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MECHANISMS OF CHRONIC COMPLICATIONS OF DIABETES WITH FOCUS ON MITOCHONDRIA AND OXYGEN SENSING

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To my parents

ABSTRACT

Chronic complications of diabetes (retinopathy, nephropathy, neuropathy, diabetes accelerated arteriosclerosis) represent a major medical and economical concern. It is an imperative need to establish the pathogenic mechanisms that contribute to development of chronic complications in order to design new therapeutic approaches.

Several pathogenic pathways are activated in diabetes and have been proposed to be responsible for the development of long-term complications of diabetes (increase polyol pathway flux, increased advanced glycation end products, activation of proteinkinase C, increased hexosamine pathway flux) including an overproduction of reactive oxygen species (ROS) by the mitochondria electron-transport chain, suggested to be a common mechanism for all the others.

Hypoxia directly and through induction of ROS has been recently suggested to have an important role in the development of chronic complications of diabetes. Adaptive responses of cells to hypoxia are mediated by the hypoxia-inducible factor-1 (HIF-1), which is an ubiquitary heterodimeric transcription factor, regulated by oxygen at the degradation level of its α subunit. The oxygen regulation of HIF is complex and involves a family of hydroxylases (HIF hydroxylases), that need Fe^{2+} or α–ketoglutarate as cofactors and regulates both HIF stability and transactivation in hypoxia. Under hypoxic conditions, HIF-1α is stabilized against degradation and upregulates a series of genes (more than 70) involved in essential processes i.e. angiogenesis, glycolytic energy metabolism, cell proliferation and survival.

ROS, produced in excess both in hyperglycemia and hypoxia can interact with different macromolecules including DNA. The close proximity of the mitochondrial DNA (mtDNA) to the ROS-generating sites makes mtDNA more vulnerable to oxidative damage. Different cellular specific antioxidants mechanisms are available but they are not always able to fully protect DNA from the oxidative damage. Beside mtDNA mutations that contribute to the maternal inherited diabetes (0.5-1%) several somatic mtDNA point mutations and deletions (classically associated with aging) have been described in patients with diabetes even though not consistent. A drawback of these studies is the heterogeneous genetic background of the patients with diabetes coupled with the lack of reliable information about the duration of the exposure to high glucose levels. The direct influence of chronic hyperglycaemia and/or hypoxia upon mtDNA stability and repair is not clear.

Hyperglycemia impairs HIF-1α stability and function and it has been suggested that by this it contributes to the development of chronic complications of diabetes (wound healing, coronary heart disease etc). We have therefore hypothesized in the first paper that the defect in wound healing present in diabetes is a result of an inhibition of HIF-1 activity. We could first demonstrate that the repression of HIF in hyperglycemia is complex and implies not only the stability of the HIF-1α in hypoxia but also the transactivation of both N-terminal transactivation domain (NTAD) and C-terminal transactivation domain (CTAD). Furthermore we show that by blocking HIF-1α hydroxylation through chemical inhibition (deferoxamine, dimethyloxaloylglycine (DMOG)) it was possible to reverse the negative regulatory effect of hyperglycemia on HIF-1 α both *in vitro* and *in vivo* in a mouse model (db/db) of diabetic wound. Local HIF-1 α induction was able to improve several processes essential for wound healing i.e. granulation, vascularisation, epidermal regeneration, and recruitment of endothelial precursors cells (EPC).

Furthermore gain of function studies, by local adenovirus-mediated transfer of two stable HIF constructs demonstrated that stabilisation of HIF-1α is necessary and sufficient for promoting wound healing in a diabetic environment and point out on the necessity to develop specific hydroxylase inhibitors as therapeutic agents for chronic diabetes wounds.

In the second paper we investigated the stability of mitochondrial DNA against ROS overproduction in diabetic condition. By using human dermal fibroblasts (HDF) we were able to confirm an increase of ROS production (by CM-H_2DCFDA) by hyperglycemia alone or in combination with hypoxia. However, mtDNA damage (evaluated by long amplification quantitative polymerase chain reaction (LQPCR)) was observed only when the cells were exposed to both hyperglycemia and hypoxia, confirming the pathogenic role of the combination of these factors. The mtDNA damage was mediated through excess production of ROS by mitochondria as far as mtDNA was fully protected when the cells were treated with inhibitors of the electron transport chain.
We have further studied the effect of diabetes on mtDNA lesions in db/db mouse that is an inbred model of type 2 diabetes. We investigated the incidence of mtDNA lesions by LQPCR in heart and kidney of two groups of db/db mice: "young prediabetic mice" (before developing diabetes) and "old diabetic mice" (34 weeks) and compared with nondiabetic mice (heterozygotes) of the same age. Unexpectedly, the "old diabetic mice" had a lower incidence of mtDNA lesions in both tissues studied, compared with "old non-diabetic" and "young prediabetic" animals even though the tissues are exposed to an excess ROS (as shown by increased protein nitrosylation). This was explained by an increase in the antioxidant capacity (expression of Mn Superoxide Dismutase (SOD_2) and Catalase) and of the mitochondrial base excision repair (mtBER) activity in "old diabetic" animals.

In conclusion, we have shown that hypoxia plays together with hyperglycemia an important role in chronic complications of diabetes by activating several pathogenic pathways. Some mechanisms are fully compensated (i.e. antioxidant defence and reparatory capacity of mtDNA) while others (HIF repression) need to be addressed therapeutically in order to be able to improve the outcome.

Keywords: diabetes, chronic complications, hyperglycaemia, hypoxia, ROS production, mtDNA, HIF.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. Stabilization of HIF-1α is critical to improve wound healing in diabetic mice. Ileana Ruxandra Botusan, Vivekananda Gupta Sunkari, **Octavian Savu**, Anca Irinel Catrina, Jacob Grünler, Stina Lindberg, Teresa Pereira, Seppo Ylä -Herttuala, Lorenz Poellinger, Kerstin Brismar, and Sergiu-Bogdan Catrina. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19426-31.

II. Stability of mitochondrial DNA against reactive oxygen species generated in diabetes. Octavian Savu, Vivekananda Gupta Sunkari, Ileana Ruxandra Botusan, Jacob Grünler, Andrej Nikolsjkov, Sergiu-Bogdan Catrina. Manuscript.

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LIST OF ABBREVIATIONS

Amino-terminal transactivation domain	NTAD
AGEs	Advanced glycozylation end products
AR	Aldose reductase
APE	Apurinic/apyrimidinic (AP) endonuclease
ARNT	Aryl hydrocarbon receptor nuclear translocator
BER	Base excision repair enzymes
BH4	tetrahydrobiopterin
bHLH	Basic helix-loop-helix
CAD	Coronary artery disease
CTAD	Carboxy-terminal transactivation domain
CAT	Catalase
CM-H_2DCFDDA	5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester
CREB	c-AMP response element (CRE)-binding protein
DAG	Diacylglycerol
DM	Diabetes mellitus
DMOG	Dimethyloxalylglycine
DFX	Deferoxamine
DSB	Double-strand breaks
EPC (CAG)	Endothelial precursor cells
FIH	Factor Inhibiting HIF
GFR	glomerular filtration rate
GAPDH	glyceraldehyde phosphate dehidrogenase
GPx	Glutathione Peroxidase
HAF	hypoxia-associated factor
HIF	Hypoxia-Inducible Factor
HRE	Hypoxia Responsive Element
IPAS	inhibitory PAS domain protein
MCP-1	Monocyte Chemoattractant Protein 1
mtDNA	mitochondrial DNA,
NAC	N-Acetyl cysteine
NO	Nitric oxyde
NF-κB	nuclear factor kappa B
ODD	Oxygen-dependent degradation domain
PARP	Poly(ADP-ribose)polymerase
$PBGD_2$	Porphobilinogen deaminase 2
PHDs	Prolyl 4-hydroxylases
PKC	Protein kinase C
Polg	nuclear-encoded mtDNA polymerase
RAGE	Receptor for AGE
ROS	Reactive Oxygen Species
SENP	SUMO specific proteases
SSB	Single Strand Break
SOD_2	Mn Superoxide Dismutase
STUbL	SUMO-targeted ubiquitin ligases
SUMO	Small ubiquitin-like modifiers
TBARS	Thiobarbituric acid–reacting substances
THF	Tetrahydrofuran
TTFA	Thenoyltrifluoroacetone
VEGF	Vascular endothelial growth factor
pVHL	von Hippel-Lindau tumor suppressor protein

1. RATIONALE

Chronic complications of diabetes (retinopathy, nephropathy, neuropathy, diabetes accelerated arteriosclerosis) represent a major medical and economical concern.

Despite of the important progress in developing different therapeutic strategies, the overall efficacy of the management is poor, since the morbidity and mortality related with diabetes is still important. Therefore, there is an imperative need for a better understanding of the pathogenic mechanisms contributing to the occurrence and progression of chronic complications of diabetes which might serve as tools for new more performant therapeutic approaches and premises for future drug development.

Several pathogenic pathways are activated in diabetes and have been proposed to be responsible for the development of long-term complications of diabetes diabetes (increase polyol pathway flux, increased advanced glycation end products, activation of proteinkinase C, increased hexosamine pathway flux) including an overproduction of reactive oxygen species (ROS) by the mitochondria electron-transport chain, suggested to be a common mechanism for all the others.

The aim of this thesis is to establish new pathogenic mechanisms that contribute to the development of chronic complications of diabetes in order to design new efficient therapeutic approaches for the benefit of the patients.

2. BACKGROUND

2.1. Mitochondrial DNA (mtDNA)

2.1.1. Structure and gene content of mammalian mtDNA. MtDNA (figure 1) was discovered in 1963 (1) and its molecule was first fully sequenced in 1981 (2).

The structure of mtDNA is highly conserved (3). Human mtDNA is a double-stranded circular molecule of about 16,5 kb located into mitochondrion. The strands are named heavy (H) and light (L) because they can be separated on a cesium chloride gradient due to differences in guanine content. mtDNA is organized in a DNA-protein structure called the nucleoid. The most important package protein into the nucleoid seems to be TFAM, a transcription factor suggested to regulate the mtDNA copy number in mammals (4). Each mitochondrion contains several copies of mtDNA. In the same normal cell, wild-type (normal) and mutated mtDNA can coexist, a condition called heteroplasmy (5). mtDNA lacks introns, containing only two noncoding (control) regions (3). The displacement loop (D-loop) region is the largest non-coding segment in human mtDNA (about 1 kb long) and it has a triple-stranded structure, in which a nascent H-strand DNA segment of 500-700 nucleotides remains annealed to the parental L-strand.

The gene content of mtDNA is reduced (3). Mammalian mtDNA genes encode for two ribosomal RNAs and 22 transfer RNAs which are components of the mitochondrial translation machinery, as well as for 13 from about 90 different polypeptides that are subunits of the respiratory chain. mtDNA maintenance and expression as well as the vast majority of

different mitochondrial proteins are encoded by nuclear DNA. Therefore, the cross-talk between the two genomes is essential for mitochondria.

Figure 1. Map of the human mtDNA.
ND1–ND6, NADH dehydrogenase (complex I) subunits 1–6; Cox1–Cox3, cytochrome oxidase subunits 1–3 (complex IV); ATP6 and ATP8, subunits 6 (Adapted from (6))

The inheritance of mtDNA is almost exclusively maternal, although some exceptions have been reported (7). The process is affected by bottleneck phenomenon by which only a small numbers of mtDNA molecules are transmitted to the offsprings (8). This phenomenon explains why mtDNA mutations are rapidly transmitted to descendents in one or few generations.

The level of expression of mtDNA mutations can differ in different cells or tissues, for several reasons. mtDNA replication is not coupled to cell cycle and therefore one of the daughter cells can have unbalanced load of mutated mtDNA. (9). Despite initial heteroplasmy, *de novo* mutations occurring in one molecule of mtDNA can increase the amount of mutated mtDNA to homoplasmy (i.e. presence of only mutated mtDNA) (10-11) There could be different levels of mutated mtDNA in different tissues and organs due to heteroplasmy, and in different cells of the same organ, due to different intracellular distribution of mtDNA, or even in mitochondria located in different intracellular sites, due to a dynamic compartmentalization of mtDNA within the mitochondria (10-11). Nevertheless, a certain threshold level of mtDNA mutation load must be overcome in order to cause mutation expression (3; 10-11).

