Linking TNF-induced dysfunction and formation of methylglyoxal derived AGEs in endothelial cells

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Thesis submitted to fulfil the requirements for achievement of the degree of Ph.D in Biomedical Sciences
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced Glycation End Product</td>
</tr>
<tr>
<td>ASK</td>
<td>Apoptosis Signal Regulating Kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-Cell Activating Factor</td>
</tr>
<tr>
<td>BCL</td>
<td>B-Cell Lymphoma/Leukaemia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAMKIV</td>
<td>Calcium/Calmodulin-Dependent Kinase</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>Cdc37</td>
<td>Cell Division Cycle 37</td>
</tr>
<tr>
<td>CEL</td>
<td>Carboxy Ethyl Lysine</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxy Methyl Cysteine</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine Rich Domain</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element-Binding Protein</td>
</tr>
<tr>
<td>Crm</td>
<td>Exportin 1, Chromosome Region Maintenance</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclo Heximide</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DD</td>
<td>Death Domain</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone Phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>DesoxyriboNucleic Acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Cell Matrix</td>
</tr>
<tr>
<td>ELKS</td>
<td>Glutamic Acid (E), Leucine (L), Lysine (K) and Serine (S)-Abundant Protein</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial NO Synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Related Kinase</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas Associated Death Domain</td>
</tr>
<tr>
<td>FADH</td>
<td>Flavine Adenine Dinucleotide</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acids</td>
</tr>
<tr>
<td>FIP-3</td>
<td>14,700 kDA Interacting Protein 3</td>
</tr>
<tr>
<td>F2,6BP</td>
<td>Fructose 2,6 Bisphosphate</td>
</tr>
<tr>
<td>GA</td>
<td>Glyceraldehyde</td>
</tr>
<tr>
<td>GAP</td>
<td>Glyceraldehyde Phosphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine DiPhosphate</td>
</tr>
<tr>
<td>GLCNAC</td>
<td>N-Acetyl glucosamine</td>
</tr>
<tr>
<td>GLO</td>
<td>Glyoxalase</td>
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<tr>
<td>GRB</td>
<td>Growth Factor Receptor Bound Protein</td>
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<td>GSH</td>
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<td>Germinal Centre Kinase</td>
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<tr>
<td>GTP</td>
<td>Guanosine TriPhosphate</td>
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<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
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</table>
HAT  Histone Acetyl Transferase
HDAC  Histone Deacetylase
HLH  Helix Loop Helix
HSA  Human Serum Albumin
Hsp27  Heat Shock Protein 27
Hsp90  Heat Shock Protein 90
ICAM  Intracellular Adhesion Molecule
IDDM  Insulin Dependent Diabetes Mellitus
IgG  Immunoglobulin G
IKK  IκB Kinase
IKKAP1  IKK Associated Protein 1
IL  Interleucine
IkB(a)  Inhibitory κB (α)

JNK  Jun N-Terminal Kinase

LPS  Lipopolysaccharide
LT  Lymphotoxin
LZ  Leucine Zipper

MAP  Mitogen Activated Protein
MAPK  Mitogen Activated Protein Kinase
MCP  Macrophage chemo-attractant Protein
MEK  MAPK/Erk Kinase
MEKK  MAPK/Erk Kinase Kinase
MG  Methylglyoxal
MG-H1  AGE of the Hydro-imidazolone type
MKK  Mitogen Activated Protein Kinase Kinase
MODY  Maturity Onset Diabetes of the Young
MOLD  Methylglyoxal Lysine Dimer
MSK  Mitogen and Stress activated Kinase

NADPH  Nicotinamide Adenine Dinucleotide Phosphate
NAK  NF-κB Activating Kinase
NBD  Nemo Binding Domain
NEMO  NF-κB Essential Modulator
NES  Nuclear Export Signal
NF-κB  Nuclear Factor κB
Niddm  Non-Insulin Dependent Diabetes Mellitus
NK CELLS  Natural Killer Cells
NLS  Nuclear Localisation Sequence
NO  Nitric oxide

OPGL  OsteoProteGrin Ligand

PAI-1  Plasminogen activator inhibitor 1
PARP  Poly(ADP-Ribose)Polymerase
PECAM  Platelet/Endothelial Cell Adhesion Molecule
PKF  Phospho Fructo Kinase
PI3K  Phosphoinositide Kinase 3
PKA  Protein Kinase A
PKC  Protein Kinase C
PLAD  Pre-Ligand Assembly Domain

Rage  Receptor For AGEs
RANK  Receptor Activator of NF-κB
RANKL  Receptor Activator of NF-κB Ligand
RAS  Rat Sarcoma
RHD  Rel Homology Domain
RIP  Receptor Interacting Protein
RNA  Ribonucleic Acid

SAPK  Stress Activated Protein Kinase
SODD  Silencer of Death Domain
SOS  Son Of Sevenless
SP1  Stimulatory Protein 1

T2K  TRAF2 Associated Kinase
TACE  TNF converting enzyme
TBK  Tank Binding Kinase
TCA  TriCarbolic Acid
THP  TetraHydroPyrimidine
TNF  Tumour Necrosis Factor
TNFR  TNF-Receptor
TPI  TriosePhosphate Isomerase
TRADD  TNFR Associated Death Domain
TRAF  TNFR Associated Factor
TRAIL  TNF Related Apoptosis-Inducing Ligand
TRANCE  TNF Related Activation-Induced Cytokine
TWEAK  TNF-Related Weak Inducer of Apoptosis

VCAM  Vascular Cell Adhesion Molecule
VEGI  Vascular Endothelial Growth Inhibitor

Yap1  Yeast Activator Protein 1
Summary

Diabetes is one of the world’s most important diseases and has taken on epidemic proportions worldwide. This multi-factorial disease is mainly characterised by hyperglycaemia and a low grade inflammation and increases the risk for developing several complications like nerve damage, high blood pressure, blindness and heart failure. Endothelial cells, which delineate the blood vessel, are the primary target for the development of complications. These specialised cells not only have a barrier function, preventing leakage from the blood vessel, but also function as an organ regulating blood flow, inflammation, blood clotting… Disturbance of any of these functions may lead to endothelial dysfunction and the development of vascular complications. Researchers all over the world are now trying to understand the development of these complications in an attempt to prevent them. It is now well established that hyperglycaemia itself induces endothelial dysfunction, for instance by modifying proteins. These modified proteins are called ‘advanced glycation end products’ or AGEs. Also, the mechanisms by which inflammation contributes to endothelial dysfunction are starting to become elucidated. For instance, the pro-inflammatory cytokine TNF is known to disturb the blood flow regulating function of endothelial cells.

Although glucose was thought to be the main precursor of those AGEs, later studies have proven that it is in fact methylglyoxal, which is the main precursor. Methylglyoxal is a cytotoxic side product of the glycolysis and is normally detoxified by the glyoxalase system, which comprises glyoxalase1 and glyoxalase2. A previous study in our lab indicated that in a fibrosarcoma cell-line, TNF is able to induce the formation of specific methylglyoxal derived AGEs (MG-AGEs) by phosphorylating glyoxalase1. Given the importance of both TNF and AGEs in endothelial dysfunction, we wanted to investigate whether TNF could also phosphorylate glyoxalase1 in endothelial cells, and whether this phosphorylation was accompanied by the formation of specific MG-AGEs.

Here we provide evidence that TNF is also capable of inducing AGE formation in endothelial cells, just like hyperglycaemia does, by upregulating the glycolytic flux which produces the AGE precursor methylglyoxal. In addition we provide evidence that TNF modifies specific proteins. Although we also observed phosphorylation of glyoxalase1 upon TNF treatment, phosphorylation of this enzyme seems not necessary to induce the formation of MG-AGEs in these cells.

Additionally we identified the transcription factor NF-κB as a possible new AGE modified protein. Our data indicate that methylglyoxal modification of this protein abolishes its DNA binding capacity, resulting in decreased transcription. Since NF-κB is known to induce the transcription of several genes, protecting cells from TNF induced cell death, our data indicate that methylglyoxal modification of NF-κB could sensitise endothelial cells to TNF induced apoptosis, resulting in vascular leakage and the development of vascular complications.
**Samenvatting**

Diabetes is één van ‘s werelds meest belangrijkste ziektes aan het worden en heeft eigenlijk epidemische proporties aangenomen. Deze ziekte heeft verschillende oorzaken maar wordt steeds gekenmerkt door hoge suikerwaarden in het bloed (hyperglycemie), en een milde ontsteking. Bovendien verhoogt diabetes de kans op vasculaire complicaties zoals zenuwschade, hoge bloeddruk, blindheid en hartfalen. Endotheelcellen, die de binnenste wand van het bloedvat vormen, zijn primaire doelwitten in de ontwikkeling van deze complicaties. Deze gespecialiseerde cellen vormen niet alleen een barrière die lekkage uit het bloedvat vermijdt maar functioneren ook als een orgaan dat bv. bloeddoorstroming, ontsteking en bloedklontering regelt. Elke verstoring van één van deze functies kan aanleiding geven tot wat men noemt endotheliale dysfunctie en de ontwikkeling van vasculaire complicaties. Over gans de wereld proberen onderzoekers nu uit te vissen hoe deze complicaties tot stand komen in een poging om de ontwikkeling ervan te verhinderen. Zo is het al geweten dat hyperglycemie op zich oorzaak kan zijn van endotheliale dysfunctie, door bijvoorbeeld eiwitten te modifieren. Deze gemodificeerde eiwitten worden dan ‘advanced glycation end products’ (AGE’s) genoemd of vrij vertaald ‘geavanceerde glycatie adducten’. Ook de mechanismen hoe inflammatie bijdraagt tot endotheliale dysfunctie beginnen stilaan opgehelderd te geraken. Zo is bijvoorbeeld aangetoond dat het pro-inflammatoire TNF de regulerende functie van endotheelcellen op het gebied van bloedstroom kan verstoren.

Hoewel oorspronkelijk gedacht werd dat glucose de voornaamste precursor van AGE’s was, hebben latere studies uitgewezen dat methylglyoxaal in feite de voornaamste precursor is. Methylglyoxaal is een cytotoxisch bijproduct van de glycolyse en wordt normaal gedetoxifieerd door het glyoxalase systeem, bestaande uit glyoxalase1 en glyoxalase2. Een vorige studie in ons labo heeft uitgewezen dat in een fibrosarcoma cellijn, TNF de vorming van specifieke methylglyoxaal afgeleide AGE’s (MG-AGE’s) kan induceren door middel van fosforylatie van glyoxalase1. Gezien zowel TNF als AGE’s bijdragen tot endotheliale dysfunctie, wilden we nagaan of TNF in endotheliale cellen glyoxalase1 fosforyleert en of dat of deze fosforylatie gepaard gaat met de vorming van specifieke MG-AGE’s.

In onderliggend werk hebben we kunnen aantonen dat TNF ook de vorming van AGE’s induceert in endotheliale cellen en dit, net zoals in hyperglycemie, door de glycolyse op te reguleren die de precursor methylglyoxaal produceert. Bovendien hebben we ook kunnen aantonen dat TNF specifieke eiwitten modificereert. Hoewel we ook in deze cellen fosforylatie van glyoxalase1 konden vaststellen na TNF stimulatie, blijkt de fosforylatie van dit enzyme niet noodzakelijk voor de vorming van de AGE’s.

