.3 UCP3 and neuroprotection by IGF-1 during hyperglycemia (Paper IV)

Paper IV demonstrates that UCP3 most likely is involved in IGF-1-mediated protection of neurons from insults induced by high glucose concentrations. These studies were performed using highly differentiated SH-SY5Y cells that, unlike their native counterparts, are vulnerable to high glucose concentrations (cf. 3.1.2). Whereas native SH-SY5Y cells responded to glucose treatment with enhanced cell proliferation, the viability of the differentiated SH-SY5Y cells was rather reduced, as demonstrated by decreased protein content in cell dishes and cellular neutral red uptake (not shown). Importantly, the differentiated cells still expressed insulin and IGF-1 receptors (Söderdahl, 2000).

First, a morphological evaluation of the cells was performed. This study showed that cells incubated with 30 and 60 mM glucose for 72 h exhibited fewer and shorter neurite processes per cell than control cells (cells incubated with 8 mM glucose, Paper IV). In line with the neuroprotective role of IGF-1, stimulation with 10 nM IGF-1 significantly prevented the degeneration of the neurites.

In order to address the possible involvement of UCP3 in this IGF-1-mediated neurite protection, the effects of high glucose concentrations on UCP3 expression, MMP, and cellular redox status were examined. After incubation with 60 mM glucose, the UCP3 expression was markedly decreased (Fig. 17). In parallel, the MMP, together with the levels of ROS and total glutathione, increased. The raise in glutathione probably reflected induction of mild oxidative stress, to which the cells adapted with increased glutathione synthesis (Shi et al., 1994; Moellering et al., 2002). The fact that reduced UCP3 expression was accompanied with increased MMP and ROS levels supports
the theory of UCPs as antioxidants, as previously suggested (Nègre-Salvayre et al., 1997; Arsenijevic et al., 2000; Nishikawa et al., 2000; Vidal-Puig et al., 2000; Brand et al., 2002; Echtay et al., 2002a). Co-incubation with IGF-1 sustained the UCP3 expression at a high level after treatment with 60 mM glucose (Fig. 17), and simultaneously, the levels of MMP, ROS, and glutathione were restored to those in controls cells. These data clearly suggest that UCP3 may be important for the IGF-1-mediated neurite protection.

Recently, regulation of MMP was identified as a promising target for prevention of diabetic complications. When a glucose-induced raise in MMP was prohibited, the mitochondrial ROS production dramatically decreased (Nishikawa et al., 2000). This, in turn, prevented mitochondrial depolarization, cytochrome C release, and mitochondrial swelling, as well as activation of major pathways believed to promote diabetic complications (Du et al., 2000; Nishikawa et al., 2000; Russell et al., 2002; Vincent et al., 2002; Kiritoshi et al., 2003; Vincent et al., 2004). The reduced UCP3 expression and increased ROS levels demonstrated in Paper IV further reinforce the importance of MMP for glucose-induced oxidative stress.

The finding that high glucose treatments decreased the UCP3 expression in SH-SY5Y cells (Paper IV) was recently confirmed in cultured embryonic DRG neurons (Vincent et al, 2004). These data indicate that neuronal UCP3 expression may be defected in states of diabetes. Indeed, UCP3 expression is disturbed in skeletal muscles of patients with type 2 diabetes (Argyropoulos et al., 1998; Krook et al., 1998; Otabe et al., 1999; Schrauwen et al., 2001), and in DRG neurons of STZ-induced diabetic rats, the UCP3 expression is reduced (Vincent et al., 2004). Defective UCP3 expression in neurons may result in increased MMP and enhanced mitochondrial ROS production, and may thereby promote progression of diabetic neuropathy. It should be noted,
however, that in the present study the ROS levels increased also in the presence of 30 mM glucose; a concentration where UCP3 and MMP were unaffected (Paper IV). This finding indicates that reduced UCP3 expression is not solely responsible for the increase in ROS following glucose treatments, but other mechanisms must be contributing.

As suggested in Paper IV, defective expression of UCP3 in the PNS during diabetes may unify the highly interrelated mitochondrial dysfunction, oxidative stress and impaired neurotrophic support, all of which are factors implicated in the development of diabetic neuropathy. Consequently, restoration of UCP3 by IGF-1 represents an attractive therapeutic regimen for diabetic neuropathy. Intriguingly, neurotrophic factors were recently shown to also regulate the MMP in vivo: administration of insulin to STZ-induced diabetic rats prevented diabetes-dependent depolarization of mitochondria in DRG neurons (Huang et al., 2003).

In conclusion, I propose that UCP3 is involved in IGF-1-mediated neuroprotection from high glucose concentrations via regulation of MMP and inhibition of mitochondrial ROS-production. This hypothesis is outlined in Fig. 18.

Fig. 18. Proposed mechanism for involvement of UCP3 in IGF-1-mediated neuroprotection from high glucose concentrations. Glucose induces mitochondrial ROS production by increasing the MMP. Subsequently, the cellular redox status is changed (indicated by increased glutathione (GSH) levels) and the neurite degeneration (ND) is increased. ROS also activate “classical” damaging pathways for diabetic neuropathy and induce mitochondrial dysfunction. Glucose reduces the UCP3 expression, resulting in enhanced mitochondrial ROS production. IGF-1 restores the UCP3 expression. In parallel, the MMP becomes stabilized, and the mitochondrial ROS production and subsequent neuronal injury inhibited. Black arrows indicate findings presented in the thesis, gray arrows findings demonstrated by others.
4.4 Future perspectives

In this thesis, I suggest that neuronal UCP3 plays an important role in the defense from oxidative stress. Furthermore, UCP3 seems to take part in IGF-1-mediated neuroprotection from hyperglycemia-induced oxidative stress. However, due to the lack of pharmaceutical tools that specifically inhibit UCP3, these assumptions are only based on indirect data. However, attempts were made to down-regulate the UCP3 expression with antisense technique, and results from initial trials are very promising. Incubation with low, subtoxic, concentrations of a peptidic nucleic acid antisense sequence specific for UCP3 mRNA reduced the IGF-1-induced UCP3 protein expression by 80% (Olsson, 2003). Thus, in the future it may be possible to specifically downregulate UCP3, and thereby determine the precise role of UCP3 in the protection of neurons from oxidative stress.
5. CONCLUSIONS

i. Native human neuroblastoma SH-SY5Y cells express UCP2, UCP3 and UCP4 (Papers I and III)

ii. UCP3 expression is regulated by IGF-1 via activation of the IGF-1 receptor and the “classical” antiapoptotic PI3-kinase signaling pathway (Papers I and II)

iii. UCP2 and UCP4 are only marginally or not at all regulated by IGF-1 (Paper III).

iv. High glucose concentrations reduce the UCP3 expression. Concomitantly, the MMP, oxidative status, and neurite degeneration are increased. This supports an antioxidant and neuroprotective role of UCP3 (Paper IV)

v. IGF-1 prevents glucose-induced reduction in UCP3 expression and changes in MMP, cellular oxidative status, and neurite degeneration. These data suggest that UCP3 is involved in IGF-1-mediated neuroprotection from glucose-induced oxidative stress (Paper IV).
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What’s past is prologue
Shakespeare
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58
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