2.1.2. Mammalian mtDNA and ageing. It has been shown that mtDNA is a very sensitive target for oxidative damage, being two-three times more damaged than nuclear DNA, since it is closely associated with the mitochondrial inner membrane where most ROS are generated (12). This is the reason why mtDNA is involved in the ageing process. Therefore, it has been suggested that accumulation of somatic mutations in mtDNA might produce cells with a reduced oxidative capacity which can be responsible for ageing (13). The damage of mtDNA can associate with functional alterations in the activity of electron transport chain complexes since they are partially encoded by mtDNA.

The importance of somatic mtDNA mutations in ageing was very recently reviewed (10-11; 14-17).

Data showing that somatic mtDNA mutations are able to induce a variety of ageing phenotypes in mammals originate from animal studies performed on genetically-modified (knock-in) mice expressing a proofreading deficient form of the nuclear-encoded mtDNA polymerase (Polg) (14-15; 17). The mutation induced profoundly reduces the exonuclease activity without influencing the DNA polymerase activity of the enzyme. mtDNA mutator mice are completely normal in phenotype expression at birth, show signs of premature ageing in early adolescence (about 25 weeks of age), and die prematurely before age of about 60 weeks. Different types of mtDNA mutations (point mutations and deletions) accumulate progressively and randomly in different levels and different tissues. The importance of one or another specific type of mtDNA mutation is debated (16).

The most frequent deletion observed in human tissues is the "common deletion" that spans 4977 nucleotides (16). It occurs at a deletion "hot spot" involving two 13 bp direct repeats and is often associated with other two deletions (mtDNA7436 and mtDNA10422). The levels of accumulation are different in different tissues and within different regions in the tissues. Analysis of human different single cells revealed that number of cells with deficiency in complex IV of the electron transport chain increases with age and that the amount of the mutations in the cells can be substantial, despite of a low overall level (16).

The accumulation of mtDNA point mutation with age in human tissues is controversial (16). Several reports describe A to G transitions (i.e. A8344G, A3243G) and transversions (T414G) accumulating with age in different tissues. Though, one mutation (A13167G) declined significantly with age in muscles of infants 1 hour to 5 weeks old (18). Murdock et al. (19), also found by a different approach no age-dependent accumulation of A3243G and A8344G mutations in skeletal muscle and brain.
Notably though, there is clear data on mtDNA point mutations accumulation with age in studies performed in different human single cells, even in human stem cells (16).

In conclusion, more experiments are needed in order to clearly establish the significance of somatic mtDNA mutations in aging.

2.1.3. Mammalian mtDNA and oxidative damage and repair. mtDNA is highly susceptible to damage induced by ROS (12). The most important source of endogenous mtDNA damage is ROS generated endogenously from electron transport chain (20). ROS can induce many types of DNA lesions, i.e. abasic (apurinic-apyrimidinic) sites (AP), oxidized DNA bases or sugars, DNA strand breaks (single-strand breaks – SSBs, double-strand breaks – DSBs) (20). An efficient repair system is therefore essential for mtDNA protection and survival.

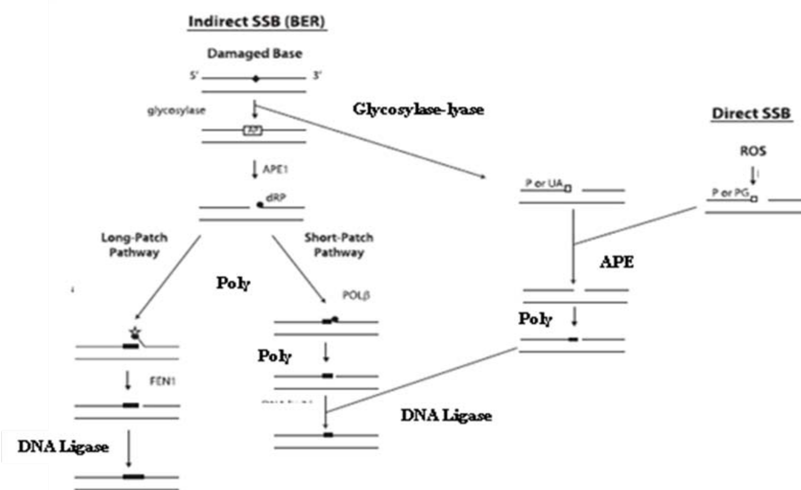

Figure 2. Single-strand break (SSBs) repair pathways.

Adapted from (20)

The base excision repair (BER) pathway (figure 2) repairs the vast majority of the mtDNA lesions induced by ROS (20). Briefly, there are described several steps, as follows (20).

1. Excision of the damaged base by a DNA glycosylase generating an abasic site (AP). AP sites result also from ROS attack, and their removal is critical because they are highly mutagenic. There are several glycosylases specific for certain lesions. One specific glycosylase is able to repair more than one type of damage. Monofunctional glycosylases have only glycosylase activity and for the 3` incision at AP site they need an AP-endonuclease (APE) for lyase activity. The left over 5`- deoxyribose (dRP) residue is removed subsequently by lyase activity of mtDNA polymerase γ. Bifunctional glycosylases have both glycosylase and lyase activity and they are able to perform both base excision and incision to the AP site. The leftover is a 3´ unsaturated aldehyde (UA), derived from deoxyribose or a phosphate (P), which is removed either by APE and mtDNA polymerase γ.
2. The cleavage of AP site and leftovers generates a SSB. SSBs can be produced also directly by ROS. The result is an AP site and a 3´ leftover (phosphoglycolate – PG) which is removed specifically by APE. The SSB is replaced further by mtDNA polymerase γ (Polγ), the only polymerase existing in mitochondria. Presence of both polymerase and lyase activities make this enzyme the most important for the repair synthesis step in BER (20).
3. The final step is the ligation of the remaining nick by a specific ligase complex. This is the short-patch BER pathway, by which only one nucleotide is incorporated instead of the damaged base.
4. If the polymerase cannot access the 5´ end and the leftover residue is resistant to its lyase activity, the process of removing the blocking fragment requires incorporation of several (from 2 to 6) nucleotides. This is long-patch BER pathway and involves several new enzymes. FEN1 (flap endonuclease 1) is a 5´ exo/endonuclease which recently has been isolated also in mitochondrial extracts from human cells (21-22).

2.2. Hypoxia-inducible factor (HIF)

2.2.1. HIF structure

HIF was first discovered during the studies performed in the mammalian Hep3B cell line on the regulation of erythropoietin (EPO) expression (23). HIF belongs to the PER-ARNT-SIM (PAS) subfamily of the basic-helix-loop-helix (bHLH) family of transcription factors (24).

HIF is a heterodimeric complex that is composed by one α-subunit and one β-subunit (25). The α-subunit is oxygen regulated whereas the β-subunit is oxygen independent and is known as aryl receptor nuclear translocator (ARNT) (26-27).

All three isoforms of the α-subunit and only two isoforms of the β-subunit (ARNT and ARNT2) are thought to be involved in the in vivo response to hypoxia (28). Whereas ARNT3 does not participate in hypoxia responses (29), HIF-3α might be a negative regulator of hypoxia-inducible gene expression in the human kidney (30). It has been shown that there is an inhibitory PAS domain protein (IPAS) which negatively regulates the HIF-mediated gene expression in mouse cornea and correlates with low levels of expression of the VEGF gene, maintaining an avascular phenotype under hypoxic condition (31).

Whereas HIF-1α is ubiquitously expressed, HIF-2α and HIF3α expression is more restricted (32). Therefore, HIF-2α has been found especially in renal interstitial cells, hepatocytes, cardiomyocites – which are tissues highly vascularized (33-35) and HIF-3α is detected mostly in kidney (31), skeletal muscle and brain (34).

Despite differences, HIF members share common characteristics for the bHLH-PAS family of transcription factors (28; 32) (figure 3).
The N-terminal part is common for all HIF members and contains bHLH/PAS domain which mediate DNA binding and induces dimerization.
The activation/degradation domain is different between HIF members.
The C-terminal region is present only in HIF1-α and HIF-2α and is involved in transactivation of HIF complex by two specific domains (N-TAD of C-TAD, transactivation domains).
N-TAD domain contains the oxygen-dependent degradation domain (ODDD) and the two proline residues (P402 and P564) involved in HIF-α degradation in normoxia.
HIF-β and HIF-3α lack C-TAD region.
In all HIF members, bHLH motif and C-terminal contain nuclear localization signals (NLS). When stable, NLS allows HIF to translocate into nucleus (36).

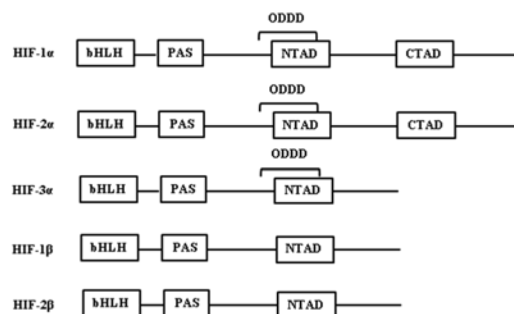

Figure 3. Schematic representation of the HIF subunits.

2.2.2. HIF α regulation.

Postrranslational hydroxylation of the ODDD at two critically proline residues (P402, from N-terminal region and P564 from C-terminal region) is the key modification for HIF α degradation in normoxia (28; 37). The reaction is catalyzed by a family of 2-oxoglutarate (2-OG) dependent dioxygenases, named as prolyl hydroxylase domain-containing proteins (PHD). Their activity is dependent on substrates (oxygen and 2-OG) and on cofactors ($Fe2^{+}$

and ascorbate). The biochemical characteristics of the PHDs are similar to the collagen prolyl-4-hydroxylases. However, these enzymes are unable to catalyze HIF proline hydroxylation (38). All of the three PHDs isoforms described in humans are able to hydroxylate HIF in vitro but it was shown that PHD2 seems to be the key limiting enzyme for HIF α stability (39). Moreover, PHD2 is able to shuttle between the cytoplasm and the nucleus, thereby contributing to HIF-1α degradation in both compartments (37).

The acetylation of lysine located in the ODDD of HIF-1α is another posttranslational modification which is involved in HIF degradation in normoxia (40). The reaction is catalyzed by Arest Defective Protein-1 (ARD-1), an acetyltransferase which is downregulated by hypoxia.

The above mentioned mechanisms induce the HIF α degradation by promoting its interaction with von Hippel Lindau protein complex (pVHL), a component of an E3 multiprotein ubiquitin-ligase complex known as VHL/elongin B/elongin C (VBC) (41). Along with E1 activating enzyme and E2 conjugation enzyme (more than 25 members), E-ligases (100 members) are components of the ubiquitinilation system (42). The pVHL E3 ligase complex is expressed in all tissues and it can shuttle between the cytoplasm and nucleus, like PHDs (28; 37). After binding to this complex, HIF is polyubiquitinilated and directed to 26S proteasome for degradation.

It has been reported that HIF α degradation can occur also through other pathways which are pVHL independent (37).

HIF α inactivation in normoxia implies an additional hydroxylation of asparagines 803 (N803) located at the end of C-TAD. The result is inhibition of HIF activation by transcriptional co-activators, such as p300, steroid receptor coactivator-1 (SRC-1) or CBP (CREB-binding protein) (28; 37). The reaction is catalyzed by Factor Inhibiting HIF-1 (FIH-1) which belongs to the dyoxygenase family of proteins. As PHDs, FIH-1 needs 2-OG and oxygen as substrates and $Fe2^{+}$ as cofactor, but the k_m for oxygen is lower. Therefore, the enzyme is more sensitive to hypoxia gradients.

Despite clear evidence that HIF hydroxylation is the main regulator, there are other mechanisms that contribute to the control of HIF activity (37; 43).