Ook hebben we aangetoond dat de transcriptiefactor NF-kB gemodificeerd kan worden tot een AGE. Onze resultaten wijzen er op dat de DNA bindingscapaciteit van dit eiwit verloren gaat wanneer dit eiwit methylglyoxaal gemodificeerd wordt, wat resulteert
in een verminderde transcriptie. NF-κB induceert de transcriptie van verschillende genen die beschermend werken in TNF geïnduceerde cel dood. Onze resultaten kunnen er dus op wijzen dat methylglyoxaal modificatie van NF-κB, endotheelcellen gevoelig kan maken voor TNF geïnduceerde cel dood wat kan leiden tot lekkage van het bloedvat en de ontwikkeling van vasculaire complicaties.
General Introduction
1. The diabetic epidemic 1-3

Diabetes is the fifth-deadliest disease in the United States and the disease has taken on epidemic proportions, worldwide. This (yet) non-curable disease is characterised by a lack of insulin production and/or impaired insulin sensitivity. The human body, and in particular fat and muscle cells, need insulin to take up glucose from the blood. As a consequence, diabetic patients suffer from high blood sugar levels. In the U.S. alone there are about 14 million people diagnosed with diabetes, and an estimated 6.2 million diabetics are unaware of their disease (Figure 1). In total that is about 7% of the U.S. population. Additionally 54 million are in a pre-diabetic state, which means that they have a high risk for developing diabetes. Worldwide there are an estimated 240 million people with diabetes, and it is predicted that this number will increase with 35 million in 2025. In Belgium, about 600,000 people have diabetes and this will probably increase to 750,000 by 2025. Given these numbers, it is not surprising that the disease represents a large portion of the health care expenditure. In the U.S., 11% of health care expenditure is due to diabetes. Direct costs, like medical costs, were about $92 billion in 2002. Indirect costs due to for example work loss or disability were about $40 billion in the same year. In Belgium, between 7 and 15% of healthcare expenditure is due to diabetes.

There is almost no difference between men and women in the risk for developing diabetes. However people with a family history in diabetes have higher risks, and also people aged over 45, people who are overweight, do not exercise regularly, have low HDL cholesterol or high triglycerides, high blood pressure and women who had a

![Figure 1: Estimated number of people with diabetes (in millions) for 2000 (top) and 2010 (bottom). The lower panels indicate the increase in %. Adopted from 3.](image-url)
baby weighing more than 4 kilograms at birth. Also certain ethnic and racial groups like Non-Hispanic Blacks, Hispanic/Latino Americans, Asian Americans, Pacific Islanders, American Indians and Alaska Natives are at higher risk.

There are generally three types of diabetes. Type 1 diabetes is characterised by a destruction of the insulin producing pancreatic beta-cells. This form of diabetes accounts for 5-10% of all cases of diabetes and usually strikes young people or adolescents (and is therefore also referred to as juvenile-onset diabetes), but it can occur at any age. There are several causes for the development of type 1 diabetes including viral infections, perinatal infections, environmental factors and auto-immune responses. Although the cause of type 1 diabetes is well studied, there is still no known way to prevent it. Patients must rely on insulin injections or insulin pumps. Type 1 diabetes is therefore also called insulin dependent diabetes mellitus (IDDM).

Type 2 diabetes accounts for 90-95% of all cases of diabetes. These patients still produce enough insulin, but they have become insensitive to the action of insulin. This form of diabetes is therefore also called non-insulin dependent diabetes mellitus (NIDDM). The disease strikes especially older people, but nowadays it is also seen in very young people (referred to as ‘maturity onset diabetes of the young’ or MODY). Type 2 diabetes is a typical welfare disease caused by inactivity, obesity, greasy food, smoking... The human body will try to compensate impaired insulin sensitivity by increasing insulin production. This may eventually lead to a destruction of the pancreatic beta cells and the development of type 1 diabetes. Type 2 diabetics are usually treated with insulin-sensitising drugs like Metformine, Rosiglitazone and Pioglitazone. Other drugs like sulfonylureas try to compensate insulin insensitivity by increasing insulin secretion. Another approach is to delay digestion of carbohydrates (with i.e. Acarbose) avoiding high glucose peaks after meals. Yet, in many cases cocktails of these drugs are necessary for survival.

A third type of diabetes is gestational diabetes. This form of diabetes only occurs with pregnancy, is due to changes in hormone levels, and disappears after giving birth. However, 5 to 10% of these patients eventually develop type 2 diabetes within the next 5-10 years. During pregnancy, gestational diabetes requires treatment to avoid complications in the fetus. Women who are obese or who have a family history in diabetes are more prone to develop this form of diabetes.

The reason why diabetes is a serious and sometimes life threatening disease is that it can eventually lead to the development of several complications including heart disease, stroke, high blood pressure, blindness, kidney disease, nerve damage, amputations, dental disease, pregnancy complications and erectile dysfunction. A typical complication is the diabetic foot which is characterised by a loss of pain perception in the foot (due to nerve damage) resulting in several injuries of the foot without the patient even being aware of it. Typical for the development of complications is dysfunction of the vascular endothelial cells. This will be discussed in the next chapter.
2. Endothelial dysfunction and vascular complications

Endothelial cells delineate the blood vessel and form a dynamic and heterogeneous organ with diverse functions. An adult human body has approximately $1 \times 10^{13}$ to $6 \times 10^{13}$ endothelial cells that weigh about 1 kg and cover 7 m$^2$. Endothelial cells are connected to an extracellular matrix (ECM) through i.e. integrins and to each other by PECAM-1, VE-cadherin, tight junctions and gap junctions. These latter prevent the vessels from leaking and help to maintain i.e. the blood-brain barrier. In other organs like the liver, spleen and bone marrow sinusoids, blood vessels are lined by discontinuous endothelial cells that allow trafficking through the gaps. Villi, endocrine glands and kidneys are lined by fenestrated endothelial cells, facilitating selective permeability.

Endothelial cells not only have a barrier function but also influence vascular haemodynamics by secreting vasodilators like nitric oxide (NO) and vasoconstrictors like endothelin (ET). The production of these two molecules is regulated by insulin. Binding of insulin to its receptor activates two pathways: the MAPK pathway leading to the production of ET and the PI3K pathway leading to the production of NO. Clearly these two pathways need to be tightly regulated to guarantee normal blood flow. However in diabetic patients, insulin signalling is abolished (due to lack of insulin production in type 1 diabetics) or disturbed by free fatty acids (FFA) and cytokines like TNF. FFA and TNF are known to selectively inhibit the PI3K pathway, resulting in decreased production of the vasodilator NO and thus vascular occlusion. This has implications for fat and muscle cells since vascular occlusion also means that less glucose is transported to these cells which may amplify hyperglycaemia (reviewed in).

In addition to vasodilation and vasoconstriction, endothelial cells also facilitate blood flow by providing an antithrombotic and fibrinolytic surface.

Endothelial cells also play an important role in inflammation. Most leukocytes constitutively express L-selectin which allows leukocytes to bind to the endothelium and to other leukocytes (Figure 2). Additionally, endothelial cells express P-selectin and upon stimulation with inflammatory mediators like TNF, also E-selectin. Both these selectins are involved in binding leukocytes. Because these selectin mediated interactions are rather weak, leukocytes do not really bind to the endothelium, but rather tether to and roll on the endothelial surface. However this brings leukocytes into close proximity to the endothelium, increasing the odds that they encounter leukocyte activating chemokines like IL8 and MCP. Once activated, integrins on leukocytes can firmly bind to cellular adhesion molecules like ICAM and VCAM on the endothelial surface. Expression of these cellular adhesion molecules is also mediated by inflammatory cytokines like TNF. Subsequently, leukocytes migrate between endothelial cells causing a local inflammation. Such transmigration requires the action of endothelial PECAM-1. In most cases, after tissue repair, the inflammatory reaction is terminated. However in some cases of massive inflammation, leukocytes produce large amounts of inflammatory cytokines, which can have detrimental consequences in distant tissues.
Endothelial dysfunction is one of the hallmarks of the development of vascular complications in diabetes. Dysfunction is considered as a state in which the properties of endothelial cells are altered in such a way that it may be damaging for tissues and organs. Several risk factors like hypertension, obesity and smoking may underlie endothelial cell dysfunction in diabetes. Also, diabetes is associated with a low grade inflammation which may also induce endothelial dysfunction. The group of Michael Brownlee however pinpoints to a role of hyperglycaemia itself and oxidative stress. Hyperglycaemia increases glycolytic flux, resulting in the formation of reactive oxygen intermediates in the mitochondria that subsequently inhibit glyceraldehyde phosphate dehydrogenase (GAPDH), a glycolytic enzyme. This results in an accumulation of all upstream glycolytic intermediates activating four pathways underlying endothelial cell dysfunction:

- Activation of the polyol pathway: In the first step, glucose is converted into sorbitol by aldose reductase. This step consumes NADPH which is needed for regenerating reduced glutathione (GSH). Reduced GSH levels may lead to increased oxidative stress in endothelial cells resulting in endothelial dysfunction.
- Activation of the hexosamine pathway: In this pathway, fructose-6-phosphate is shunted from the glycolysis yielding glucosamine-6-phosphate and UDP-N-Acetylgulosamine (GlcNac). Modification of transcription factor SP1 by GlcNac increases activation of SP1 resulting i.e. in increased transcription of PAI-I which is prothrombotic. Also glucosaminylation can inhibit eNOS (endothelial NO synthase), resulting in decreased NO production and vascular occlusion.
General Introduction

- Activation of PKC: The PKC family is activated by the secondary messenger diacylglycerol (DAG). DAG can be de novo synthesised from dihydroxyacetone phosphate (DHAP), a glycolytic intermediate. Activation of PKC has been shown to mediate blood flow abnormalities by decreasing NO production and increasing ET1. Also PKC can activate NF-κB resulting in increased expression of cellular adhesion molecules and subsequent endothelial dysfunction.

- Increased formation of AGEs: In this pathway, a side product of the glycolysis, methylglyoxal, binds and modifies several proteins, leading to i.e. altered function of the protein or crosslinking of proteins. This pathway is discussed in detail in another chapter (see page 23).

Figure 3: Potential mechanism by which hyperglycaemia induced mitochondrial superoxide activates four pathways underlying endothelial dysfunction. Overproduction of superoxide in the mitochondria inhibits GAPDH resulting in accumulation of all upstream intermediates. Glucose is shunted into the polyol pathway, consuming NADPH and depleting GSH. Excess fructose-6-P enters the hexosamine pathway resulting in GlcNac protein modifications. DHAP can be converted into DAG, an activator of PKC. Finally, from GAP, methylglyoxal is produced, the main AGE precursor. Adapted from 11.
3. Tumour Necrosis Factor

More than a century ago, P. Bruns reported the regression of tumours after bacterial infection. Later, in 1944, this phenomenon was ascribed to LPS present in bacterial extracts. Yet, O’Malley showed that tumour regression was not a direct effect of LPS but was mediated through the induction of a factor in the serum. This factor was later named tumour necrosis factor or TNF(-α)\(^1\). In 1984, the group of Aggarwal was the first to purify and sequence the protein and to clone the complementary DNA\(^13,14\). At that time, it also became clear that TNF-α was related to lymphotoxin (LT, TNF-β or LTA3), another cytotoxic cytokine that also kills tumour cells.

3.1. The TNF ligand family

TNF is expressed in a wide variety of cells (like macrophages, B cells, T cells, NK cells, monocytes and dendritic cells, lymphoid cells, mast cells, fibroblasts, neuronal tissue and endothelial cells) and is considered to be a major pro-inflammatory cytokine which has different activities on different cells\(^15\). In many auto-immune diseases, TNF plays an important role. For instance in rheumatoid arthritis and Crohn’s disease, TNF neutralising agents have been proven beneficial\(^16,17\). Also, TNF knockout mice, or mice administered with a soluble TNF receptor IgG, suffer less from obesity induced insulin resistance\(^18\).

TNF is produced as a trimeric transmembrane protein\(^19,20\) from which soluble TNF (sTNF, hereafter referred to as TNF) is produced through cleavage by the metalloprotease TACE (TNF-α converting enzyme)\(^21\). TNF belongs to the TNF superfamily, composed of 19 members (Figure 4). All of these ligands, except for LT and VEGI (vascular endothelial cell-growth inhibitor), are type II transmembrane proteins, with a C-terminal extracellular domain, an amino terminal intracellular domain and a single transmembrane domain. The C-terminal extracellular domain (also called TNF homology domain) has 20-30% amino acid identity with the superfamily members and is also responsible for receptor binding. Other members belonging to this family are: TRAIL (or APO2L), receptor activator of nuclear factor-κB ligand (RANKL, also known as TRANCE or OPGL), VEGI, B-cell activating factor (BAFF) and TNF-like weak inducer of apoptosis (TWEAK)\(^12\).