Various cytokines, growth factors, hormones or other active substances have been implicated in HIF regulation in an oxygen-independent manner (37).

Direct phosphorylation of the C-TAD, most probably at threonine 796 in HIF-1α and 844 in HIF-2α, is suggested to increase the transcriptional activity in an oxygen-independent manner (37; 44).
S-nitrosylation of the thiol group of cysteine 800 in HIF-1α has controversial effects on transcriptional activity (44)

HIF-1α activity can be mediated by SUMOylation, a system which is very similar to ubiquitin pathway (45), where the small ubiquitin-like modifier (SUMO) is the most known component. The SUMOylated proteins are specifically degraded by a de-SUMOylation system consisting of different cysteine proteases (SUMO specific proteases – SENP, 1-6 in humans). Very recent data (46) suggest more complex relations between SUMOylation and

ubiquitinilation where a novel family of E3 ubiquitin ligases (SUMO-targeted ubiquitin ligases – STUbL) seems to play an important role.

There are studies showing the importance of SUMOylation on HIF -1α degradation but the results are still contradictory (47-48). Further experiments are needed to show if other members of HIF-α family are regulated by SUMOylation/deSUMOylation.

A new oxygen-independent regulator of HIF-1α activity has been recently described (49). The hypoxia-associated factor (HAF), also known as SART1800 (squamous cell carcinoma antigen recognized by T cells), is a novel E3-ubiquitin ligase which is expressed in proliferating cells. HAF overexpression decreased HIF-1α levels, whereas HAF knockdown increased HIF-1α levels independently of pVHL or oxygen. The levels of HIF-2α remained unchanged, suggesting HAF as a potential mediator of a new degradation pathway specific to HIF-1α.

2.2. 3. HIF function

Under hypoxic conditions both PHDs and FIH are inactive, HIF-α translocates into nucleus, dimerizes with HIF-β and binds to the hypoxia response elements (HREs) located in the promoter gene of the target genes (44). More than 60 well defined genes involved in different processes (i.e angiogenesis, tumor development, erytropoiesis, metabolism, inflammation etc) are either upregulated (50) or repressed (51). Much less is known about HIF-3α isoform, which has been reported to have a dominant negative activity (28; 31). HIF target gene expression is also conditioned by C-TAD and N-TAD activity (28; 44). Despite both domains can interact with different transcriptional co-activators, it has been reported that N-TAD confers specificity for target gene expression either for HIF1-α and HIF-2α. Nevertheless, a sequential activation of the two domains in gradients of hypoxia is described. In mild hypoxia HIF stability is rescued but only N-TAD activity is preserved, whereas in full hypoxia both N-TAD and C-TAD activities are present. The explanation resides in a higher affinity for oxygen for FIH when compared with PHDs.

2.3 Mechanisms of chronic complications in diabetes mellitus: role of chronic hyperglycaemia and hypoxia

2.3.1. Pathophysiology of chronic complications of diabetes. Role of chronic hyperglycaemia and mitochondria ROS production.

Diabetes mellitus (DM) is a serious global health problem. A recent study by the World Health Organization estimated the worldwide prevalence of this disease is expected to grow from 171 million in 2000 to 366 million by 2030 (52). Both type 1 and type 2 diabetes markedly increase the risk of microvascular and macrovascular complications.
Microvascular complications, which center on dysfunction in the capillary bed of tissues, are wide-ranging, and include the retinopathy, nephropathy, and neuropathy that eventually affect nearly all patients with diabetes. Diabetic retinopathy is the major cause of adult blindness in many geographic areas (53). Diabetic neuropathy, which affects roughly half of all diabetic patients, is the most common cause of nontraumatic amputations (54) and diabetic nephropathy is the major cause of end-stage renal disease (55).
Macrovascular complications due to atherosclerosis remain the leading cause of death in diabetic patients. Myocardial infarction, stroke, and peripheral vascular disease are two to four times more prevalent in these patients. Moreover, atherosclerosis occurs earlier, and

follows a more aggressive course (56). Thus, the cardiovascular event rate in diabetic patients without documented coronary artery disease (CAD) is equivalent to that of nondiabetic patients with CAD (56). Diabetic patients have higher mortality following myocardial infarction than nondiabetic subjects (56). Women with diabetes lose their premenopausal cardioprotection, and are vulnerable to CAD at the same rate as men (57).
Several pathogenic mechanisms have been proposed to be responsible for the development of long-term complications of diabetes: increase polyol pathway flux (58), increased advanced glycation end products (AGEs) (59), activation of proteinkinase C (PKC) (60-61), increased hexosamine pathway flux (62). Hyperglycaemia-induced overproduction of reactive oxygen species (ROS) by the mitochondrial electron-transport chain has been suggested to be a common mechanism for all the others (63). Moreover, it has been shown that hyperglycemia-induced overproduction of mitochondrial superoxide is responsible for an important reversible decrease in glyceraldehyde phosphate dehidrogenase (GAPDH) activity. Superoxide induces this effect either direct (64) or indirect, via poly(ADP-ribose) polymerase (PARP) activation by oxidative lesions of mitochondrial DNA (65). Despite the modulation, superoxide GADPH inhibition induces the above mentioned mechanisms.
In diabetes, two major sources of excessively ROS (i.e. superoxide) production are described in mitochondrial respiratory chain: complex I (NADH:ubiquinone oxidoreductase or NADH/$FADH_2$ dehydrogenase) and complex III (ubiquinol:cytochrome c oxidoreductase) (66-69).

2.3.1.1. Cardiovascular disease is the major cause of morbidity and mortality in diabetes. About 80% of all diabetics die from cardiovascular events. 75% of such deaths are due to CAD and the remaining – due to cerebrovascular, peripheral or other macrovascular disease (56). The common link in these complications is atherosclerosis, a process which is accelerated in diabetes (70).

Oxidative stress in diabetic patients leads to many proatherogenic events such as endothelial dysfunction, LDL oxidation, or abnormalities of NO synthesis, monocytes, macrophages and vascular smooth muscle cells (VSMC) (70).

a. Overproduction of superoxide anion seems to be the major mechanism responsible for increased oxidative stress in endothelium (70-71). Two sources of superoxide are probably the most important: activation of NAD(P)H oxidases and uncoupled eNOS (70). Oxidized LDL (oxLDL) and the interaction between AGE epitopes and their receptors are also contributors (70-71).
Several subunits of NAD(P)H oxidase are more expressed in vessels isolated from diabetic patients and the presence of uncoupled eNOS in diabetic vasculature is supported by *in vivo* and *in vitro* studies (70). Activation of the poly(ADP-ribose) polymerase (PARP) by oxidative DNA damages in diabetes contributes also to eNOS inactivation through NAD(P)H depletion (71). It has been shown that a PARP inhibitor can maintain normal vascular responsiveness, despite the persistence of severe hyperglycemia (71). Moreover, activation of the hexosamine pathway by superoxide anion in hyperglycemia is responsible for a specific modification of serine 1177 on eNOS which prevents phosphorylation and impairs its activation Oxidized LDL has itself been shown to produce oxidative stress in endothelial cells via activation of a NADPH oxidase (72).

b. It has been suggested that hyperglycemia causes oxidative stress in monocyte/macrophages, resulting in increased production of proatherogenic agents (70). For example, a single oral dose of glucose has been shown to increase ROS generation in

monocytes of healthy volunteers (73). Furthermore, monocytes isolated from diabetic patients produce increased levels of superoxide through PKC dependent activation of NAD(P)H oxidase (74). Monocyte invasion and VSMC migration may be facilitated by the ROS-mediated expression of MMP-9, a 92 kD metaloproteinase degrading collagen IV from extracellular matrix, which has been shown to be induced by glucose. Specific activity and expression of MMP-9 were significantly increased in vascular tissue and plasma of two distinct rodent models of DM, when compared with their controls (75). When exposed to chronic hyperglycemia, monocytes secrete tumor necrosis factor-α (TNF- α) via ROS-dependent activation of NF-kβ (76).

c. Superoxide production can be increased by hyperglycemia in VSMC via NAD(P)H oxidase activation (77-79). Excess of superoxide could further react with NO from endothelial cells, thus limiting its effect on VSMC relaxation. The production of NO in VSMC themselves may be affected since high glucose concentrations can inhibit iNOS activity in these cells through a PKC-dependent mechanism (80). It has been shown in humans and animals that both hyperglycemia and oxidative stress further impair endothelial vasodilation by shifting VMSC from a contractile to a proliferative phenotype (70). Despite increased VMSC migration and proliferation observed in diabetic endothelium, atherosclerotic lesions contain very few VMSC in these patients. The explanation could be that in aorta and coronary arteries from diabetic patients there is an increased rate of apoptosis and necrosis which are mediated by high level of hydrogen peroxide radicals and oxLDL (81-82).

d. The role for supplemented antioxidants in the prevention of atherosclerosis is highly controversial, many clinical trials showing lack of protection or even deleterious effects with vitamin C, E or beta carotene (83). The most claimed reason is inappropriate patient selection. In this respect, only one very recent study (84) showed a significantly decrease in cardiovascular events in a subgroup of diabetic patients with very increased oxidative stress (haptoglobin 2-2 genotype) after 18 months of natural alpha tocopherol vitamin, 400 UI/day.

2.3.1.2. Diabetic cardiomiopathy is a specific entity first described by Rubler and colab. in 1972 (85). In most of the cases, the disease is diagnosed as an impaired diastolic function in presence of hypertension or myocardial ischemia. The exact cause of the diabetic cardiomiopathy remains unclear, and the patophysiology is multifactorial. Recent studies suggest a pathogenic role of free-radical mediated apoptosis (86-87). Up regulation of the local renin-angiotensin system (RAS) and the synthesis of angiotensin II (Ang II) are implicated in the induction of myocyte apoptosis in both type 1 diabetic animals and type 2 diabetic patients (86-88). Ang II binds to Ang II receptor 1 (AT1), activating a series of responses including the generation of reactive oxygen and nitrogen species (ROS and RNS) through activation of the NADH/NADPH oxidase. The evidence that myocardial cell death in diabetes could be prevented by application of AT1 blockers confirmed the involvement of this mechanism. ROS and RNS also activate other proapoptotic mechanisms in cardiomiocytes, i.e. mitochondrial cytochrome c - caspase-3 pathway, TNF-α, PKC, p38 MAP kinase, and PARP (86).

2.3.1.3. Diabetic nephropathy is characterized by persistent albuminuria, confirmed on at least two occasions 3–6 months apart, declining glomerular filtration rate (GFR), and hypertension. About 30% of all diabetics develop nephropathy at some point. Albuminuria correlates with risk of renal failure, as well as with cardiovascular events and mortality in type 2 DM patients (87; 89).

There are several experimental data showing the presence of the markers of oxidative stress in diabetic nephropathy. Increased levels of 8-OHdG were found in kidneys of streptozotocin-induced diabetic rats (90) and also in urine of type 2 diabetic patients where were correlated to the severity of glomerular and tubulointerstitial lesions (91). Moreover, a 5-year prospective trial documented the urinary level of an 8-OHdG metabolite as a strong predictor of the development of nephropathy in type 2 diabetic patients (92). Increased ROS production was demonstrated in streptozotocin-induced diabetic rat glomeruli (93) and in proximal tubular cells from db/db mice compared with their age-matched heterozygotes (94). Protein carbonyls are significantly increased in plasma and urine from type 2 diabetic patients with nephropathy (95).

ROS overproduction in diabetic nephropathy results from several important sources: mitochondria, activation of NAD(P)H oxidases, uncoupled eNOS and dicarbonyl products (87; 96-97). It has been also shown that ROS can mediate high glucose-induced PKC activation in mesangial cells and diabetic glomeruli, whereas PKC mediates high glucose-induced ROS overproduction (97).

a. Mitochondrial ROS overproduction in diabetic nephropathy has been validated *in vitro.* Therefore, it has been shown that increase dichlorofluorescein (DCF) – sensitive ROS overproduction is induced with time by high glucose in rat mesangial cells through activation of several pathways, i.e. mitochondrial electron gradient, NAD(P)H oxidase and PKC (97). Moreover, ROS from mitochondria induce cyclooxygenase-2 gene overexpression in human mesangial cells in diabetic nephropathy (98). A role for mitochondria in the development of diabetic kidney disease is also suggested (99) by the recent observation that up to 50% of children with mitochondrial diseases have renal impairment (100). Some of these subjects have demonstrated renal disease as their primary pathology, and associate a newly described mitochondriopathy involving a deficiency in coenzyme Q10 (101). Moreover, treatment with new generation of antioxidants with selective uptake into mitochondria (i.e. idebenone or MitoQ) in these cases seems to be efficient (99). However, the efficacy of these relatively selective mitochondrial antioxidants in diabetic nephropathy remains to be determined (102-103).

b. In addition to residing in phagocytic cells, NAD(P)H oxidase is present in nonphagocytic renal cell types such as mesangial and proximal tubular cells, vascular smooth muscle cells, endothelial cells, and fibroblasts. When oxidative stress is present, i.e in diabetes, all these sources generate high amounts of free radicals, and NAD(P)H system became an important extra mitochondrial source of ROS (99; 104).

c. In animal models of diabetes it has been suggested that uncoupling of NOS and NADPH oxidase provides two major sources of glomerular superoxide (105). There are controversies about how the expression of NO-synthases (NOS) is regulated in diabetes (106). eNOS seems to be upregulated during natural course of diabetic nephropathy, whereas iNOS has differential expression. It is therefore agreed that in early nephropathy NO production is increased and is mediated by constitutively expressed isoforms of NOS, and – at least in type 2 diabetes – by iNOS, too. During advanced renal disease NO deficiency occurs and probably involves all NOS isoformes (105).

d. It has been shown that serum and skin levels of AGEs increase as nephropathy progresses in diabetic patients (87; 107). It is also shown that AGEs accumulates in diabetic kidney (108-109). Two recent studies (99; 110) conducted on diabetic rats validate the generation of ROS

as potential source of AGEs in kidney. Superoxide production was significantly increased in mitochondria extracts from renal cortex of diabetic rats when compared with their controls, due to glycosilation of complex I and III proteins.
The interaction between AGE epitopes and their receptors are also contributors for ROS overproduction in diabetic kidney. Therefore, transgenic diabetic mice over expressing RAGE rapidly develop glomerulosclerosis (111),whereas AGEs neutralization (either by RAGE knock-out or long-term administration of a RAGE-neutralizing antibody) confers renoprotection (112-113). In addition, in the glomeruli of patients with diabetic nephropathy, RAGE expression is upregulated and positively correlates with AGE accumulation (114).

e. The importance of antioxidant therapy in diabetic nephropathy needs more investigations. Diabetic animals overexpressing either Cu/Zn SOD or renal CAT have different degrees of improvement of renal disease (97). Intensive glycemic control and treatment with different inhibitors of angiotensin II prevent the onset and delayed the progression of nephropathy both in type 1 and type 2 diabetic patients. It can be speculate that these effects are due to prevention of ROS overproduction (97).

2.3.1.4. Retinopathy is one of the most severe ocular complications of diabetes and is a leading cause of acquired blindness in young adults (53). **Diabetic retinopathy** is the most frequent cause of new cases of blindness among adults aged 20–74 years (53). Its prevalence correlates with duration ofdiabetes. Diabetic retinopathy occurs very seldom in patients under 10 years of age, and it bursts after puberty. During the first 20 years of the disease, virtually all patients with type 1 diabetesand >60% of the patients with type 2 DM develop retinopathy (115). The strongest predictor for development and progression of retinopathy is the duration of diabetes itself. (115-117).

The retina is highly rich in polyunsaturated lipid membranes, and oxygen consumption and glycolysis rate are increased. These particularities make the retina extremely vulnerable to free radical-mediated lipid peroxidation. Thiobarbituric acid–reacting substances (TBARS) assay have been found to be increased in type 1 and type 2 diabetic patients (87; 118-120).

Presence of oxidative stress in diabetic retinopathy is highly documented both *in vivo* and *in vitro* (121). Superoxide production was increased in leucocytes from diabetic patients and in normal leucocytes after incubation in serum from diabetic patients. Furthermore, significantly more superoxide was released by leucocytes incubated with serum from patients with diabetic retinopathy than from patients without retinopathy. Another study found that SOD activity was decreased in the anterior chamber and vitreous in diabetic patients, suggesting that it may be involved in the development of cataract and diabetic retinopathy. 8-OHdG was found highly increased in retinas and other tissues from diabetic rats. Retinas of diabetic rats and retinal endothelial and Muller cells exposed to high glucose exhibit elevated production of superoxide, which originated largely from mitochondria than from NAD(P)H oxidase (121). Diabetic mice overexpressing MnSOD were able to inhibit overproduction of superoxide from retinal mitochondria, to normalize diabetes-induced increase in mitochondria permeability and restore the activity of the complex III in retina (121).

These data suggest that in diabetic retina superoxide overproduction originates mainly from mitochondria and probably from impairment of antioxidant defense system. (120-121). The complex III activity is reduced in the retinal mitochondria of diabetic mice and rats suggesting that it is the origin of excessive ROS production in retina (122).

Peroxynitrite production is also increased in diabetic retinopathy (121). In this respect, increased formation of nitrotyrosine was observed in the retina of diabetic mice and rats, in retinal endothelial cells cultured in high glucose or in plasma, platelets and skin of diabetic patients (121). The source is the excess of superoxide mainly from mitochondria which reacts with NO generated in excess mainly by iNOS and eNOS (121).

ROS production can be also increased in diabetic retinopathy by biochemical pathways excessively activated in diabetic condition (120-121; 123).

a. Activated PKC regulates the activity of several growth factors, i.e. VEGF, IGF-1, and transforming growth factor β (TGF-β). Retinal expression of VEGF is elevated by ROS, and VEGF can also interact with other metabolic pathways important to the development of retinopathy apart of PKC, such as: AGEs and the polyol pathway (123). IGF-1 can also modulate the expression and activity of VEGF (123). Although the exact role of IGF-1 in the pathogenesis of diabetic retinopathy remains to be elucidated, it is possible that IGF-1 can be modulated by oxidative stress via PKC pathway (123).
b. AGEs accumulate in retina as diabetic retinopathy progresses and increase nitrosative stress in the retinal vascular cells which initiatesretinal capillary apoptosis via activation of NF-kβ and caspase-3 (123).
c. Inhibition of glyceraldehyde-3 phosphate dehydrogenase (GADPH) is observed in retina of animal models of diabetes from early stages of retinopathy (120). The mechanism behind it is still unclear, but main candidates are GADPH glycosilation, nitrosilation or PARP activation (120).
d. The hyperglycemic pseudohypoxia theory, stating that the increase in the activity of sorbitol dehydrogenase results in an increase in the intracellular concentration of NADH and an increase in redox ratio of NADH-to-NAD in highly controversial in diabetic retinopathy (124).

PARP activation has been described in retinas of diabetic animals, but the origin of activation is controversial (121).

Oxidative stress contributes to both functional and structural changes in the retina microvasculature (120). Peroxinitrite has a particular importance since induces apoptosis by direct oxidative alterations on internal mitochondrial membrane. Oxidative stress can induce apoptosis by multiple mechanisms (120), activation of NF-κB and caspases (i.e. 3 and 9) being the most studied in diabetic retinopathy.

Supplementation with antioxidants, such as vitamin C, vitamin E, and magnesium suggests no beneficial effect (125).

Oxidative stress also significantly contributes to the pathogenesis of diabetic cataract (87). The major contributors in the genesis of free radicals in diabetic lens are glycoxidation and glutathione depletion. A role for aldose reductase (AR) and sorbitol dehydrogenase (SDH) has been also documented in the pathogenesis of slowly evolving diabetic cataract (87).

2.3.1.5. The diabetic neuropathies are heterogeneous, affecting different parts of the nervous system that present with diverse clinical manifestations. Most common among the neuropathies are chronic sensorimotor distal symmetric polyneuropathy and the autonomic neuropathies. Diabetic polyneuropathy is a diagnosis of exclusion (126). Although exact prevalence depends on the diagnostic criteria used to identify neuropathy, most studies

suggest that 50% of patients with a 20 years history of diabetes, of both type 1 and type 2, have neuropathy (127). Around 10% of these cases of neuropathy are associated with abnormal sensations and pain. The prevalence of diabetic autonomic neuropathy varies between 1,6 and 90% in accordance with diagnostic modalities and diabetic population. Big trials (i.e. DCCT, Stockholm Diabetes Study, or Oslo Study – for type 1 DM, and Kumamoto Study for type 2 DM) show that the incidence of neuropathy increases with duration of diabetes and is accelerated by poor glycemic control. On the other hand, a good glycemic control improves diabetic polyneuropathy, especially in type 1 DM.

The role of oxidative stress in neuronal degeneration has been documented in multiple studies, especially in animal models (87). Oxidative stress is present in peripheral nerve, dorsal root and sympathetic ganglia, and the vasculature of the peripheral nervous system (128). In a cell culture model from dorsal root ganglion (DRG) neurons elevated levels of oxidative stress are present within 2 h of hyperglycemia (129). This may explain in part the underlying mechanism responsible for neuropathy in diabetic patients with good overall control as well as patients with impaired glucose tolerance, who also develop neuropathy (129-130).

The source for ROS overproduction in diabetic neuropathies is mainly complex III of the mitochondrial respiratory chain (131). Peroxinitrite is also produced in excess due to reaction between superoxide anion and NO (131). NO is produced also in excess in diabetic neuropathies due to increased activity of mitochondrial NOS (131). Peroxinitrite promotes release of cytochrome c from mitochondria inducing apoptosis (131).

Several metabolic pathways become overactivated in the nerve after chronic exposure to high glucose (131-132). The distal-proximal axon length-dependent manner of progression of diabetic neuropathy suggests that damage is initiated into axon. Axons are prone to hyperglycemic damage due to the direct access to nerve blood supply and to the large numbers of mitochondria (132).

a. Growing evidence indicates that aldose reductase (AR), the limiting enzyme in polyol pathway, has a key role in oxidative stress production in diabetic neuropathy(131). Sorbitol accumulation induces compensatory depletion of taurine and myoinositol. There is a strong experimental support for sorbitol accumulation and compensatory depletion of taurine and myoinositol. Studies with specific aldose reductase inhibitors (ARIs) in experimental diabetic neuropathy have shown that ARIs ameliorate nerve-fiber damage and loss, and diabetic mice over-expressing human AR develop severe functional and structural abnormalities in peripheral nerves (128). However, most of the early clinical trials with ARIs have been disappointing, with a lack of efficacy and unacceptable side effects. The promise shown with the newer ARIs is being currently explored. The potent ARI fidarestat has shown positive effect in STZ rats and promising effects in humans. Fidarestat-treated diabetic patients showing significant improvement in symptoms and electrophysiological measures compared with the placebo group (128). The SDH-mediated NAD-redox imbalances (so-called ‘pseudo hypoxia’) effects are controversial (128).
b. The activation of the PKC pathway exerts its negative action in diabetic neuropathy through its effects in vascular blood flow and microvascular disease rather than directly in neuronal cells (127-128). In animal studies, treatment with a PKC inhibitor prevented the activation of NF-κB and subsequent ROS formation (133) and reduced the nerve conduction defects and increased endoneurial perfusion (134).

c. It has been shown that the glycation process is enhanced in the peripheral nerve in both diabetic humans and animals As in case of other chronic complications, binding interactions between AGEs and their specific receptors have been suggested as a source of oxidative stress, by depleting intracellular GSH and vitamin C, resulting in the induction of nuclear factor kappa B (NF-κB) in endothelial cells (135). RAGE activation in neurons induces NADPH oxidase activity, which promotes mitochondrial oxidative stress and dysfunction (131).
d. Endothelial poly(ADP-ribose) polymerase (PARP) activation is considered as essential for the development of endothelial dysfunction in DM (136). Oxidative-induced DNA single-strand breaks under hyperglycemia condition activate PARP and deplete NAD^+ and ATP storages. Glicolysis and mitochondrial respiration are depressed and free radical production is increased. Reactive oxygen and nitrogen species activates PARP in diabetic endothelial cells. This is sustained by the fact that hyperglycemia mediated PARP activation is depressed by SOD, NOS inhibitors or NO chelators. Importantly, pharmacological PARP inhibition can also restore sensory and motor neuronal conduction in already established diabetic neuropathy, at least in murine models of the disease (137-138).
e. Human studies using α-lipoic acid in diabetic patients generally report significantly improvements in antioxidants profile and decreases of oxidative stress, even in subjects with poor glycemic control (139). Larger multicenter, randomized, double-blind placebo trials have demonstrated limited effects on neuropathic symptoms and electrophysiological testing, proposing that a longer-term assessment of neuropathic deficits is merited (i.e. longer than 7 months) (140-141). Slight improvements in cardiac autonomic neuropathy were also demonstrated (142). A recent phase III clinical trial, the SYDNEY trial, demonstrated that i.v. administration of lipoic acid rapidly and significantly improves several neuropathic symptoms and nerve function in patients with stage 2 of diabetic polyneuropathy (143).