3.2. The TNF receptor family

The 19 currently known ligands bind to 29 receptors (Figure 4) also belonging to a larger family (TNFR family)\(^12,22\). Some ligands are able to bind several receptors, and vice versa\(^12\). All these receptors have one to six cysteine-rich domains (CRD) in their extracellular region with each three disulfide bridges. TNFR1 (p55, CD120a) and TNFR2 (p75, CD120b) have 3 and 4 CRDs respectively\(^12,23\). The receptors are roughly divided into two groups: the ones containing a death domain (DD, of approximately 80 AA) like TNFR1, and those containing a docking site for TRAF proteins, like TNFR2. This DD is important for adaptor protein recruitment and signalling to cell death (cfr. infra)\(^24,25\). Neither TNFR1 nor TNFR2 possesses any enzymatic activity in their cytosolic region.
Virtually every cell in the human body expresses TNFR1 (55kDa), whereas TNFR2 (75 kDa) is mainly expressed in immune cells and endothelial cells. The predominant TNF induced pathway in endothelial cells however, is the one signalling via TNFR1. Interestingly, in most cells, including endothelial cells, TNFR1 is mainly expressed in the Golgi apparatus and has little surface expression, as opposed to TNFR2 which is mainly expressed on the plasma membrane. The role of this Golgi pool remains largely unexplored.

Figure 4: Overview of the TNF ligand family (right hand side) and the TNF receptor family (left hand side). The arrows indicate which ligand can bind to which receptor. The number of cysteine rich domains and the presence of a death domain and/or binding sites for TRAF proteins in the TNF receptors is indicated. The cleavage site where the TNF ligands are cleaved yielding soluble TNF ligands is also indicated. Adopted from 12.
unknown, but it could function as a pool from which TNFR1 is delivered when needed, or alternatively, TNFR1 in the Golgi may be responsive to endogenous produced TNF.

3.3. TNF binding

Originally, it was thought that binding of TNF to one of its receptors leads to trimerisation of the TNFR, which then signals to various pathways. However, this view has been challenged by the discovery of a pre-ligand-binding assembly domain (PLAD) in the TNFRs, which mediates assembly of both TNFR1 and TNFR2 even in the absence of a ligand. This domain brings TNFR1 or TNFR2 chains in close proximity and it is speculated that ligand binding can strengthen this interaction.

There is also evidence for interplay between the two TNF receptors. For instance, Tartaglia and colleagues proposed a mechanism of ligand passing. In this model TNF initially binds to TNFR2 (which binds TNF 20 times as fast as TNFR1) after which TNF is rapidly released again resulting in increased concentrations of TNF at the plasma membrane. This way TNF can easily bind to TNFR1. Another example of a co-operative effect between TNFR1 and TNFR2 is that TNFR2 can increase intracellular levels of TNF, which then could bind to TNFR1. And a final example is the formation of heteroduplexes between the two receptors.

3.4. The signalling complex

In unstimulated cells, signalling from the pre-assembled TNFR1 is hindered by the 60 kDa death domain binding protein SODD (silencer of death domains). Upon binding of TNF to TNFR1, SODD is rapidly released from the TNFR1 receptor, unmasking TNFR1’s DD and admitting binding of TRADD (TNFR1 associated death domain) to the receptor. Like TNFR1, TRADD also contains a DD at its C-terminus, responsible for interaction with the DD of TNFR1. TRADD then serves as a docking site for several other proteins like FADD (fas associated death domain), TRAF2 (TNFR associated factor) and RIP (receptor-interacting kinase) (Figure 5). This complex signals to various pathways including the NF-κB, JNK, ERK, p38 and apoptosis pathway. These pathways are discussed below with emphasis on the NF-κB pathway.

3.5. Signalling to NF-κB

3.5.1. Classical and alternative pathway

NF-κB is a heterogeneous collection of transcription factor dimers of the NF-κB/Rel family. To date, five mammalian Rel proteins were identified: RelA (p65), RelB, c-Rel, NFκB1 and NFκB2. NFκB1 (p105) and NFκB2 (p100) can be processed into p50 and p52 respectively. Rel proteins form dimers, and the most abundant one is the p65/p50 dimer. This is also the most studied dimer and is therefore often simply referred to as NF-κB.

In the ‘classical’ (sometimes referred to as ‘canonical’ or ‘consensus’) pathway, which is induced by TNF, NF-κB mainly resides in the cytoplasm bound to IκBa (Figure
5). This IkB protein masks the nuclear localisation signal (NLS) of NF-κB keeping NF-κB in the cytosol. Upon stimulation, IkBa is phosphorylated at serines 32 and 36 by IKKβ and subsequently ubiquitinated, marking IkBa for proteasomal degradation by the 26S proteasome. Once IkBa is degraded, the NLS of NF-κB is exposed which allows NF-κB to enter the nucleus. NF-κB then binds to a specific DNA consensus site which allows transcription of several anti-apoptotic genes and genes like IL1, ICAM, E-selectin... NF-κB also induces the transcription of its own inhibitor IkBa, resulting in the termination of the signal (reviewed in 38).

There are three other pathways known to activate NF-κB (reviewed in 39). The first is the alternative pathway, triggered by CD40 ligand, LTAβ and BAFF. In this pathway, p100 is phosphorylated at two specific serines by IKKa, leading to poly-ubiquitination...
and degradation of the C-terminal part of p100, resulting in the formation of p52. P52 mainly forms dimers with RelB. The second pathway is triggered by UV light. This pathway resembles the classical pathway in this way that IκBα phosphorylation, ubiquitination and breakdown is also essential, but as opposed to the classical pathway, phosphorylation doesn’t take place on serines 32 and 36 and the kinase involved is not IKK, but probably casein kinase II. In a third pathway, which is activated by double stranded DNA breaks, NEMO (part of the IKK complex; see further) is first sumoylated, subsequently phosphorylated and ubiquitinated. This leads then to activation of the IKK complex and NF-κB. In what follows, I will focus on the ‘classical’ pathway, since this pathway is triggered by TNF.

3.5.2. IKKs: link between TNFR and NF-κB

The most important IκB kinases are IKKα and IKKβ, both part of a larger complex which also comprises IKKγ (alternative names: NEMO, IKKAP1 or FIP-3) and probably also ELKS. Additionally, it has been shown that Cdc37 and Hsp90 are also required for IKK recruitment and activation.

IKKα and IKKβ both contain N-terminal protein kinase domains and C-terminally, a leucine zipper (LZ) and a helix-loop-helix domain. Despite the fact that both of these kinases have a high degree of similarity (50% in sequence, 70% in protein similarity), they signal in different pathways. IKKα mainly signals to NF-κB activation in response to TNF family members like BAFF, CD40 ligand and LTαβ. IKKβ is on the other hand involved in NF-κB activation by TNFα, IL-1 and Toll-like receptor agonists like LPS. Also, IKKα is mainly involved in the alternative pathway leading to NF-κB activation while IKKβ is involved in the classical pathway.

In addition to IKKα and IKKβ, two IKK related proteins have been identified: IKKε (or IKK-i) and TBK1 (also called NAK or T2K). They probably do not play a role in IκBα breakdown, because, as opposed to IKKβ, IKKε and TBK1 phosphorylate (preferentially) only one of the two IκBα phospho-acceptor sites which is not enough to induce proteolytic breakdown. Also, the role of IKKγ (NEMO) is still not clear. However IKKβ and also IKKα, have a NEMO binding domain (NBD) which is essential for IKK assembly. A plausible role for IKKγ could be that it brings IKKα and IKKβ into close proximity, such that these two can activate each other by trans-autophosphorylation.

There is considerable evidence that IKKs need to be phosphorylated to become active. IKKβ is phosphorylated at two specific serines on a so called activation loop which is essential for IKKβ activation (Figure 6). IKKα also has an activation loop, and phosphorylation of IKKα is essential for the activation of the alternative pathway and for NF-κB activation in mammary epithelial cells upon RANK stimulation. While phosphorylation at the activation loop promotes IKK activity, phosphorylation at the helix-loop-helix domain instead has a negative regulatory function and may thus serve as a negative feedback mechanism.

There is still some doubt on how IKKs are phosphorylated. As mentioned above, there is the possibility of simple autophosphorylation. Yet, several other mechanisms are
possible, and they are probably not mutual exclusive. TRAF2 is shown to be recruited to the TNF signalling complex by TRADD and both TRAF2 and TRADD can recruit RIP1 (Figure 5) 36,37. RIP1 has, besides a death domain and a RIP homotypic interaction motif, also a kinase domain. However, the kinase activity of RIP1 is dispensable for NF-κB activation excluding the possibility that RIP1 directly phosphorylates the IKK complex 56. MEKK3 is shown to interact with RIP1, and the catalytic activity of MEKK3 is shown to be essential for NF-κB activation 57, suggesting that MEKK3 is responsible for IKK phosphorylation and activation, and that RIP1 might serve as an adapter protein. Phosphorylation by MEKK3 is probably facilitated by the fact that TRAF2 is able to recruit the IKK complex through interaction with the leucine zipper motifs of IKKa or IKKβ 58.

Additionally, TAK1 also seems involved in activating NF-κB 59,60 but its activity depends on expression of TAB2 and TAB3 which contain a zinc finger motif, typical for ubiquitin binding proteins 61. It has been shown that TNF induces poly-ubiquitination on RIP1 and TNFR1 62. Since TRAF6 and maybe also TRAF2 and TRAF5 may co-operate with Ubc13/ Uev1A forming ubiquitin chains on RIP1, NEMO and possibly also on themselves 63-65, a
model is proposed in which TRAF2 and/or TRAF5 mediate ubiquitination of RIP1 and themselves, which then serve as docking sites for TAB2 and TAB3. TAB2 and TAB3 in turn recruit TAK1 to the signalling complex which subsequently activates the IKK complex \(^{41,65}\). In accordance with this model, Li et al demonstrated that ubiquitination of RIP1 is necessary in TNF induced NF-κB activation \(^{66}\).

### 3.5.3. IκB proteins

The IκB family includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and NF-κB precursors p100 and p105. IκBγ corresponds in fact to the C-terminal part of p105 and it was predicted that similarly, there must also exist an IκBδ which corresponds to the C-terminus of p100. All IκBs contain six or seven ankyrin repeats, needed for interaction with NF-κB and masking its NLS. IκBα, IκBβ and IκBε also contain an N-terminal regulatory domain. For IκBα it has been shown that IKK phosphorylates this protein at serines 32 and 36 which targets the protein for poly-ubiquitination and subsequent degradation by the 26S proteasome, liberating the NLS of NF-κB \(^{38,67}\).

Once in the nucleus, NF-κB initiates transcription of several genes, one of which is IκBα itself. IκBα also possesses a nuclear export signal (NES) and is thus able to export newly bound NF-κB out of the nucleus into the cytoplasm, terminating the NF-κB response. IκBβ and IκBε are degraded more slowly and are probably involved in a persistent activation of NF-κB \(^{67}\).

### 3.5.4. Rel proteins

To date, five mammalian Rel proteins have been identified: RelA/p65, c-Rel, RelB, NF-κB1/p50 and NF-κB2/p52 and they form dimers in nearly any combination. As mentioned above, p50 and p52 are synthesised from precursor molecules p105 and p100 respectively \(^{38,39}\). Similar to IκBs, p100 is inducibly phosphorylated by IKKa and subsequently ubiquitinated \(^{68}\). However, unlike IκBs, proteolytic breakdown is limited to the C-terminal IκB like region yielding mature p52. P50 is also generated by limited proteolysis of the p105 precursor. It was demonstrated that proteolysis of p105 is ubiquitin dependent \(^{69}\), but a recent paper argued that there may be also an ubiquitin independent mechanism involved \(^{70}\).