2.3.2. Pathophysiology of chronic complications of diabetes mellitus. Role of chronic hypoxia.

2.3.2.1. Atherosclerosis. Hypoxia in the arterial wall has for many years been implicated in the development of atherosclerosis, and has been extensively demonstrated in animal models, both by direct identification of hypoxic regions (144) and by gene expression studies of several hypoxia-induced genes in atherogenesis (145). Endothelial hypoxia has been proposed as the major initiator of the atherosclerotic plaque more than twenty years ago (146). Hence, a direct evidence of hypoxia in human atherosclerosis has only very recently emerged (147). Intimal thickening due to atherosclerosis increases diffusion distances exceeding the diffusion limit for oxygen. Hypoxia results from the combination of increased oxygen demands with insufficient oxygen supply, particularly in macrophage-rich zones from atherosclerotic lesions (144).
Nowadays, hypoxia is considered a key factor in the progression of atherosclerosis, promoting lipid accumulation, inflammation, ATP depletion and angiogenesis (148).
a. Therefore, in humans, hypoxia stimulates formation of foam cells, increasing the triglyceride content of cytosolic lipid droplets (149). Thus, it has been suggested that foam cells could be a link between the high levels of circulating free fatty acids and the development of atherosclerosis in patients with type 2 diabetes (150). Moreover, in vivo studies (148) showed that the lipid droplets turn-over correlates with severity of atherosclerosis.
b. It has been postulated that hypoxia promotes inflammation (148). Hypoxia stimulates the recruitment of T cells and monocytes to atherosclerotic lesions by inducing chemokine secretion from macrophages. Moreover, inhibition of chemokine interactions attenuates

monocyte recruitment and reduces atherosclerosis in hyperlipidemic mice. There is accumulating evidence that cytosolic lipid droplets, particularly in macrophages, are important mediators of inflammation in atherosclerosis (148).
c. It has been shown that ATP depletion occurs in rabbit atherosclerotic plaques in vivo (151). Macrophages are the main cells maintaining ATP levels of hypoxic zones within the plaque, even for advanced lesions, because of their efficient adaptation to hypoxia by a compensatory increase in anaerobic ATP production. The ATP depletion is probably caused by several factors (i.e. decreased diffusion from the lumen and vasa vasorum, increased demand for oxygen and nutrients within the plaque, especially high glucose consumption). ATP depletion contributes to formation of necrotic core and plaque destabilization.
d. Hypoxia, inflammation and/or reactive oxygen species are considered as stimuli for angiogenesis in atherosclerosis (152) by inducing the expression of growth factors (i.e. VEGF) or transcription factors (i.e. HIF) in certain cell types involved in atherogenesis (i.e. monocytes/macrophages, smooth muscle cells etc). The critical importance of hypoxia and angiogenesis in atherosclerosis has been recently reviewed (152), and new data just emerged showing a strong correlation between hypoxia and angiogenesis in human atherosclerotic lesions (147). Angiogenesis is primarily promoted within the plaque to compensate for low tissue oxygenation and ATP levels (148). There are increasing *in vivo* data showing that angiogenesis may be also an inductor for lesion instability(148). Therefore, angiogenic vessels in mice plaques provide additional route for inflammatory cells to enter the plaque, and in human lesions are structurally defective.
Recently, the importance of hypoxia for atherosclerosis has emerged as a new theory pointing out a functional hypoxia in vasa vasorum, which might explain the initiation of the atherosclerosis in the deep layer of intima (153). It is assumed that any modification into the architecture of the vasa vasorum reflects in blood flow abnormalities and hypoxia in the inner layer of the media. The development of atherosclerosis initiates according to this scenario from the opposite direction, the end arteries of vasa vasorum.

2.3.2.2. Diabetic nephropathy. Despite detection of hypoxia in kidney *in vivo* is a challenging task, chronic hypoxia has been evidenced in diabetic nephropathy both in animal and human studies (154-155). Chronic kidney disease is characterized by intensified regional hypoxia, especially in medulla, a region physiologically characterized by reduced oxygen availability (154). Chronic hypoxia is present in medulla from streptozotocin-induced diabetic mice at early stages in diabetic nephropathy despite no change in regional blood flow, as it has been shown by the blood oxygen level-dependent (BOLD)-magnetic resonance imaging (MRI) technique (156).
The importance of chronic hypoxia of the renal tubulointerstitium as an alternative common mechanism for the progression of chronic kidney disease, including diabetic nephopathy, has relatively recently emerged (157-158). The basic concept proposed a decade ago (159) suggested that chronic oxygen deprivation to the tubulointerstitial compartment leads to a scarring process consisting of tissue fibrosis and obliteration of the renal microvasculature, which perpetuates hypoxia and compromise the postglomerular capillary circulation. Therefore, a primary glomerular lesion leads to injuries to the peritubular capillary network due to decreased capillary flow, increased vasoconstriction and decreased vasodilation from affected glomeruli. The initial injury is exacerbated by the adjacent previously unaffected nephrons which are exposed to an elevated pressure and an increased blood flow generating endothelial damages and further hypoxia.
Since the hypothesis of chronic hypoxia has been proposed, accumulating data *in vivo* from animal models demonstrated hypoxia as a primary mediator of progressive scarring in the

kidney (158). In humans there are still limited clinical data. An increased expression of HIF has been reported in biopsies of patients with diabetic nephropathy.
Diabetic glomerular damage reduces the amount of functional peritubular capillaries. Thus, oxygen diffusion to tubulointerstitial cells decreases, leading to tubular dysfunction and fibrosis (160). It has been previously established that diabetic nephropathy affects primarily the glomeruli (161). In the early stages, in type 1 diabetic patients, only an increased glomerular filtration rate is present at diagnosis and the first histological modification detected is glomerular basement membrane thickening and mesangial volume expansion. Type 2 diabetic patients are more homogenous. Glomerulosclerosis, tubulointerstitial lesions and microalbuminuria are often present (162). Hypoxia plays an important pathogenic role even before the development of structural tubulointerstitial injury. It is supposed that hypoxia per se is a profibrogenic stimulus (157; 160) for tubular epithelial cells, interstitial fibroblasts and renal endothelial cells leading to apoptosis or epithelial-mesenchymal transdifferentiation. Low oxygen tension induces accumulation of extracellular matrix proteins, both by increase synthesis and decreased degradation. The mechanisms behind seems to involve a HIF dependent and independent regulation (157).
In the late stages, renal fibrosis is the hallmark of chronic kidney diseases regardless of the etiology, and therefore glomerulosclerosis and tubulointerstitial fibrosis are the best indicators for progression to end-stage disease (158; 160).
It became obvious that chronic hypoxia of the tubulointerstitium has an important pathogenic role not only in early stages of chronic renal diseases, but also is a common pathway to end-stage renal failure.

2.3.2.3. Diabetic retinopathy. Recent work suggests that diabetic retinopathy involves more than microvascular lesions and a significant neuroglial dysfunction is also present at very early stages of the disease (163). It has been shown a decade ago than retinal neurodegeneration occurs early in diabetes (164) and glial reactivity is an early feature of diabetic retinopathy (165). Chronic hypoxia could be involved in these processes since *in vitro* studies showed that Muller cells have lost their trophic effect under hypoxic conditions (166).
Chronic hypoxia could be related with retinal hypoperfusion suggested to occur in early stages of diabetes, but data are controversial (167-168). It is a general agreement that retinal blood flow changes early in diabetes and, despite many confounders (168), majority of studies suggest that patients with short duration of diabetes (<5 years) show a decrease in retinal blood flow and that blood flow increases with the severity of retinopathy (167). Data from retinal P_{O2} measurements are also inconclusive, especially in humans (168). Recently it has been shown by immunohystochemistry that retinal hypoxia occurs in mice after 5 months of diabetes (167). Despite controversy (168), lower vitreal P_{O2} was reported on diabetic patients undergoing vitrectomy (169).
Indirect evidence for chronic hypoxia occurring early in diabetic retinopathy is the observation that patients with very incipient lesions improved ocular function under pure oxygen atmosphere (167). Furthermore, intermittent chronic hypoxia is associated with retinopathy since sleep apnea syndrome has been related with diabetic macular edema and higher levels of circulating levels of retina-specific rhodopsin mRNA were observed in obese type 2 diabetic patients with more than 5 dips/hr (170).
It has been speculated that a certain threshold of hypoxia may overcome the early retinal vasoconstriction in diabetes, therefore mediating the shift to hyperperfusion (167). Hence, some of the lesions which define microvascular disease in diabetic retinopathy, i.e. basement membrane thickening, breakdown of the inner blood-retinal barrier and endothelial damage,

could be related to hyperperfusion. Increased retinal blood flow contribute directly to the occurrence of diabetic maculopathy (167).
Hypoxia mediates diabetic dysregulation of retinal blood flow in advanced retinopathy, also (168). Patients with proliferative diabetic retinopathy exposed to hypoxia show significantly increase in diameter of retinal vessels in the perifoveal circulation after improving of retinal oxygenation by laser treatment.
Strong body of evidence for importance of hypoxia as pathogenic factor in progression of diabetic retinopathy comes from studies both *in vivo* and *in vitro* using growth factors (171-173). VEGF is most extensively studied, since it has been shown to accumulate in the aqueous and vitreous of patients with all grades of retinopathy, especially in those with high levels of neovascularization (174-175). Hypoxia is considered to be the main regulator of VEGF in retinal cells (176). Moreover, VEGF represents one of the target genes for hypoxia-inducible factor 1 alpha (HIF-1α) which is the most important mediator of chronic hypoxia responses in organism (177). A very recent report (178) show that intravitreous VEGF in diabetic patients with proliferative diabetic retinopathy is increased and correlates with the severity of retinopathy and angiogenesis.
All these data suggests increased importance of chronic hypoxia for the pathogenesis of diabetic retinopathy, both in early and advanced stages.

2.3.2.4. Diabetic neuropathy. Diabetic neuropathies are the most heterogeneous and remain the least understood group of chronic complications of diabetes mellitus. Peripheral polyneuropathy is the most common and extensively studied form of diabetic neuropathy.
Hypoxia and reduced nerve blood flow are constant findings of *in vivo* studies in established diabetic polyneuropathy, both in humans and animals, even in early stages, before any structural changes occur (179-180). The role of hypoxia in nerve dysfunction has been documented by direct and indirect evidence (179). Endoneurial microangiopathy has been observed in diabetic patients with chronic diabetic neuropathy and could be a maladaptative response to hypoxia, reducing the oxygen diffusion, therefore exacerbating hypoxia (179; 181). An indirect evidence for the role of chronic hypoxia as a contributing factor in the development of diabetic neuropathy comes from numerous vasodilator studies, performed both in experimental diabetes and in humans, showing the benefits of vasodilator treatment on nerve conduction velocity (NCV) or blood flow (179; 182). Therefore, several alterations observed in diabetic neuropathy were reproduced in animals subjected to hypoxic conditions (183) or prevented by treatment with vasodilators (184). Furthermore, peripheral neuropathy develops in non-diabetic patients with chronic hypoxia, i.e. chronic obstructive pulmonary disease (185).
Hypoxia-inducible factor (HIF) mediates the response of the organism to the chronic hypoxia and several recent *in vivo* studies were performed to evaluate its contribution to the development and progression of diabetic neuropathy (186-187).
In sciatic nerve homogenates from type 1 diabetic rats it has been observed a transient increase in the expression of HIF-1α and its target genes, VEGF and eritropoietin, 4-6 weeks after the induction of diabetes with a decline at 8 weeks. Moreover, transient activation of HIF-1α did not induced any apoptotic event, as bNip3, a pro-apoptotic member of the Bcl2 family of proteins induced during hypoxia by HIF dependent pathway, has no change in the expression in diabetic nerves. The protective effects of HIF-1α activation are not sufficient during prolonged or severe hypoxia, and cell death occurs (188). VEGF expression decreases in chronic stages of the disease, as it has been shown in 6-months old diabetic rats (189). It has been therefore suggested a possible role for VEGF in nerve regeneration processes in long term diabetes.

Recently, the relevance of VEGF and HIF-1α in the maintenance of peripheral nerve integrity in diabetic patients with mild and severe neuropathy was assessed (187). A reduction of epidermal VEGF and its receptor expression was found by immunostaining in diabetic patients with more severe neuropathy. Despite increased blood vessel density, no change in HIF-1α expression, neither significant blood flow changes were documented. Another recent study has been quantified the wound-healing response in relation with VEGF and HIF-1α expression evaluated by immunostaining in type 2 diabetic patients with acute wound on the dorsum of the foot (190). A normal wound healing rate was found in diabetic patients despite an impaired maximal hyperemic response, a low level of VEGF and normal level of HIF-1α in skin. The studies were performed in either unwounded or acute wounded skin from diabetic patients, therefore the lack of difference in HIF-1α expression is not surprising, since the mains stimulus for HIF-1α is hypoxia (191) These findings suggest an important role of vascular factors in the development of painful symptoms and provide an argument for the use of vasodilating drugs in painful diabetic neuropathy. It has been also speculated a possible repressive effect of hyperglycemia on epidermal VEGF expression.
Is therefore clear that chronic hypoxia is a contributor to the development of diabetic neuropathy. New findings, at least *in vivo,* raise controversies for the relevance of hypoxic effects of the hyperglycemia in diabetic microangiopathy (hyperglycemic pseudohypoxia) (124). Recent studies performed on animals with both type 1 and type 2 diabetes, revealed the importance of insulin and peptide C deficiency as pathogenetic factors in diabetic neuropathy (180).

3. AIMS

3.1 General aim

The general aim of present thesis was to investigate two pathogenic mechanisms involved in chronic complications of diabetes and to identify new therapeutic approaches.

3.2 Specific aims

1. To characterize the mechanisms of glucose-dependent HIF-1α destabilization and decrease of function.
2. To describe the therapeutic effect of HIF induction in diabetic wounds.
3. To investigate the effect of hyperglycemia and hypoxia on the somatic mutations of mtDNA.
4. To investigate the effect of mitochondrial ROS production on mtDNA integrity in diabetes.

4. MATERIAL AND METHODS

Animals (Paper I and II). Female C57BL/KsJm /*Leptdb* (*db_/db_*) mice (Stock 000662) and their normoglycemic heterozygous littermates at different ages (paper I – 24 weeks; paper II – "old" 34 and "young" 10 weeks, respectively) originated from breeding pairs obtained from Charles River (Belgium). The female knock-in mice expressing an exonuclease-deficient version of the mitochondrial DNA polymerase γ (PolgD257A) ($PolgA^{mut}/PolgA^{mut}$) (15) and their heterozygous knock-in ($+/PolgA^{mut}$) mice at different ages (9 and 24 weeks respectively) were kindly offered by dr. N-G. Larsson, Karolinska Institutet, Stockholm (Sweden)**.** Animals were kept under controlled light and humidity conditions, with free access to standard laboratory food and water. The experimental procedure was approved by the North Stockholm Ethical Committee for Care and Use of Laboratory Animals.

Wound Model (Paper I). Following blood glucose control, general anesthesia was performed with 3% isoflurane (Abbott). The hair of the back was shaved with an electric clipper followed by a depilatory cream. The skin was rinsed with alcohol and two full-thickness wounds extending through the panniculus carnosus were made on the dorsum on each side of midline, using a 6-mm biopsy punch. A transparent dressing (Tegaderm; 3M) was applied to cover the wounds after topical application of 100 µl of DMOG (2 mM), DFX (1 mM), or vehicle alone. When specified, a viral suspension was injected intradermally into the wound edges using a 30-gauge needle. Four injections of 20 µl per wound were administrated (109 pfu/ml) containing HIF-1 V-N, HIF-1 V-NC or LacZ-expressing adenoviruses. Following the surgical procedure, the animals were individually housed. During the first 2 days, the animals received s.c. buprenorphine (0.03 mg/kg) twice a day for relief of any possible distress caused by the procedure. The wounds were harvested at 7 days after surgery (~50% closed). Treatment with freshly-made 100µl DMOG (2mM), DFX (1mM), or control was applied through the dressing using a 30-gauge needle every other day. Viruses (HIF-VN, HIF-V-NC, LacZ) were inoculated once at the beginning of the experiment. Each treatment was evaluated in 10 animals per group.

Wound Analysis (Paper I). Digital photographs were recorded at the day of surgery and every other day after wounding. A circular reference was placed alongside to permit correction for the distance between the camera and the animals. The wound area was calculated in pixels with ImageJ 1.32 software (http://rsb.info.nih.gov/ij), corrected for the area of the reference circle and expressed as percentage of the original area.

Tissue Preparation and Histological Analysis (Paper I). After fixation in formalin, the samples were embedded in paraffin and sectioned (5 µm). For histological evaluation, sections were deparaffinized and rehydrated followed by hematoxylin and eosin staining. All slides were then evaluated by light microscopy by two independent observers unaware of the identity of the biopsy, using a semiquantitative score to evaluate vascularity, granulation, and dermal and epidermal regeneration as previously described (38) and internally validated in our laboratory.
We used four-point scales to evaluate vascularity (1, severely altered angiogenesis with one or two vessels per site and endothelial edema, thrombosis, and/or hemorrhage; 2, moderately altered angiogenesis with three to four vessels per site, moderate edema and hemorrhage, but absence of thrombosis; 3, mildly altered angiogenesis with five to six vessels per site, moderate edema, but absence of thrombosis and hemorrhage; and 4, normal angiogenesis with

more than seven vessels per site with only mild edema but absence of thrombosis and hemorrhage) and granulation tissue formation (1, thin granulation layer; 2, moderate granulation layer; 3, thick granulation layer; and 4, very thick granulation layer) and a three-point scale to evaluate dermal and epidermal regeneration (1, little regeneration; 2, moderate regeneration; and 3, complete regeneration).

Immunohistochemistry Staining and Evaluation (Paper I). We evaluated microvessel density by semiquantitative, double-blind analysis of the specific binding of GS-1 isolectin B4 to microvascular structures using a four-point scale (0, no positive vessels; 1, low number of positive vessels; 2, moderate number of positive vessels; and 3, high number of positive vessels). Isolectin B4 binding was performed using biotinylated isolectin B4 (diluted 1:25). Expression of the adenovirus-mediated transfer of _-galactosidase was evaluated by immunohistochemistry using anti-β-galactosidase antibody (1:500) from Abcam. Matched IgG isotype controls were included for each marker. The hypoxia level within the wounds was evaluated using the Hypoxiprobe kit (Natural-Amersham Pharmacia) following the instructions of the manufacturer.

Cell culture (Paper I and II). Human dermal fibroblasts (HDFs, PromoCell, Heidelberg, Germany) were maintained in a humidified atmosphere with 5% CO_2 at 37°C in commercially supplied growth media for fibroblasts (Gibco, Stockholm, Sweden). Only cells between passages 4 and 9 were used. The human SKRC-7 cell line, originating from renal carcinoma from a patient with point mutated VHL, was kindly provided by E. Oosterwijk (Nijmegen, The Netherlands) and maintained as described (192). 3T3 cells (Promocell) were maintained like HDFs. Primary mouse skin fibroblasts (MSF) culture was established as below.

Establishing fibroblast primary cell culture (Paper I). Primary mouse skin fibroblasts (MSF) culture were establish using the skin explant technique. Skin samples from db/db or from control mice, cleaned from subcutis when needed, were cut in small pieces (2-3 mm) and placed in 6 wells plate under sterile coverslips. Two ml of complete DMEM were added to each well.

Hypoxia induction (Paper I and II). Hypoxia studies were run in a Modular Incubator Chamber (Billups-Rothenberg) flushed with a gas mixture of 95% N_2 and 5% CO_2 until the concentration of O_2, measured with an oxygen meter (Dräger) decreased to 1%.

Adenovirus preparation (Paper I). Adenoviruses expressing full-length mouse HIF-1α either stabilized against degradation by point mutations (P402A/P563A) only (V-N) or additionally with C-terminally truncated and VP16 replaced (V-NC) were cloned by homologous recombination into an E1- and partially E3-deleted serotype 5 adenovirus plasmid. Viruses were produced in 293 cells (193) and tested to be free of microbiological contaminants, mycoplasma, and endotoxin. Biological activity of the virus constructs was confirmed by an *in vitro* assay. LacZ adenovirus was used as a control (193).

Reporter Gene Assay (Paper I). 3T3 cells were transfected with 500 ng of a GAL4-driven luciferase reporter gene plasmid and 10 ng of NTAD residues (pFLAG-GAL4-mHIF1α-[531–584]) or CTAD residues (pFLAG-GAL4-mHIF1α-[772–822]) using Lipofectamine 2000 (Invitrogen) in 60-mm plates, following the instructions of the manufacturer. After 12 h, the cells were trypsinized and transferred in 12-well plates. After another 12 h, the cells were exposed to different glucose concentrations (5.5 mM or 30 mM) for 48 h and to different O_2

tensions for the last 24 h. The luciferase activity was determined (BioThema) in the cell extract and expressed on the total protein concentration.

VHL siRNA (Paper I). Human dermal fibroblasts were transiently transfected with 200pmol per well of either pVHL-siRNA (Hs_VHL_5HP validated siRNA SI02664550 for gene pVHL) or scrambled siRNA from Qiagen using HiPerFect. transfection reagent (Qiagen). After 48 h, cells were exposed to different glucose concentrations (5.5 mM and 30 mM) for another 48 h when the RNA was prepared as described.

Immunoblot Analysis (Paper I and II). Western blot analysis was performed for both HIF expression studies (paper I) and in vivo intracellular formation of reactive oxygen species (ROS) (paper II). The cell pellet was resuspended in 70 µl of RIPA buffer (50 mM Tris HCl pH 8.8, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS supplemented with protease inhibitors cocktail plus freshly-added 0.5 M Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 4 C for 20 min at 20 000 g. A total of 100 µg of total cellular proteins (measured by Bradford's assay with BSA standards) were blotted after SDS-PAGE to a nitrocellulose filter and blocked at room temperature for 1 ½ h with 5% nonfat milk in PBS. The membrane was then incubated with the primary antibody diluted in blocking buffer overnight at 4 C (paper II) or 2 h at room temperature (paper I). After several washes, the membrane was incubated with the secondary peroxidase-conjugated antibody from Amersham Biosciences (Sweden) diluted in PBS that contained 1% nonfat milk for 1.5 h. After extensive washing with PBS, the complexes were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. Membrane was then stripped in the 62.5 mM Tris-HCl, pH 6.7, buffer containing 2% SDS and 100 mM β-mercaptoethanol, and reprobed with β-actin antibody (1:5000) to confirm equal protein loading. Image analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij).