Rel proteins are characterised by the presence of a Rel Homology Domain (RHD), which mediates dimerisation, DNA binding and interaction with IκB proteins. The RHD also contains a NLS sequence which is masked when IκB is bound to the RHD. Once IκB is degraded, the NLS of NF-κB is exposed allowing translocation to the nucleus. Different NF-κB dimers all have affinity for a conserved sequence, the κB motif \(\text{[GGGRNNYYCC]}\) \(^{38}\).

Activation of NF-κB is not only regulated by upstream kinases or ligand binding, but also at the level of NF-κB itself. For this purpose NF-κB proteins RelA/p65, c-Rel and RelB, but not p50 and p52 have a transactivation domain. Additional regulation of NF-κB activity is mediated by phosphorylation, acetylation and ubiquitination at the level of NF-κB itself. The precise effect of ubiquitination on NF-κB is not clear, as opposed to the
well studied acetylation and phosphorylation. Most of the knowledge comes again from RelA/p65, which will be discussed here (Table 1).

The conserved Ser536 residue can be phosphorylated by five known kinases, one of which is IKKa/β. Ser536 Phosphorylation of p65 is believed to weaken, or even inhibit interaction with nuclear IκBα, resulting in a drastic decrease of nuclear export of NF-κB and thus enhanced transcription.

Ser535 is targeted by calmodulin dependent kinase IV (CaMKIV). This modification is thought to be involved in increasing transcription, especially of anti-apoptotic genes.

Ser468 phosphorylation appears to have negative effects. Ser468 phosphorylation by GSK3β inhibits basal activity of p65. However, another group showed that GSK3β deficient embryos cannot activate NF-κB even if IκBα breakdown is unaltered suggesting a positive role for GSK3β.

Ser311 is phosphorylated by PKCζ upon TNF stimulation resulting in increased interaction with cAMP response element binding (CREB) binding protein (CBP) and
recruitment to the IL6-promotor together with RNA polymerase II. CBP is, like p300, a histone acetyltransferase (HAT) which facilitates transcription. Ser311 phosphorylation thus increases transcriptional activity.

Ser276 is phosphorylated by PKAc and MSK1 and results in enhanced recruitment of histone acetyltransferases such as CBP and p300, and removal of inhibitory deacetylase-1 (HDAC1) complexes from the DNA.

Besides phosphorylation on serine residues, NF-κB is also phosphorylated at three threonine residues. Phosphorylation on both Thr435 and Thr505 has a suppressive effect on RelA activity. Thr254 phosphorylation results in a conformational change of p65, decreasing its affinity for IκBα.

Upon TNF stimulation RelA is also acetylated at five lysine residues: Lys122, -123, -218, -221 and -310. Acetylation at Lys218 and Lys221 decreases interaction with IκBα and acetylation at Lys310 enhances transactivation of p65. Acetylation at Lys122 and Lys123 decreases RelA DNA binding capacity.

3.6. **MAP-kinase pathway**

TNF is also able to induce activation of extracellular receptor-activated kinase 1 and 2 (ERK1/2), but to observe the effects, endothelial cells must be made quiescent by withdrawal of serum and growth factors. The MAP kinase pathway normally starts with the recruitment of Grb-2 to the receptor (either directly or by a Shc-adaptor protein), whereafter Grb-2 recruits the nucleotide exchange factor Sos (Figure 5). Sos activates Ras by facilitating GDP/GTP exchange, and GTP•Ras then activates serine-threonine kinase Raf. Activated Raf in turn phosphorylates MEK-1 which activates Erk1 and Erk2. It has been shown that Sos is part of the TNF complex and that Grb-2 associates with TNFR1 via an adapter protein.

3.7. **SAPK p38 and JNK**

TNF also induces activation of kinases of the stress activated protein kinases (SAPK)/c-Jun N-terminal kinase (JNK) group (Figure 5). Upon activation, JNK translocates to the nucleus and enhances transcriptional activity of transcription factors c-Jun and ATF2. Several pathways have been suggested for activation of JNK. In one pathway, TRAF2 recruits MEKK1 which activates MKK7. JNK is in turn activated by MKK7. Additionally MEKK1 can also be activated by members of the germinal centre kinase (GSK) family. In another pathway, TNFR1 stimulation can result in TRAF2 dependent increase in ROS production and recruitment of Ask1. Ask1 is bound to thioredoxin which inactivates Ask1. However, upon ROS production, thioredoxin is released from Ask1 which is then able to activate MKK7 and subsequently JNK.

Activation of p38 is not completely understood, but there is evidence for interplay between p38 and the JNK pathway. For instance TRAF2, ASK1 and MEKK1 are also strong inducers of p38 activation. However it is clear that JNK and p38 are also activated by distinct kinases as activation of p38 is believed to involve RIP and MKK3. Dependent
on the cell type, p38 can be pro- or anti-apoptotic. However in endothelial cells, p38 is most likely involved in an anti-apoptotic pathway.

3.8. Induction of apoptosis

Induction of apoptosis starts with the recruitment of FADD to TRADD after which caspase-8 is also recruited to the receptor complex (Figure 5). Once caspase-8 is autocatalytically processed, activated caspase-8 results in activation and cleavage of a series of other caspases culminating in the activation of effector caspases like caspase-3. Effector caspases are responsible for cleavage of several other proteins, like PARP. Caspase-8 may directly activate caspase-3 or via cleavage of Bid and the release of cytochrome c from the mitochondria. However, in endothelial cells activation of caspase-3 is probably dependent on direct cleavage by caspase-8 and not on cytochrome c release. Normally endothelial cells are fairly resistant to TNF induced apoptosis, but can be sensitised when cultured in the presence of serum.

3.9. TNF and the endothelium

In the late nineties, it has been suggested that low-grade inflammation is involved in the pathogenesis of type 2 diabetes. Several studies supported this notion, and studies like the EPIC-Potsdam report even showed a positive correlation between TNF levels and the incidence of developing type 2 diabetes. Morohoshi and colleagues additionally showed that hyperglycaemia increases TNF secretion in human peripheral monocytes. Another group on the other hand, showed that serum TNF levels are not influenced by the glycaemic state, but are instead increased with body fat accumulation (obesity) which is a risk factor for the development of type 2 diabetes. In either case it is obvious that TNF plays an important role in the development and pathogenesis of diabetes.

TNF has diverse effects on the vasculature. It plays e.g. a role in the misbalance of vasoconstrictive and vasodilatory mediators. TNF stimulates on the one hand the production of vasodilatory substances like nitric oxide and prostaglandins and on the other hand the production of vasoconstrictive mediators like platelet activating factor, endothelin-1 and also prostaglandins. Yet, it is shown that TNF is able to inhibit endothelium dependent relaxation and that it favours contraction of mesangial cells which may pinpoint to a general vasoconstrictive effect of TNF. This view is supported by the fact that TNF induces insulin resistance in endothelial cells resulting in vascular occlusion.

Several papers also point to a role of TNF in endothelial permeability involving cytoskeleton re-arrangements, re-distribution of VE-cadherin, production of NO and oxidative stress. SAPK p38 is shown to be involved in TNF induced permeability and Nwariaku and colleagues additionally showed that ERK is also implicated, but only in lung endothelial cells and not in umbilical vein cells (HUVEC). Endothelial permeability is of course also facilitated by TNF induced apoptosis. Moreover, apoptotic endothelial cells become pro-coagulant and adhesive to leukocytes and platelets.
TNF may also facilitate leukocyte and monocyte extravasation. In unstimulated cells, the interaction between leukocyte and endothelium is normally too weak to tether the leukocytes. However upon TNF stimulation, endothelial cells produce several cellular adhesion molecules (CAM) like E-selectin, ICAM-1 and VCAM-1 to which leukocytes can firmly bind whereafter the leukocyte transmigrates across the endothelium (see page 10). TNF induced CAM expression is mainly mediated by NF-κB, but also by c-Jun. SAPK p38 and ERKs are shown to be required for p65 transactivation and may thus enhance upregulation of leukocyte binding molecules. However, it has also been suggested that in endothelial cells ERK suppresses NF-κB activity by inhibiting IKK phosphorylation.

Many of the effects of TNF are mediated through activation of NF-κB. Besides CAMs, NF-κB also induces the transcription the vasoconstrictor endothelin and even TNF itself. All of these molecules contribute to the development of vascular complications. However, NF-κB also has beneficial effects by initiating the transcription several anti-apoptotic genes like Bcl and A20, protecting the cell from TNF induced cell death.
4. Methylglyoxal and Advanced Glycation End Product Formation (AGE)

AGEs are a type of posttranslational modification in which proteins, lipids or even nucleic acids become non-enzymatically glycated and oxidised after interaction with an aldehyde or ketone group from a sugar like fructose or glucose, resulting for instance in protein-protein crosslinks and formation of brown pigments (e.g. in foods) (reviewed in 110). This process was already studied in the early 1900’s by L.C. Maillard, and is therefore also known as the Maillard reaction. Remarkably, Maillard already speculated that AGE formation could be important in diabetes. It’s only later however, that it became clear that AGEs are major contributors to the development of diabetic complications.

4.1. Chemistry: The Maillard Reaction

Reducing sugars like glucose, fructose, galactose, mannose and ribose are very reactive towards nucleophilic nitrogen bases of amino acids. Although glucose is the most abundant sugar in vivo, it is in fact the least reactive one, which probably explains why it has been selected as the principal free sugar. These sugars react with free amino acid groups such as a free N-terminus or an amino group from lysine or arginine forming a Schiff base (Figure 7). Like sugars, this Schiff base exists in a cyclic form (glycosylamine) or an open-chain state which is thermodynamically disfavoured. The formation of a Schiff base is very fast and highly reversible. In time, the Schiff base will undergo additional molecular re-arrangements resulting in so called Amadori products, believed to occur via an intermediate open-chain enol form. This reaction is slower than Schiff base

Figure 7: Formation of Schiff bases and Amadori products from glucose. These structures also exist in a cyclic form which is more stable than the open chain form which is depicted here. In a first step, glucose or another reducing sugar, binds to a free amino group from a protein forming a Schiff base. Through further re-arrangements this Schiff base is converted into an enaminol intermediate and Amadori product subsequently. The formation of an Amadori product from the enaminol intermediate is faster than the opposite reaction, and as a consequence Amadori products tend to accumulate. Adapted from 110.
formation, but since it is faster than the opposite reaction, Amadori products tend to accumulate. Schiff bases and Amadori products are called ‘early glycation products’. Through further chemical re-arrangements these Amadori products then form ‘late-stage’ or ‘advanced glycation end products’ (AGEs). Despite intensive research, the exact mechanism is not fully understood but it may include dehydration and oxidation (called glycoxidation). These AGEs are a heterogeneous group of fluorescent and non-fluorescent structures that may or may not form crosslinks (reviewed in 110).

The best known Amadori product is HbA1c (glycated haemoglobin). Levels of HbA1c correlate very well with glucose levels in diabetics 111 and HbA1c is therefore used to monitor glucose levels in diabetic patients. This way, HbA1c serves as a marker in the treatment of diabetes.

4.2. Methylglyoxal

Although glucose was thought to be the most important sugar in AGE formation, the work of Shinohara et al showed that it is in fact not glucose that is mainly responsible for AGE formation, but methylglyoxal (MG), a cytotoxic side product from glycolysis (Figure 8) 112. Methylglyoxal (also called 2-oxopropanal or pyruvaldehyde) is a highly reactive dicarbonyl that can react directly with amino acids forming AGEs, omitting the formation of Schiff bases and Amadori products.

4.2.1. Methylglyoxal production from glycolysis

Glycolysis is one of the most important energy sources in the cell. Briefly, glucose is converted through several cytosolic enzymatic reactions into pyruvate (Figure 9), which is transported to the mitochondria. There, pyruvate is further oxidised in the Krebs or tricarboxylic acid (TCA) cycle yielding ATP, NADH and FADH₂. In the mitochondrial respiratory chain, electrons from NADH and FADH₂ are passed on through an electron transport system. Transporters that catalyse these reactions also have the ability of creating a proton gradient across the mitochondrial membrane, which allows the mitochondrion to generate ATP, a universal energy source 113.