Intracellular reactive oxygen species (Paper II). The intracellular formation of reactive oxygen species (ROS) was detected *in vitro* using the fluorescent probe CM-H_2DCFDA (Molecular Probes, Sweden), according to the manufacturer's recommendations. At the end of the incubation period with different glucose or inhibitors concentrations and oxygen tensions, cells ($1x10^6$/ml) were loaded with 10 µM CM-H_2DCFDDA in pre-warmed phosphate buffer, incubated for 20 min at 37°C, washed and then re-incubated in normal growth media 15-20 min at 37°C for recovering. Cell lysates (70 µl) were analyzed in a Labvision Fluorescent Plate Reader (BMG Labtech, Germany) using the Fluostar software according to manufacturer's instructions. ROS production was expressed in fluorescence units as percentage of control. *In vivo* detection was performed by immunoblotting against anti–nitrotyrosine polyclonal primary antibody (Biomol, Plymouth Meeting, PA) as described (15; 194).

Mitochondrial DNA damage assays (Paper II). ROS-mediated mtDNA damage load was evaluated by long DNA targets quantitative polymerase chain reaction (QPCR) (195). The method is based on the premise that lesions in DNA will slow down or block the progression of DNA polymerase on template resulting in the decrease of DNA amplification in the damaged template when compared to the undamaged DNA. DNA extracted from cells or tissues QIAGEN Genomic DNA kit, QIAGEN, Sweden), was accurate quantified using PicoGreen (Invitrogen, Sweden). Long mtDNA fragments were amplified as described (195) using GeneAmp XL PCR kit (Applied Biosystems, Sweden). An additional reaction was performed to amplify a short mtDNA fragment used to normalize for mitochondrial copy

number. The number of mitochondria was also evaluated by QRT-PCR (see below). Different primer sets (Invitrogen, Sweden) were used for either human targets (Large mito 5999/14841, Small mito 14620/14841) or mouse targets (Large mito 3278/13337, Small mito 13688/13597). Optimal Mg^{++} final concentrations were 1,2 mM and 0,9 mM for human and mouse material, respectively. Normalized amplification of large mtDNA fragment was obtained by the ratio between absolute amplification values for both large and small mtDNA fragments. A mean of normalized amplification for non-damaged template was then calculated. Normalized damage load was finally obtained using the Poisson expression for a randomly normal distribution ($D=-\ln A_D/A_C$, where D is the number of damages per fragment, and A_D/A_C is the ratio between normalized amplification of target – damaged and control – undamaged template).

Preparation of mitochondrial fractions (Paper II). Mitochondrial protein fractions were isolated from HDFs and mice heart using Mitochondria Isolation Kit (PIERCE), according to manufactures' recommendation. For cells, isolation of mitochondria was performed using Reagent based method. Each animal sample was first homogenized (Dounce homogenizer, 7-8 strokes/sample, on ice), and treated afterwards with 0.3 mg/ml trypsin in PBS for 3 minutes on ice to facilitate cell structural breakdown and sample homogenization. The reaction was stopped by an equivalent amount of bovine trypsin inhibitor. Depending on the size, the mitochondrial pellet was resuspended in about 0.15 or 0.35 ml suspension buffer (2% CHAPS in Tris Buffered Saline, pH 7,2). The protein concentration was determined spectrophotometrically (NanoDrop, ThermoScientific). The purity of the mitochondrial fractions was evaluated both by lactate dehydrogenase (LDH) activity assay (196), and by Western blot using antibody against the nuclear – c-AMP response element (CRE)-binding protein (CREB, 1:500, Santa Cruz Biotechnology) (197). Briefly, for enzymatic activity studies, 10 μl of sample was 50 or 500-fold diluted in substrate buffer [Trisma HCl 0.2 M pH 8.5, 7.6 μM NAD^+, 0.244 M lactic acid (Sigma, Sweden)], and absorbance was measured by a spectrophotometer in kinetics mode for 120 sec at λ=340 nm, r.t. Specific enzymatic activity was measured [mol/(ml*min*μg tissue)]. Western blot analysis of CREB was performed as above. A whole cell lysate from stimulated HepG2 cells (TF CREB Phospho-Ser^{133}, Cayman Chemical) was used as positive control.

AP endonuclease activity assay (Paper II). Mitochondrial base excision repair (mtBER) activity was investigated using abasic site-containing oligonucleotides as substrates (25). 20 pmol of single-stranded 21-mer oligonucleotide with a tetrahydrofuran residue (THF) at the 10th position (The Midland Certified Reagent Company, Inc, TX) was end-labeled (45 min at 37°C followed by 10 min at 68°C) in a total volume of 20 μl with 5 pmol of γ[33P]ATP, 10 U of T4 polynucleotide kinase (Promega, Madison, WI), and appropriate kinase buffer. Complementary oligonucleotide (20 pmol) was added at room temperature to form duplex DNA. 80 ng of mitochondrial extracts or 2.5 units of control APE enzyme (Trevigen, Gaithersburg, MD) and 2 μl of 10x REC buffer (10 mM HEPES, pH 6.5, 100 mM KCl, 10 mM $MgCl_2$) were then incubated 1 h at 37°C with 1 pmol of labeled duplex oligonucleotide in a total volume of 20 μl. Formamide (80%) was added to the mix to stop the reaction, and the reaction products were analyzed by electrophoresis in soaked 25% polyacrylamide, 8 M urea gels. Wet gels were scanned using Fuji BAS-1500 Bioimaging Analyzer System (FUJIFILM Life Science, Stamford USA).

Quantitative RT-PCR (Paper I and II). Total RNA was extracted from cells or animal tissues (Micro-to-midi total RNA purification system, Invitrogen). cDNA was obtained by

reversely transcribing total RNA with SuperScript III and first-strand synthesis supermix for qRT-PCR according to the manufacturer's recommended protocol (Invitrogen, Sweden). The primers used in paper II (Invitrogen, Sweden) were: catalase forward 5'-GCGTCCAGTGCGCTGTAG-3' and reverse 5'-CCTTTGTGTAGAATGTCCGCAC-3'; SOD_2 forward 5'-ACCCAAAGGAGAGTTGCTGGAG-3' and reverse 5'-CTGAACCTTGGACTCCCACAGA-3'; $PBGD_2$ forward 5'-ACTCTGCTTCGCTGCATTG-3' and reverse 5'-AGTTGCCCATCTTTCATCACTG-3'; cyclophiline forward 5'-GGCTCCGTCGTCTTCCTTTT-3' and reverse 5'-ACTCGTCCTACAGATTCATCTCC-3' , cytochrome b forward 5'-TGTTCGCAGTCATAGCCACAG-3'and reverse 5'-TCGGGTCAAGGTGGCTTTGTCTA-3'; $PBGD_2$ forward 5'-GTGTTGCACGATCCTGAAATC-3' and reverse 5'-GTTGCCCATCCTTTATCACTGTA-3', and cyclophiline forward 5'-CAAATGCTGGACCAAACACAA-3'and reverse 5'-GCCATCCAGCCATTCAGTCT-3'. The primers for paper I (see *SI* text) were selected from Harvard University primer bank (primer 1-4) (http://pga.mgh.harvard.edu/primerbank), used as previously published (primers 5-9) (198). Real time PCR was performed in an Applied Biosystems Instrument 7300 using Platinum SYBR Green qPCR supermix-UDG with ROX reference dye (Invitrogen). After incubation 2 minutes at 50ºC and 2 minutes at 95ºC a two-step cycling protocol (15 s at 95ºC, 30 s at 60ºC) was used for 40 cycles. The melting curve analysis was done using the program supplied by Applied Biosystems. The quality of qPCR run was determined by standard curves and melting curve analysis.

Statistic analysis (Paper I and II). Differences between groups were computed using one-way analysis of variance (ANOVA), with *Tukey* or *Bonferoni* post hoc tests. A $p<0.05$ was considered statistically significant.

5. RESULTS AND DISCUSSION

This thesis investigated two pathogenic mechanisms involved in chronic complications of diabetes in order to identify new therapeutic approaches.
Therefore, in the first paper, we focused our investigation on the effects of hyperglycemia and hypoxia on hypoxia-inducible factor (HIF) 1α stability and function, as a molecule with a pivotal role in wound healing in diabetes (199), whereas in the second paper we further investigated the influence of chronic hyperglycemia and hypoxia in relation with reactive oxygen species (ROS) production on mitochondrial DNA (mtDNA), as main target for oxidative damage mediated by ROS (12).

5.1. Paper I. Stabilization of HIF-1α is critical to improve wound healing in diabetic mice

5.1.1. Hyperglycemia has repressive effects on HIF-1α stability

The repressive effect of hyperglycemia on HIF-1α stability observed in db/db mouse primary fibroblasts (Fig. 1A) was confirmed in diabetic wounds of the db/db mice (Fig. 3A). The effect of glucose was dependent on the VHL-mediated degradation mechanism, since hyperglycemia did not modulate HIF-1α stability in renal carcinoma cells that constitutionally lack functional pVHL (Fig. 1B Left) and VEGF expression in human dermal fibroblasts where VHL expression was down-regulated by treatment with siRNA (Fig. 1B Right). Hyperglycemia did not induce VHL (Fig. 1B) suggesting that it can make HIF-1α more sensitive to the VHL machinery.

5.1.2. Hyperglycemia impairs HIF function

We further investigate the effect of hyperglycemia on HIF-1α function. The negative effect of glucose affected both transactivation domains of HIF-1α (Fig. 1C) and several HIF-1α target genes essential for wound healing (Fig. S1) were down-regulated by hyperglycemia *in vitro*. The repressive effect of hyperglycemia on HIF-1α function was confirmed in diabetic wounds. Therefore, HIF-1α expression was much lower in db/db wounds than in control heterozygote mice (Fig. 3A), despite more pronounced hypoxia in db/db wounds (as evaluated by binding of the hypoxia dye pimonidazole hydrochloride; Fig. 3B). Moreover, HIF-1 target genes essential for wound healing cell motility (i.e., HSP-90), angiogenesis (i.e., VEGFA and VEGF-R1), and recruitment of CAG (i.e., SDF-1α, SCF, and Tie-2) were also repressed in the diabetic wounds, as compared with the wounds in normoglycemic heterozygote mice (Fig. 3C). Previous studies reported a delayed expression of VEGF-A (200) and a low expression of SDF-1α in diabetic wounds (201), both mechanisms being suggested as good candidates for therapeutic interventions. Our results show that repression of HIF is the common causative link between hyperglycemia and suppression of VEGF-A and SDF-1 expression, thereby allowing the proposal of a potentially more effective therapy.

5.1.3. HIF stabilization counteracts the repressive effect of hyperglycemia on its stability and function

The role of prolyl and asparaginyl hydroxylation for the regulation of HIF-1α stability and repression is critical (38; 202-203). Therefore, we further studied if inhibition of hydroxylase

activity could counteract the negative regulatory effect of hyperglycemia. We used two structurally different hydroxylase inhibitors, i.e., the 2-oxoglutarate analogue dimethyloxalylglycine (DMOG) and the iron chelator deferoxamine (DFX), which stabilize and activate HIF-1 by different mechanisms (204). In primary db/db mouse fibroblasts both inhibitors stabilized the HIF-1α in hyperglycemia and normoxia to levels which were very similar to those produced by hypoxia in normal glucose concentrations (Fig. 2A). Moreover, the compounds induced expression of HIF target genes essential for motility, angiogenesis, and recruitment of CAG (Fig. 2B) even in the cells exposed to high glucose concentrations. The effect can be observed even in hypoxia as illustrated for VEGF (Fig. S2). Both treatments improved wound healing process in db/db mice (Fig. 4 A and B), and the effect persist despite persistent chronic hyperglycemia, without interfering with the animal's weight. We might expect a better effect for DFX treatment, considering its antioxidant proprieties (205) and the essential role of reactive oxygen species in the pathogenesis of chronic complications of diabetes (206). However, DMOG was at least equally as efficient as DFX, if not more effective (Fig. 4E), in promoting wound healing (both used as the highest efficient doses as titrated in pilot experiments). This could possibly be attributed to the known antiproliferative effects of DFX (207), which we also observed *in vitro* (data not shown). Despite the mechanism of hyperglycemia-induced HIF-1 destabilization is still unclear (38; 40; 202; 208-209), here we provide strong evidence that it can be satisfactorily overcome by blocking the prolyl hydroxylation.