Two triose intermediates of glycolysis are of particular interest in regard to methylglyoxal production: DHAP (dihydroxyacetone phosphate) and GAP (glyceraldehyde-3-phosphate). In solution, it has been shown that these two intermediates can produce methylglyoxal through non-enzymatic phosphate elimination of a common 3-phospho-1,2-enediol intermediate 114 (Figure 10). When TPI (triosephosphate isomerase, a glycolytic

\[
\text{H}_3\text{C} \quad \text{CH} \quad \text{O} \\
\text{O} \quad \text{H} 
\]

Figure 8: Chemical structure of methylglyoxal.
Figure 9: Schematic overview of the glycolytic pathway.

Figure 10: Subsequent steps in the formation of methylglyoxal from dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) through phosphate elimination. Adapted from 159.
enzyme that catalyses the conversion of DHAP in GAP and vice versa) is added to the reaction, it raised methylglyoxal formation. It has therefore been suggested that phosphoenol intermediates may ‘leak’ from the active site of the enzyme and thereafter eliminate phosphate resulting in methylglyoxal formation. Another possible explanation is that DHAP is converted to GAP which eliminates phosphate more efficiently. In red blood cells, it has been calculated that 0.089% of trioses are converted into methylglyoxal. This amount may seem rather modest, but considering that glycolysis is a major pathway in the cell, methylglyoxal may accumulate. Additionally, in diabetic settings, glycolytic rate is increased (except in the liver where gluconeogenesis is promoted) producing even more methylglyoxal. It is thus not surprising that Chaplen and co-workers found up to 310 µM methylglyoxal in CHO cells. They also estimated that at this concentration 5 to 10% of the proteins could be modified by methylglyoxal. This urges the need for efficient detoxification mechanisms.

4.2.2. Minor methylglyoxal producing pathways

Methylglyoxal can also be formed enzymatically by a methylglyoxal synthase from DHAP. This enzyme has already been described in E. coli, but to my knowledge, there is only one report describing methylglyoxal synthase in mammalian cells, namely in goat liver cells. So, it remains to be shown that this enzyme is functional in other mammalian cells.

Lipids can also be a source of methylglyoxal (Figure 11). The human body breaks down tri-acylglycerols into glycerol and fatty acids. The glycerol part can be phosphorylated and converted in DHAP, from which methylglyoxal is produced. The fatty acids are converted into acetone by the β-oxidation pathway and subsequent ketogenesis. Acetone is then converted into hydroxyacetone (acetol) by an acetone mono-oxygenase and from this intermediate methylglyoxal is produced via an acetol mono-oxygenase.

Methylglyoxal can also be produced from amino acid metabolism. Amino-acetone is an offshoot of glycine and threonine metabolism and can also be converted into methylglyoxal by an amino-oxidase.

In diabetic settings, up to 33% of all glucose is shunted into the polyol pathway. In this pathway, aldose reductase catalyses the conversion of glucose into sorbitol which is then further metabolised by the sorbitol dehydrogenase into fructose. Phosphorylation then results in fructose-6-phosphate, which enters glycolysis, or in fructose-1-phosphate which can be cleaved into DHAP and glyceraldehyde (GA). The latter can be phosphorylated yielding GAP. As mentioned above, GAP and DHAP are major sources for methylglyoxal production.

Lastly, glycation itself can be a source for methylglyoxal production. The group of Thornalley et al has shown that α-oxo-aldehydes, like methylglyoxal, are formed early in the glycation process from degradation of glucose and Schiff bases.
Methylglyoxal (but also other dicarbonyls) is particularly reactive towards lysine, arginine and cysteine residues. Methylglyoxal could be expected to react with proteins most rapidly at cysteine residues, since it reacts with reduced glutathione to form a hemithioacetal, the substrate for the glyoxalase system (see page 32). However, studies with MG-modified BSA show that arginine is the most important target for methylglyoxal modification. Methylglyoxal modification of proteins can be both reversible and irreversible and several epitopes have been described both in vivo and in vitro (Figure 12): Methylglyoxal reacts with lysine forming MOLD (Methylglyoxal-derived lysine dimer, 1,3di(Nε-lysine)-4-methyl-imidazolium) and CEL (Nε-(1-carboxyethyl)lysine), and with arginine forming MG-H1 (Nδ-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine), argpyrimidine and tetrahydropyrimidine (THP, Nδ-(4-carboxy-4,6dimethyl-5,6dihydroxy,1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine). Finally, the reaction of methylglyoxal with the thiol group of cysteine was thought to be mainly reversible but it has been shown that methylglyoxal can also form an irreversible adduct, CMC...
In addition, it is shown that methylglyoxal can inhibit cysteine proteases by modifying the cysteine residue, possibly resulting in a decreased clearing and accumulation of modified proteins. The most important biomarkers for diabetes are hydro-imidazolones which can modify up to 3% of total arginine and up to 7% of total proteins in extracts of rat brain. In accordance, Ahmed and co-workers showed that 90% of all epitopes formed on HSA are of the hydro-imidazolone type. Besides MG-H1 there are other structurally related hydro-imidazolones but one of them is later identified as being identical to argpyrimidine.

The Amadori re-arrangement of a lysine-glucose Schiff base is thought to be facilitated if there is a histidine side chain or another lysine present. Yet, for methylglyoxal modification there is no consensus site known. However, a study with HSA showed that methylglyoxal can modify specific residues since it preferentially modifies Arg410 of HSA. This can have very damaging consequences, since arginines are commonly found in ligand and substrate recognition sites in receptors and active enzymes. It’s also worthwhile mentioning that it is predicted that about 3 to 13% of all proteins have at least 1 methylglyoxal modified epitope.

Of most proteins the turnover is high enough to avoid non-enzymatic browning. Yet, some proteins like collagen and lens crystalline are extremely long-lived and are therefore major sites for glycation. Since scientists became aware that methylglyoxal is an important glycating agent, more and more MG modified proteins are described,
although the number of studies is still rather limited. In the following a few examples are given:

(i) YAP1 in yeast is a functional analogue of transcription factor AP1 and transcribes several genes involved in oxidative stress defences. YAP1 has a NES sequence in its C-terminal domain and Crm1 (exportin) binds to YAP1 exporting YAP1 from the nucleus into the cytoplasm under non-stress conditions. Upon stress, critical cysteine residues in the C-terminus of YAP1 become modified, inhibiting binding of Crm1, resulting in a translocation of YAP1 to the nucleus. Maeta and colleagues showed that when methylglyoxal modifies any of the three cysteine residues in the C-terminus, YAP1 is constitutively translocated to the nucleus, activating transcription of several anti-oxidative genes. They speculated that this is due to inhibited binding of Crm1. As expected, since this involves methylglyoxal modification of cysteine residues, the modification was reversible. Additionally, they also showed that methylglyoxal dependent YAP1 activation occurs in yeast under physiological circumstances. To my knowledge, this is the first report on the formation of a stable, yet reversible, methylglyoxal modification on a cysteine residue, in vivo.

(ii) Schalkwijk et al. have shown that in endothelial cells, Hsp27 is a major argpyrimidine modified protein. Hsp27 is found throughout the entire body and plays a role in apoptosis, actin polymerisation, damaged protein repair or destruction and it also inhibits caspase-3 activity, thus promoting cell recovery. Because methylglyoxal modification of Hsp27 enhances its chaperone function, it has been speculated that methylglyoxal could protect cells against hyperglycaemia-induced damage in diabetes. This is one of the few reports in which methylglyoxal has a positive effect.

(iii) Enhanced chaperone function upon methylglyoxal modification is also seen with α-crystallin. In contrast, glycation inhibits its chaperone function but addition of methylglyoxal during or before glycation not only prevents loss of chaperone function but actually enhanced chaperone function.

(iv) Again in Yeast, Gomes and colleagues showed that enolase2 is a primary target for methylglyoxal modification. Additionally they identified two other glycolytic enzymes: aldolase and phosphoglycerate mutase. None of these modifications had any effect on the glycolytic flux. Three heat shock proteins were also identified as being methylglyoxal modified: Hsp71/72 and Hsp26.

4.4. Pathological effects of AGEs in diabetes

Vascular complications are very common in diabetic patients and are the leading cause of the development of several pathologies like blindness, stroke, limb amputation, kidney failure, nerve failure... AGEs are thought to be important mediators of vascular
complications and this view is supported by the following observations:
- Concentration of AGE residues is higher in sites of complications development
- Experimental exposure to AGEs induces diabetes-like vascular disease
- Studies of AGE-modified proteins in vascular cells implicate cellular dysfunction
- Inhibitors of AGE formation also inhibit vascular complications

AGEs can exert their effect directly by modifying proteins or through an AGE receptor. These two mechanisms are discussed below.

4.4.1. Interaction with AGE receptors

Most of the effects of AGEs are mediated through binding to AGE receptors including AGE-R1 (OST-48), AGE-R2 (80K-H), AGE-R3 (Galectin3) and RAGE. The first three are poorly characterised AGE binding receptors. Yet, there is growing evidence for a role of these receptors in diabetic complications. Galectin3 for example could have a protective role in nephropathy. The most important receptor for AGEs is however RAGE. RAGE signalling is believed to occur via activation of ERK1/2, p38 and JNK. In addition, RAGE may induce formation of reactive oxygen species. All of these pathways can result in activation of NF-κB. In addition, Bierhaus and co-workers showed that in diabetes, RAGE activation can result into sustained NF-κB activation. They found that de novo synthesis of p65 results in a large pool of p65 and that there is probably not enough IκBα to compensate. Since NF-κB can induce the transcription of RAGE, this results in the upregulation of the receptor and amplification of the sustained NF-κB activation. As mentioned in the previous chapter, NF-κB induces the expression of several proteins, involved in endothelial dysfunction including the pro-inflammatory cytokine TNF, the vasoconstrictor endothelin and the leukocyte binding proteins ICAM-1 and VCAM-1. This way, AGEs may contribute to vascular complications indirectly by binding to the RAGE receptor and activating NF-κB.

4.4.2. Direct effects

Several studies have indicated that methylglyoxal induces oxidative stress by modifying enzymes that are important in regulating or inducing oxidative stress. For example Cu,Zn-superoxide dismutase and glutathione peroxidase, two anti-oxidant enzymes, are shown to be modified by methylglyoxal resulting in a decreased enzymatic activity. It its worthwhile mentioning that in addition to the direct inhibition of anti-oxidant enzymes, hyperglycaemia and AGE formation itself are also known inducers of oxidative stress. Lastly, methylglyoxal also induces oxidative stress indirectly by modifying and inhibiting proteins of the mitochondrial respiratory chain.

AGEs induce the expression of several extracellular matrix proteins, like type IV collagen and laminin. These proteins have a slow turnover and are easily glycated. Crosslinking of these AGEs in the extracellular matrix leads to a decreased matrix breakdown and arterial stiffness.

Also, it is reported that AGEs are able to block the activity of NO. Normally, nitric oxide activates smooth muscle cell guanylate cyclase leading to muscle relaxation.
and vasodilation. Blocking the activity of NO may thus disturb vasodilation, leading to increased blood pressure and eventually endothelial dysfunction. Several mechanisms by which AGEs block or reduce NO have been proposed. A first mechanism suggests that AGEs reduce the half-life of endothelial NO synthase (eNOS) through mRNA degradation. Another mechanism proposes that binding of AGEs to AGE receptors causes a decreased phosphorylation of eNOS, resulting in a decreased activity of the enzyme. Lastly, AGEs may also simply quench NO.
5. Detoxifying pathways: Focus on the glyoxalase system

Due to the cytotoxic activity of methylglyoxal, Nature has developed several detoxifying pathways. At least four enzymes are known to detoxify methylglyoxal: aldose reductase, betaine aldehyde dehydrogenase, 2-oxoaldehyde dehydrogenase and the ubiquitous and highly efficient glyoxalase system. This chapter will mainly focus on this last system, since the glyoxalase pathway is the major detoxifying pathway.

5.1. The glyoxalase system

The glyoxalase system (reviewed in \[159\]) catalyses the conversion of methylglyoxal into D-Lactate. The system consist of only two enzymes, glyoxalase1 (lactoylglutathione lyase, E.C.4.4.1.5.) and glyoxalase2 (hydroxyacylgluthathione hydrolase, E.C.3.1.2.6.) and a catalytic amount of reduced glutathione \[160\]. In a first step, reduced glutathione binds non-enzymatically via its thiol group to the aldehyde group of methylglyoxal forming a hemithioacetal \[159\] (Figure 13). The latter is then enzymatically converted into S-D-Lactoylgluthathione by the action of glyoxalase1 (GLO1). This reaction is almost irreversible, since the reverse reaction rate is only 0.5% of the forward reaction \[161\]. Glyoxalase2 (GLO2) then converts this intermediate into D-lactate, with regeneration of glutathione \[159\].

Although methylglyoxal is the main substrate of the glyoxalase system, glyoxalase1 also shows substrate specificity for hydroxypyruvaldehyde, hydroxypyruvaldehyde phosphate, 4,5-doxovalerate, glyoxal, phenylglyoxal and other alkyl- and aryl-glyoxals \[159,162\].

![Figure 13: The glyoxalase system. Methylglyoxal first binds with glutathione, yielding a hemithioacetal. Glyoxalase1 converts this hemithioacetal into S-D-lactoylgluthathione, the substrate for glyoxalase2. Glyoxalase2 then produces D-Lactate.](image)

5.1.1. Glyoxalase1

The metalloprotein glyoxalase1 consists of two domains (residues 31-104 and 124-183) which are linked by 20 residues and preceded by a long N-terminal arm. The first domain consists of a ‘βαββαβ’ conformation and the second domain of a ‘βαβββα’ structure (Figure 14). The high structure similarity between domain1 and 2 indicates that the glyoxalase1 gene has arisen by gene duplication \[163\].

Glyoxalase1 is mostly active as a dimer of 43 kDA. In this dimer, the first strand of the first domain of one monomer contacts the first strand of the second domain of the second monomer. This sheet then combines with an extra helix of domain 1 and the C-terminus...
of domain 2 to form a barrel structure with the active site at the dimer interface. It is also speculated that the two monomers can exchange equivalent domains in a process called ‘3D domain swapping’ \(^{163}\).

As opposed to humans, plants and bacteria where glyoxalase functions as a dimer, the active enzyme is functional as a monomer in yeast. Yeast glyoxalase1 consists of two similar segments, each resembling a subunit of dimeric glyoxalase1, indicating that yeast glyoxalase1 probably folds into two covalently linked subunits, mimicking dimeric glyoxalase1. This also indicates that in yeast, the glyoxalase1 gene has probably undergone a second gene duplication \(^{164}\).

The shape of the active site can vary from species to species, but there is nearly always a Zn\(^{2+}\) ion present. Studies have shown that this Zn\(^{2+}\) ion can be replaced by either Fe\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\) or Mn\(^{2+}\) \(^{165}\). Monomeric yeast glyoxalase1 has two active sites and the metal ions bound in these sites are probably one Fe\(^{2+}\) and one Zn\(^{2+}\), although the Zn\(^{2+}\) ion can be replaced by Mn\(^{2+}\) \(^{166}\).

5.1.2. Glyoxalase2

Glyoxalase2 is an 18-30 kDA monomeric enzyme, found in a wide variety of species including mammals, birds, reptiles, plants and yeast \(^{167}\). It is a member of the zinc metallohydrolase family and has a broad substrate specificity for glutathione thiol esters \(^{168}\). Remarkably, glyoxalase2 is inhibited by hemithioacetal, the substrate of glyoxalase1. However, in vivo, MG levels are probably too low to induce inhibition \(^{167}\).

Several isoforms of glyoxalase2 have been described which all differ in molecular weight, iso-electric point and cellular distribution \(^{169,170}\). Glyoxalase2 also exists in a cytosolic and several mitochondrial forms \(^{170}\). While in yeast and higher plants, the mitochondrial form is encoded by different genes \(^{171,172}\), in mammals only one gene encodes both isoforms by making use of alternative start codons \(^{168}\). Proteins transcribed from the first start codon include a mitochondrial targeting sequence, while proteins
transcribed from a downstream start codon, lack this sequence and remain cytosolic. Up to now, there is no evidence why the mitochondrial form is produced, especially since glyoxalase 1 is not found in mitochondria.

5.1.3 Biological relevance of the system

Although discovered in 1913, the exact function of the glyoxalase system is still largely unknown. Nobel prize winner Szent Györgyi was one of the first to describe a role for the glyoxalase system. And although his theory has still influence on scientific thinking, it suffers from several uncertainties. Nowadays it is widely accepted that the main function of the system is probably the detoxification of α-oxo-aldehydes like methylglyoxal. Since the latter is of particular interest in diabetes, it is not surprising that the glyoxalase system gained a lot of attention from researchers in the field of diabetes. Yet, the glyoxalase system also seems important in other diseases.

Both glyoxalase 1 and glyoxalase 2 are significantly elevated in symptomatic IDDM. It remains however unclear whether elevated levels of glyoxalase 1 and glyoxalase 2 are the cause or consequence of diabetes. It therefore remains doubtful whether glyoxalase 1 is prognostic for the development of diabetes especially since increased expression is only significant when patients start to develop complications.

Although the glyoxalase system is normally very effective in detoxifying methylglyoxal, Staniszewska et al. reported that in mouse lenses, hyperglycaemia induced upregulation of glyoxalase 1 is not sufficient to normalise levels of methylglyoxal. They speculated that the rate of methylglyoxal production exceeded the metabolism by the glyoxalase system or alternatively that glyoxalase 1 is compartmentalised so that it does not come in contact with all methylglyoxal. The same group however also showed that glyoxalase 1 is essential for human retinal pericytes survival under hyperglycaemic conditions. Accordingly, Shinohara and co-workers showed that in a bovine endothelial cell-line, overexpression of glyoxalase 1 completely prevented hyperglycaemia induced AGE formation also indicating a protective role for glyoxalase 1 under hyperglycaemic conditions.

Our group has previously described a novel function for the glyoxalase system in a necrotic cell death model, namely in the formation of specific AGEs. In a fibrosarcoma cell-line, we showed that TNF phosphorylates glyoxalase 1 and that phosphorylated glyoxalase 1 is involved in the formation of specific AGEs. This may be of extreme importance in diabetes.

The glyoxalase system has also been implicated in other pathologies. Increased expression, transcription or activity of glyoxalase 1 has been seen in several cancers like colon tumours, breast cancer, lung tumours, prostate cancer and kidney tumours. Also, increased expression of glyoxalase 1 is considered to be a marker for resistance against chemotherapeutics.

There is also evidence that the C419A SNP of glyoxalase 1 (which causes an Ala111Glu exchange) could play a role in autism as well as in multiple sclerosis. In addition,
the polymorphism is also associated with the levels of sRAGE and also predisposes to the development of vascular complications in haemodialysis patients.  

5.2. Minor detoxifying pathways  
Without any doubt, methylglyoxal is mainly detoxified by the glyoxalase system. However, three other detoxifying enzymes have been described: Aldose reductase, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. Aldose reductase catalyses the conversion of methylglyoxal into 95% acetol and 5% D-lactaldehyde. Intriguingly, methylglyoxal elevates aldose reductase expression and enzymatic activity. Betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase both convert methylglyoxal into pyruvate with NAD and NADP as co-factors respectively.
6. References


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Scope of the Thesis
The development of vascular complications is a major concern in the field of diabetic research. One of the first signs for the development of these complications is endothelial dysfunction and both hyperglycaemia and inflammation have been shown to be causative factors. Hyperglycaemia induces, amongst others, the formation of advanced glycation end products (AGEs) which are known to perturb cellular function. These AGEs were thought to arise from the binding of glucose or other reducing sugars to free amino groups. However the major precursor for AGEs is later shown to be methylglyoxal, a cytotoxic sideproduct of glycolysis.

Previously, we have shown that the pro-inflammatory cytokine TNF can induce the formation specific methylglyoxal derived AGEs (MG-AGEs) in a fibrosarcoma cell-line by phosphorylating glyoxalase1. The latter enzyme is normally involved in the detoxification of the AGE precursor methylglyoxal. Since TNF is known to contribute to the development of vascular complications and since the extreme importance of AGEs in diabetes, we wanted to further investigate the role of TNF and glyoxalase1 in the formation of AGEs in endothelial cells.

Objective 1: A first objective was to verify whether TNF also induced formation of specific MG-AGEs in endothelial cells, similar as we observed in L929 cells. Therefore we wanted to examine the formation of AGEs in endothelial cells upon TNF treatment, and we will also investigate whether there is a role for phosphorylated glyoxalase1 in this process. If our hypothesis is true, AGE formation could be a new mechanism by which TNF contributes to endothelial dysfunction and the development of vascular complications.

Objective 2: Since methylglyoxal is a sideproduct of the glycolysis, we also wanted to evaluate the role of the glycolysis in TNF induced MG-AGE formation. After all, previous findings in our lab hinted us to a role for TNF in the regulation of the glycolytic flux. Therefore we hypothesised that TNF possibly increases the glycolytic flux, giving rise to increased formation of the AGE precursor methylglyoxal. This could represent a second pathway by which TNF induces the formation of MG-AGEs.

Objective 3: Previous findings in our lab also indicated that methylglyoxal is synergistic with TNF induced cell death. Since endothelial cell death is one of the pathways leading to diabetic complications, we wanted to explore the mechanism responsible for this synergistic effect.

TNF normally triggers the activation of NF-κB which protects cells from TNF induced cell death. We therefore hypothesised that methylglyoxal possibly inhibits NF-κB’s protective function. Furthermore we will try to unravel the mechanism by which methylglyoxal inhibits NF-κB. Our assumption was that methylglyoxal directly binds to NF-κB, which would then lose its protective function.
TNF stimulates Glycolysis and induces Methylglyoxal Modification of specific target Molecules in Aortic Endothelial Cells
1. Abstract

OBJECTIVE – Formation of AGEs is one of the pathways that contribute to the development of diabetic vascular complications. Emerging evidence has highlighted the important role of methylglyoxal (MG) in the formation of AGEs. We have previously shown that, in a tumor cell death model, TNF modulates the glyoxalase system by phosphorylating glyoxalase I (GLO1) and induces MG-derived AGE-modification of specific target molecules. The purpose of this study was to investigate whether TNF induces similar effects in bovine aortic endothelial cells (bAEC).

RESEARCH DESIGN AND METHODS – bAEC were treated with TNF for various times. Phosphorylation of GLO1 was assessed by 2-dimensional gel electrophoresis and Western blotting. MG-modification of specific proteins was investigated by cell labeling with $^{14}$C-MG.

RESULTS – TNF rapidly induced a transient phosphorylation of GLO1 in bAEC. This was not accompanied by significant changes in GLO1 activities. TNF induced a substantial increase in the concentration of D-lactate, suggesting an increased flux of MG through the glyoxalase system. TNF induced a significant increase in glucose uptake and in the levels of fructose-2,6-bisphosphate, which suggests that TNF stimulates carbohydrate metabolism presumably to induce an increased production of MG. Accordingly, TNF induced an increase in methylglyoxal-derived AGEs in bAEC. By labeling cells with $^{14}$C-MG, we could show that TNF induced methylglyoxal-modification of specific proteins.

CONCLUSIONS - TNF increases the intracellular levels of methylglyoxal and induces methylglyoxal-modification of specific proteins in endothelial cells. This may represent another pathway by which TNF can contribute to the development of vascular diseases.