5.1.4. HIF stabilization is critical for improving defective wound healing in diabetic mice

In order to obtain a direct evidence whether the wound healing-promoting effects observed after treatment with the hydroxylase inhibitors were mediated through HIF, we performed gain-of-function studies by adenovirus-mediated expression of constitutively stable forms of HIF-1α (V-N and V-NC) in which both the critical proline residues have been substituted with alanines (Fig. S2). In the V-NC construct CTAD was additionally substituted with the potent VP16 TAD. We provided this evidence by observing the same positive effect on wound healing in diabetic animals as pharmacological inhibitors (Fig. 4C). The V-NC construct had no additive effects compared with the V-N construct, strongly suggesting that CTAD repression is not critical for wound healing (Fig. 4D). Our result might suggest a central pathogenic role for the NTAD without any additional regulatory effect from the CTAD which has also recently been reported for HIF-2α-driven renal carcinogenesis (210). An alternative explanation for lack of superiority of V-NC compared with VN is the squelching effect (211) of a highly active transactivation activity classically described for VP16. Our observation is highly relevant for future development of hydroxylase inhibitors as potential therapy.

5.1.5. Stabilization of HIF-1α activates several processes involved in wound healing

Histological analysis of diabetic wounds revealed that treatment with either hydroxylase inhibitor (DMOG or DFX) or inoculation of the stable V-N HIF-1α adenovirus construct was followed by an improvement in all of the essential steps of wound healing. Wound healing rate is delayed in db/db mice as a result of a decrease in granulation formation and neovascularization (212). We observed a well structured granulation tissue (Fig. 5A) and a significant improvement of the total number of vessels, as evaluated by classical histology or by staining with GS-1 lectin (Fig. 5A and B) in diabetic wounds were HIF-1α was either pharmacology stabilized or over-expressed in a stable form. Local treatment with DMOG, DFX, or V-N in wounds of db/db mice increases the expression of HIF-α target genes

encoding angiogenic cytokines (Fig. S4), which is in an excellent agreement with the histological changes we observed here, and with previous reports in the literature (198; 213). The positive effect on angiogenesis was not followed by edema, which is in agreement with the phenotype of transgenic mice over-expressing HIF (214) and in contrast to the mice over-expressing VEGF (215) in the skin. Lack of homing of CAG has been suggested to be an important factor in delayed wound healing in diabetes (201), but the intimate mechanisms for it have not been established. We indirectly evaluated the recruitment of angiogenic cells to the wounds by measuring the levels of the cytokine receptor expression typically present on these cells. This approach circumvents the technical problem to detect the small numbers of angiogenic cells usually present in tissues (198). We found that HIF-1α stabilization was followed by an increase at the wound level of these cytokine receptors (i.e., CXCR4, C-Kit, Tie-2) (Fig. 5*C*).

In conclusion, we show here that hyperglycemia-induced destabilization and inhibition of HIF-1α is a central pathogenic mechanism for delayed wound healing in diabetes. HIF stabilization is essential and sufficient for improving wound healing in diabetes.

5.2. Paper II. Stability of mitochondrial DNA against reactive oxygen species (ROS) generated in diabetes

5.2.1. High glucose and/or hypoxia increase the mitochondrial ROS production in HDFs and induce mtDNA damage.

We first evaluated ROS in human dermal fibroblasts (HDF) exposed to hyperglycemia and hypoxia, considering their pathogenic relevance. We used the carboxy-methyl-H_2DCFDA probe (DCF), which has the ability to detect a wide spectra of ROS (216). High glucose and hypoxia alone were able to increase the DCF formation whereas their combination induced the highest amount of ROS in HDF (Fig. 1A). The excessive ROS production originates in mitochondria since it is normalized if the HDF are treated with thenoyltrifluoroacetone (TTFA), an inhibitor of complex II (figure 1A) that blocks the mitochondrial respiratory chain.

Therefore, we measure the normalized lesions load in long fragments of mtDNA from HDFs cultured in different glucose concentrations and oxygen tensions. Only the combination between high glucose and hypoxia increased the mtDNA normalized damage load in HDFs when compared with normal conditions (normoglycemia and normoxia) (Fig. 1B). The sensitivity of the method to detect mtDNA lesions was validated by obtaining a dose dependent increase of the normalized damage frequency in mtDNA when exogenous H_2O_2 was used as donor in HDFs (*Insert*). There is no excess of mtDNA damage accumulation in HDF exposed to the combination of hyperglycemia and hypoxia if the respiratory chain is blocked with TTFA (Fig. 1C) showing that mtDNA lesions in these conditions is a consequence of the increased ROS production by mitochondria.

The levels of ROS seem to be critical for inducing mtDNA lesions since only the combination of hyperglycemia and hypoxia was able to induce mtDNA damages along with the highest concentration of ROS. The relevance of the levels of ROS for mtDNA lesion load was also observed in other context (12) and is confirmed here by the dose-dependent effect of H_2O_2 on the mtDNA damage. In the same direction is the observation that glucose alone is able to affect mtDNA but only at extreme high glucose concentrations (217) or when the time of exposure is long enough to permit accumulation of sufficiently high levels of ROS (218).

Other factors with potential relevance for complications in diabetes as palmitate have been shown to induce mtDNA lesions *in vitro,* probably ROS mediated (219).
Even though ROS have been previously reported to have a detrimental effect on mtDNA (220) we here made the first observation that pathogenic mechanisms relevant in diabetes (hyperglycemia and hypoxia) can induce mtDNA lesions through mitochondrially produced ROS.

5.2.2. Db/db mice accumulate less mtDNA lesions in heart and kidney despite increase in ROS levels.

Unexpectedly, the mtDNA damages *in vivo* were lower in diabetes. Significantly less mtDNA lesions were found in both heart and kidney from "old" db/db mice when compared either with "young" (before developing diabetes) animals or with non-diabetic "old" littermates ($p<0.01$) (Fig. 2A1 and A2). We performed an additional validation of the long fragment QPCR method to evaluate the mtDNA damage *in vivo* by measuring the widespread lesions load of mtDNA in hearts of $PolgA^{mut}/PolgA^{mut}$ mice. An increased mtDNA damage load ($p=0.025$) was observed (Fig. 2A3). The model chosen for this study (db/db mice at different age) has several advantages over previous observations (221-222). The genetic background is homogenous (it consists of an inbred strain) and there is a definitive control over the time of tissue exposure to diabetes. We also obtained consistent results in two tissues (hearts and kidney) with different content of mitochondria, traditional targets for chronic complications of diabetes. It is therefore highly relevant compared with previous observations in peripheral blood mononuclear cells (PBMC) (221; 223). Our results are in line with the finding of low mutagenicity of ROS in ageing cells (224) and increasing evidence that susceptibility of mtDNA to the accumulation of oxidative base substitution have been overestimated (225). It is probably also important to mention that we could observe a decrease of the mtDNA damages in diabetic animals at an enough early age since no typical age-dependent mtDNA lesions were reported in non-diabetic animals (226-227). We cannot exclude that the method used to evaluate the mtDNA damages can have a lower sensitivity to detect the expected age-dependent mtDNA lesions in controls but this does not change the significance of the mtDNA protection registered in "old" diabetic mice.

Despite the lower incidence of mtDNA lesions, the ROS production in "old" diabetic mice was increased as expected. Therefore, the levels of nitrosylation were higher (Fig. 2B) as a direct consequence of increased levels of peroxynitrite production resulting from the reaction between nitric oxide and superoxide (228). Similarly, the expression of the two major mitochondrial antioxidant enzymes SOD_2 and CAT (Fig. 3) was also reactively increased in "old" db/db mice. It has been reported that cells chronically exposed to high glucose concentrations expressed an increased enzymatic activity for both CAT and SOD_2 (229). We have, however, observed an increase of CAT in both tissues studied while SOD_2 was up-regulated only in hearts (Fig. 3). Some other tissue specific modulation of the antioxidant enzymes were reported in other animal models (230-231). We, however, did not observe a difference in the tissue accumulation of mtDNA mutations dependent on different antioxidant enzymes expression as it was previously reported in a different scenario (230).

5.2.3. The mitochondrial base excision repair (BER) system is increased in the "old" db/db mice.

We therefore analyzed mtDNA repair, as a second line of defense against oxidative stress. The activity of apurinic/apyrimidinic (AP) endonuclease (APE) was investigated, since the

enzyme repairs AP sites generated by both ROS and excision steps along base excision repair (BER) pathway (232). Activation of APE in mitochondrial extracts from the hearts of the "old" db/db mice (Fig. 4A1) was observed. That could explain the lower amount of mtDNA lesions accumulated in these mice. The observation is even more interesting as a decrease of the BER activity with age has been reported both in mice and humans (233-234). Mitochondrial aggression by ROS in diabetes could trigger a retrograde signaling from the mitochondria to the nucleus as it was described for the antiapopotic response (235). The induction of the APE needs probably a chronically exposure to ROS since it is not induced after 48 hours exposure to hyperglycemia and 48 hours additional exposure to hypoxia (Fig. 4A2). The mechanism of APE activation in diabetes needs further investigation considering the potential therapeutic applications for other agents that induce mtDNA damages, i.e. UV, chemotherapy etc.

In conclusion, our study shows for the first time that hyperglycemia and hypoxia were able to induce mtDNA damages through induction of mitochondrial ROS production. The pathogenic mechanism was however successfully compensated in diabetic animals by the repairing mechanisms, therefore making it probably not contributing to the development of chronic complications in this model of diabetic animals.

6. PERSPECTIVES

We focused our investigation on HIF and mtDNA, two factors of increased impact for the understanding of the pathophysiological processes involved in chronic complications of diabetes.

HIF is critical for adaptative responses to hypoxia and therefore for regulation of all processes involved in tissue repair and wound healing. We observed a different regulatory effect of the two transactivation domains of HIF in an animal model of diabetic wounds. This observation is highly relevant for future development of hydroxylase inhibitors as potential therapy.

mtDNA accumulates somatic mutations with age which probably induces a variety of aging phenotypes. Therefore, understanding the mechanisms behind stability of mtDNA might be important for the evolution of chronic complications of diabetes.

We show that accumulation of mtDNA lesions was successfully compensated in a model of diabetic animals, suggesting as possible explanation the activation of APE. The mechanism by which APE is activated in diabetes warrants further investigation considering the potential therapeutic applications for other agents that induce mtDNA damages such as UV or chemotherapy.

7. CONCLUDING REMARKS

7.1. HIF-1α is an essential factor and a potential therapeutic target for improving the defective wound healing process in diabetes.

7.1.1. High glucose impairs HIF-1α stability and function in fibroblasts and wounds of the db/db mice.

7.1.2. The repressive effect of high glucose involves the VHL-mediated degradation mechanism.

7.1.3. Blockade of the HIF hydroxylation, either pharmacologically (by treatment with DMOG or DFX) or by adenovirus-mediated experiments (constitutively stable forms of HIF-1α) is a highly relevant potential therapy for improving wound healing in diabetes.

7.1.4. HIF stabilization is critical for improving defective wound healing in diabetic mice, activating all the essential steps of this process.

7.2. High glucose and hypoxia together, are able to induce mtDNA damages through induction of mitochondrial ROS production in HDFs.

7.2.1. The levels of ROS is critical for inducing mtDNA damages in HDFs since only the combination of high glucose and hypoxia induced highest concentrations of ROS and significant mtDNA damages.

7.2.2. mtDNA repair system evaluated by APE activity needs a chronically exposure to ROS since it is not modulated after 48 h exposure to high glucose and 48 h additional exposure to hypoxia.

7.3. Db/db mice accumulate less mtDNA lesions in heart and kidney despite increase in ROS levels.

7.3.1. Increased ROS levels in “old” db/db mice were associated with high expression of both mitochondrial SOD and CAT.

7.3.2. mtDNA repair system evaluated by APE activity was upregulated in “old” db/db mice and could explain the lower amount of mtDNA lesions accumulated in this mouse model of diabetes.

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