2. Introduction

Inflammation is being increasingly regarded as an important factor in the pathophysiology of diabetes, and especially the accelerated development of macrovascular disease. Epidemiological studies indicate that diabetic patients have increased serum levels of several pro-inflammatory cytokines, including IL-6 and TNF. In addition, there is evidence that obesity, the most important risk factor for the development of insulin resistance and type 2 diabetes, may be an inflammatory condition. Excess adiposity leads to elevated production of adipo cytokines, such as TNF and IL-6. These cytokines not only directly promote insulin resistance, but also stimulate endothelial production of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). These molecules are critical mediators of endothelial dysfunction, which is one of the hallmarks in the development of diabetic complications. Furthermore, chronic hyperglycemia further aggravates inflammation by inducing the expression of pro-
inflammatory cytokines, such as TNF, IL-1β and IL-6 in monocytes. Moreover, chronic hyperglycemia has detrimental effects on endothelial cell function and is the primary etiologic factor in the majority of diabetic complications. One of the pathways that contribute to endothelial damage by hyperglycemia is the increased formation of advanced glycation end products (AGEs). In endothelial cells, the highly reactive dicarbonyl compound methylglyoxal (MG) has been identified as a major precursor in the formation of intracellular AGEs. MG is formed during normal cellular metabolism, primarily by dephosphorylation of triose phosphate intermediates of glycolysis, namely dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. MG is efficiently catabolised to D-lactate by the glyoxalase system, consisting of glyoxalase I (GLO1), glyoxalase II and the cofactor glutathione.

We have previously discovered a phosphorylated form of GLO1 and, in a tumor cell death model, we have shown that the TNF-induced phosphorylation of GLO1 is required for cell death. Furthermore, phosphorylated GLO1 is not involved in the detoxification of MG, but instead mediates the cytotoxic effects of MG via a pathway that leads to MG-modification of specific target molecules (MG-derived AGE formation). However, the actual biological function of phosphorylated GLO1 remains to be determined.

Vascular endothelial cells are among the principal physiological targets of TNF. Therefore, the purpose of this study was to investigate whether TNF modulates the glyoxalase system, and whether TNF also induces MG-modification of proteins in aortic endothelial cells under normal and hyperglycemic conditions.

3. Research Design and Methods

3.1. Cell cultures

Primary bovine aortic endothelial cells (bAEC) were purchased from Cambrex. The bovine endothelial cell line GM7373 was obtained from Coriell Cell Repositories. Both cell types were cultured in MEM supplemented with 10% fetal calf serum, penicillin (100U/ml), streptomycin (0.1mg/ml) and non-essential amino acids at 37°C in a humidified incubator in a 5% CO₂ atmosphere.

3.2. Reagents

Murine TNF was obtained from Roche. Methylglyoxal, glutathione, D-lactate dehydrogenase and the PKA inhibitor H89 were from Sigma. ¹⁴C MG was custom made by Scynexis (Durham, North Carolina) and 2-deoxy-D-[¹³H]-glucose was from Amersham Biosciences. The polyclonal anti-human glyoxalase I antibody has been described previously. The mAb6B was a kind gift of Prof. K. Uchida.

3.3. Preparation of cell lysates

bAEC and GM7373 cells were grown to confluency and hyperglycemia treatment
lasted for at least 48h. After TNF incubations, cells were rinsed three times with ice-cold PBS buffer and lysed. For Western blots and glyoxalase assays, cells were lysed in a CHAPS containing cytosol extraction buffer. Protein concentration was determined by the Bradford method (Biorad). For the NF-κB assays, cells were lysed in Totex buffer (20mM Heps/KOH pH 7.9; 350mM NaCl; 20% glycerol; 1% NP-40; 1mM MgCl2; 0.5mM EDTA pH 8.0; 0.1mM EGTA; 5mM DTT; 10mM NaF; 40mM β-glycerophosphate; Protease inhibitor cocktail).

3.4. 2-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out on 18 cm IPG strips, pH 4-7 (GE Healthcare) according to the manufacturer’s instructions. The buffer used for IEF contained 7M Urea, 2M Thio-Urea, 10mM DTT, 30mM CHAPS and carrier ampholines (GE Healthcare). For the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE, 12%).

3.5. Assay of glyoxalase1 activity

GLO1 activity was determined by using a spectrophotometric method, which monitors the initial rate of change in absorbance at 240 nm caused by the formation of S-D-lactoylglutathione. The standard assay mixture contained 2mM MG and 2mM GSH in a sodium phosphate buffer (50 mM, pH 6.6, 20°C). The reaction mixture was allowed to stand for 10 min at 37°C before the addition of the cytosolic protein fraction to ensure the equilibration of hemithioacetal formation.

3.6. Measurement D-Lactate

A fluorimetric method was used as previously described by McLellan et al. Briefly, D-Lactate formation is measured by the formation of NADH during oxidation of D-Lactate by a D-lactate dehydrogenase.

3.7. Fru-2,6-P2 Measurement

Fru-2,6-P2 was measured as previously described with slight modifications. After TNF stimulation, cells were lysed in 200mM NaOH. The lysate was then sonicated, boiled for 15 minutes and centrifuged. Fru-2,6-P2 was assayed by the stimulation of pyrophosphate-dependent phosphofructokinase (PPi:PFK) from potato tubers. All other enzymes and Fructose 6-phosphate and NADH were from Roche. Briefly, the cell lysate containing Fru-2,6-P2 was mixed with 50mM Tris-acetate pH 8, 2.5mM Mg-Acetate, 14.2mg/ml NADH, 1mM Fructose-6-phosphate, 3mM Glucose 6-phosphate, 50µg/ml aldolase, 10µg/ml triosephosphate isomerase/glycerol dehydrogenase and PPi:PFK. After pre-incubation for 5 minutes, the reaction was started with sodium pyrophosphate (final concentration 1mM) and the reaction was performed at 25°C.
3.8. Western blotting

Proteins were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Hybond P, Amersham). The blots were incubated with a polyclonal anti-human glyoxalase I antibody, followed by enhanced chemi-luminescence (ECL)-based detection (Amersham Pharmacia Biotech).

4. Results

4.1. TNF induces a transient phosphorylation of GLO1 that is mediated by PKA

We recently described that most cell types analysed so far express multiple isoforms of glyoxalase 1 (GLO1), including a NO-responsive form and a non-NO-responsive form, which we designated as the α-isoform. These isoforms can be separated by 2-dimensional gel electrophoresis (2-D) and detected by Western blotting using an anti-GLO1 antibody. Furthermore, multiple phosphorylation of GLO1 can occur either on the NO-responsive form or the α-isoform and this can be monitored in 2-D gels by a shift of the isoform to the acidic side of the gel.

TNF-induced phosphorylation of GLO1 in bovine aortic endothelial cells (bAEC) was investigated by 2-DE under normo- as well as under hyperglycemic conditions (5mM and 30mM glucose, respectively). bAEC were grown to confluency and stimulated with 1000 U/ml TNF for the indicated time (Fig. 1). In control cells, we could easily detect the same isoform pattern of GLO1 in normo- and hyperglycemic conditions that we observed previously in several other cell types. Under the hyperglycemic conditions, we also observed phosphorylation of the NO-responsive form of GLO1 (indicated by an arrow). However, this modification was not always present. TNF treatment resulted in a rapid shift of the α-isoform to the acidic side of the gel, indicating phosphorylation of GLO1. The phosphorylated spots of the α-isoform of GLO1 are indicated by arrowheads in Fig. 1. This phosphorylation was apparent from 10 minutes after TNF treatment onwards under normoglycemic conditions and from 5 minutes onwards under hyperglycemic conditions. Note also the TNF-induced transient disappearance and re-appearance of the NO-responsive form of GLO1 (the isoform on the far right) under both conditions. After 30 minutes of TNF treatment, the isoform pattern of GLO1 returned to that of the control cells, suggesting its de-phosphorylation. This indicates that the TNF-induced phosphorylation of GLO1 is transient and not sustained in bAEC. A similar TNF-induced phosphorylation of GLO1 was also observed in the endothelial cell line GM7373 (data not shown).

We previously reported the involvement of PKA in the TNF-induced phosphorylation of GLO1 in the fibrosarcoma cell line L929. Therefore, we investigated whether PKA might also be implicated in the TNF-induced phosphorylation of GLO1 in bAEC. Cells were pre-incubated for 1h with the PKA inhibitor H89 (5µM) and treated with TNF (1000 U/ml) for 30 min. As shown in Fig. 1B, H89 completely abolished the TNF-induced phosphorylation
TNF induced AGE formation in Endothelial Cells

Fig. 1. TNF-induces phosphorylation of GLO1 in bAEC, which is inhibited by the PKA inhibitor H89.
A. TNF-induces transient phosphorylation of GLO1 in normoglycaemic (5mM) as well as in hyperglycaemic (30mM) conditions. Western blots of 2-DE gels (pH 4-7) are developed with an anti-human GLO1 antibody. The NO-responsive form of GLO1 is indicated by an arrow. The non NO-responsive form of GLO1 is designated with α. The phosphorylated forms of GLO1 are indicated by arrowheads and appear to the left of the α-isoform, which indicates that TNF induces phosphorylation on the non NO-responsive form of GLO1. Note also the TNF-induced disappearance of the NO-responsive form of GLO1 and re-appearance of this form after 30 min of TNF treatment.
B. The TNF-induced phosphorylation is inhibited by the PKA inhibitor H89. bAEC were pre-incubated with H89 (5 μM) for 1h and then treated with TNF for another 20 min.

Fig. 1. TNF-induces phosphorylation of GLO1 in bAEC, which is inhibited by the PKA inhibitor H89.
A. TNF-induces transient phosphorylation of GLO1 in normoglycaemic (5mM) as well as in hyperglycaemic (30mM) conditions. Western blots of 2-DE gels (pH 4-7) are developed with an anti-human GLO1 antibody. The NO-responsive form of GLO1 is indicated by an arrow. The non NO-responsive form of GLO1 is designated with α. The phosphorylated forms of GLO1 are indicated by arrowheads and appear to the left of the α-isoform, which indicates that TNF induces phosphorylation on the non NO-responsive form of GLO1. Note also the TNF-induced disappearance of the NO-responsive form of GLO1 and re-appearance of this form after 30 min of TNF treatment.
B. The TNF-induced phosphorylation is inhibited by the PKA inhibitor H89. bAEC were pre-incubated with H89 (5 μM) for 1h and then treated with TNF for another 20 min.
of GLO1, indicating that PKA might be involved in the phosphorylation of GLO1 in bAEC. Note that H89 also prevented the TNF-induced disappearance of the NO-responsive form of GLO1.

4.2. TNF does not inhibit GLO1 activity and the MG-detoxification capacity of the glyoxalase system in bAEC

To investigate whether the TNF-induced transient phosphorylation of GLO1 affected the MG-detoxification capacity of the glyoxalase system, we measured GLO1 activities as well as D-lactate formation (the end product of the glyoxalase system) in TNF-treated and untreated bAEC.

GLO1 activity in cell lysates derived from control and TNF-treated bAEC was determined by a spectrophotometric method, which monitors the initial rate of the GLO1-catalyzed production of S-D-lactoylglutathione from the hemithioacetal that is formed spontaneously by incubating MG in glutathione (see M&M). As shown in Fig. 2A, there was no significant change in GLO1 activity in cell lysates from TNF-treated cells in several separate experiments. Hyperglycemia alone did not significantly alter GLO1 activity, which agrees with previous other reports from retinal pericytes and red blood cells [27,28]. These findings suggest that phosphorylation of GLO1 does not affect its MG-detoxification activity, in agreement with our previous findings in L929 cells [20].

To further investigate whether TNF affected the MG-detoxification activity of the glyoxalase system in endothelial cells, we measured the formation of D-lactate by a fluorimetric enzyme assay as described by McLellan et al [25]. The concentration of D-lactate was determined by the formation of NADH during oxidation of D-Lactate by D-lactate dehydrogenase. As shown in Fig. 2B, D-lactate concentrations rapidly increased upon TNF treatment. After only 10 minutes of TNF treatment, D-lactate was increased by about 40%. However, D-lactate concentration did not increase further after 3h of TNF treatment but remained at higher levels. These results indicate that TNF induces an increased flux of MG through the glyoxalase system, which cannot be attributed to changes in the activity of GLO1 because the latter was not altered in TNF-treated bAEC. However, the increased flux of MG through the glyoxalase system could simply be due to an increase in the intracellular concentrations of MG induced by TNF. Likewise, it has been shown that increased flux into D-lactate can be attributed to increased MG formation in red blood cells [28].

The major intracellular source of MG production is via phosphate elimination from the two glycolytic intermediates GA3P and DHAP [17]. Furthermore, it has been well documented that oxidative stress induced by hyperglycemic conditions results in increased intracellular levels of MG [29]. This results in the formation of AGEs, which is one of the pathways that contributes to the development of vascular complications in diabetes (reviewed in [14]). Therefore, we investigated whether TNF increased glucose metabolism in bAEC, which could then explain the increased flux of MG through the glyoxalase system.
4.3. TNF stimulates glucose uptake and increases Fru-2,6-P$_2$ levels in bAEC.

To investigate whether TNF affected glucose metabolism, we measured glucose uptake and Fru-2,6-P$_2$ concentrations in bAEC. Fru-2,6-P$_2$ is a potent allosteric stimulator of phosphofructo-1-kinase (PFK1), the rate limiting enzyme in glycolysis. Glucose uptake was measured by 2-deoxy-D-[1-\textsuperscript{3}H]-glucose uptake. Deoxyglucose is readily taken up by cells but is not further metabolized by glycolysis. bAEC were incubated with TNF (1000 U/ml) for the indicated times and then briefly treated with 2-deoxy-D-[1-\textsuperscript{3}H]-glucose for 1 min. To stop the reaction quickly, the cells were washed four times with ice-cold PBS and lysed in NaOH with 0.1% Triton X-100. The amount of 2-deoxy-D-[1-\textsuperscript{3}H]-glucose in the cell lysate was measured by scintillation counting and corrected for protein concentration. As shown in Fig. 3A, after 45 min. of TNF treatment, glucose uptake was already significantly increased by about 20%, both in normal and hyperglycemic conditions. After 3h of TNF
Part 3

Treatment, glucose uptake further increased by about 40% compared with control cells (Fig. 3A). This indicates that TNF stimulates glucose uptake in endothelial cells regardless of whether the cells have been exposed to high glucose.

Fru-2,6-P$_2$ levels were measured in cell extracts via the stimulation of plant PPI:PFK$^{26}$ (see methods). As shown in Fig. 3B, TNF induced increased Fru-2,6-P$_2$ levels and, after 3h of TNF treatment, its concentration was increased by about 30% compared with control cells under normo- as well as under hyperglycemic conditions.

In conclusion, TNF stimulates glucose uptake and increases the levels of F2,6BP, which would be expected to increase the flux through PFK-1. It has been reported that the levels of F2,6BP usually reflect glycolytic activity$^{30}$ — therefore, TNF-induced stimulation of glycolysis could be responsible for the increased production of MG.
4.4. TNF increases MG levels and induces MG-modification of specific proteins in bAEC

We investigated whether TNF indeed increases the intracellular MG levels in bAEC. Because we did not have the equipment to measure MG concentrations directly, we used a monoclonal antibody (mAb6B) raised against in vitro MG-modified keyhole limpet hemocyanin to monitor intracellular MG levels. This antibody recognizes MG-modified proteins in arterial walls of diabetic kidneys, and we have previously shown that this antibody recognizes specific MG-derived AGE epitopes induced during TNF-induced cell death in L929 cells. bAEC were incubated with TNF for the indicated times. After incubation, cell lysates were analysed by Western blotting using the mAb6B. A number of protein bands were recognized by this antibody in bAEC. However, TNF did not induce an increased MG-derived AGE-modification of all proteins, but only of a particular protein (Fig. 4A, indicated by an arrow) under normo- as well as under hyperglycemic conditions. It is noteworthy that the MG-AGE modification of the upper band is already increased in control cells under hyperglycemic conditions compared to normo-glycemic conditions. This is in agreement with the fact that hyperglycemia itself leads to increased AGE formation. These data suggest that TNF increases the intracellular concentration of MG and consequently induces MG-AGE modification of specific target proteins.

To further examine whether TNF induced MG-modification of specific proteins in bAEC, we labeled intact cells with $^{14}$C-MG and visualized MG-modified proteins by auto-radiography. To obtain sufficient protein labeling, we used 250µM $^{14}$C-MG. No significant cell death was observed with this concentration of MG over the incubation period (3h). Also, this MG concentration might be in the physiological range, because plasma concentrations of 400 µM MG have been reported in diabetes. bAEC were grown to confluency and then cultured for two days in culture medium with either 5mM glucose (normo-glycemic) or 30mM glucose (hyperglycemic). Cells were labeled for 3h with 250µM $^{14}$C-MG in the presence or absence of TNF (1000 IU/ml). After labeling, cells were washed 4 times with ice cold PBS and lysed in a buffer containing CHAPS. The proteins were separated by SDS-PAGE and electroblotted on PVDF membranes. Blots were then exposed for one week, and an auto-radiogram is shown in Fig. 4B. Several proteins were labeled with $^{14}$C-MG. However, TNF did not induce a global increase in all MG-modified proteins. Under normo-glycemic conditions, TNF induced MG-modification of a specific protein with an apparent M.W. of 120 kDa (indicated by the arrow), which was not significantly present in control cells. Under hyperglycemic conditions, TNF also induced MG-modification of this 120 kDa protein. We frequently observed a limited amount of this MG-modified protein in control cells, which indicates that the protein is prone to MG-modification by hyperglycemia itself. The TNF-induced MG-modification of this protein was observed in several independent experiments. As shown in Fig. 4B, TNF also induced an increased MG-modification of a protein with an apparent M.W. of 45 kDa, which was already present in control cells (indicated by the arrowhead). The latter further confirmed the fact that TNF induces an increase in the intracellular levels.
Part 3

It is worth mentioning that, in this experimental set-up, we could only detect one TNF-induced MG-modified protein, which indicates that this is a targeted process. It cannot be excluded that other MG-modified proteins might be induced by TNF, but these may be below the detection limit for this type of analysis.

TNF is a potent inducer of gene transcription in endothelial cells and, therefore, the induction of the 120 kDa MG-modified protein could have been due to either TNF-induced synthesis of this protein or TNF induced specific MG-modification of a pre-existing protein. However, TNF still induced this MG-modified protein in the presence of the protein synthesis inhibitor cycloheximide (2µg/ml; data not shown). Control
experiments indicated that this concentration of cycloheximide prevented the TNF-
induced synthesis of IκBα, the NF-κB inhibitor. This suggests that this MG-modified protein
induced by TNF is specifically due to MG-modification of the protein and is not due to
a protein synthesis effect.

We have previously shown that, in a TNF-induced tumor cell death model, the
phosphorylated form of GLO1 is no longer involved in the detoxification of MG, but
contributes, directly or indirectly, to a pathway leading to MG-modification of specific
proteins 20. Therefore, we investigated whether phosphorylation of GLO1 might be
involved in the TNF-induced MG-modification of the 120 kDa protein in bAEC. For this,
we used the PKA inhibitor H89, which also inhibits the TNF-induced phosphorylation of
GLO1 in bAEC. As shown in Fig. 4C, the TNF-induced MG-modification of the 120 kDa
protein could not be significantly inhibited in the presence of H89. This suggests that
GLO1 phosphorylation does not play a major role in MG-modification of the 120 kDa
protein.

5. Discussion

Epidemiological studies have confirmed that hyperglycemia is the most important
factor in the onset and progress of vascular complications, both in Type 1 and Type
2 diabetes 34,35. Hyperglycemia causes oxidative stress, which is responsible for the
induction of several signal transduction pathways that lead to endothelial dysfunction,
one of the early indicators of the development of vascular complications 29. One of these
pathways is the formation of AGE-modified proteins (reviewed in 36-38). Over the last few
years, emerging evidence has highlighted the important role of the reactive dicarbonyl
AGE precursor methylglyoxal in the formation of AGEs 14. Methylglyoxal formation is
accelerated in conditions associated with hyperglycemia 39,40, and it has been shown
that MG-modification of transcription factors and growth factors in endothelial cells and
other cell types can directly affect their function 41-44. In addition, methylglyoxal also
affects the insulin-signaling pathway by impairing the insulin-stimulated insulin receptor
substrate-1 tyrosine phosphorylation 45. Therefore, methylglyoxal might not only contribute
to the development of diabetic vascular complications, but it might contribute to the
general pathophysiology of diabetes.

Besides its direct role in tissue damage, hyperglycemia also induces the production
of pro-inflammatory cytokines from monocytes, such as TNF 11-12, which further
aggravate diabetic complications. These pro-inflammatory cytokines contribute to the
development and progression of diabetes and its associated complications through
several mechanisms. For instance, TNF plays a direct role in insulin resistance (reviewed
in 46) and also contributes directly to endothelium dysfunction by inducing the expression
of adhesion molecules that promote the adhesion of monocytes to the endothelium,
one of the key early events in the pathogenesis of atherosclerosis 47,48.

In our present work, we demonstrate for the first time that TNF may also contribute
to endothelial dysfunction by another molecular mechanism: namely, by modulating
glucose metabolism, the glyoxalase system and inducing methylglyoxal-modification of specific target molecules in endothelial cells. In our study, TNF induced a rapid (10 to 20 min.) and transient phosphorylation of the non-NO responsive form of GLO1. This was accompanied by a transient disappearance of the NO-responsive form of GLO1. These TNF-induced modifications of GLO1 did not result in a significant change of GLO1 activity in TNF-treated bAEC. However, TNF rapidly induced an increase in the concentration of D-Lactate, the end product of the glyoxalase system. This suggests that TNF accelerates the production of MG in endothelial cells, which consequently results in an increased flux of MG through the glyoxalase system. Furthermore, TNF induced a significant increase in glucose uptake and in the levels of fructose-2,6-bisphosphate, an allosteric activator of the rate-limiting enzyme in glycolysis PFK1. These data suggest that TNF stimulates carbohydrate metabolism, presumably to induce an increased production of MG. This was further evidenced by an increase in the methylglyoxal-derived AGEs in TNF-treated bAEC. Furthermore, by labeling cells with $^{14}$C-MG, we could show that TNF induced methylglyoxal-modification of a specific protein with an apparent M.W. of 120 kDa. The identity of this protein remains to be determined. TNF still induced this 120 kDa MG-modified protein in the presence of the protein synthesis inhibitor cycloheximide, which suggests that TNF specifically induced the MG-modification of this protein rather than inducing its synthesis. The TNF-induced MG-modification of this 120 kDa protein could not be significantly inhibited by the PKA inhibitor H89, while the TNF-induced transient phosphorylation of GLO1 in bAEC could be inhibited by H89. This contrasts with our results from the TNF-induced tumor cell death model in which the TNF-induced formation of a specific MG-derived AGE could be significantly inhibited by inhibitors that also block the TNF-induced phosphorylation of GLO1. However, there are some fundamental differences in the pathways induced by TNF in the two cell types, which may explain this different effect. First of all, the two cell types have completely different biological responses to TNF: namely, cell death characterized by necrosis in L929 cells, and primarily gene activation and cell survival in endothelial cells. Second, the TNF-induced modifications of GLO1 in the tumor cell death (L929 cells) model and those induced in bAEC are different: L929 cells show sustained phosphorylation (longer than 1.5h) primarily on the NO-responsive form of GLO1; whereas in bAEC, the phosphorylation is very early and transient (< 20 min.) and occurs on the non-NO-responsive form of GLO1. In addition, concomitant with the phosphorylation, TNF also induces a transient disappearance of the NO-responsive form of GLO1. Third, the M.W. of the specific MG-modified proteins that are induced by TNF in these different cell types is different, indicating that the proteins are cell-type specific. But, for the moment, we cannot exclude the possibility that the phosphorylation of GLO1 might be involved in the TNF-induced MG-modification of other specific target proteins in bAEC that were not detected in our type of analysis. This will require a more detailed comparative analysis of MG-modified proteins in TNF-treated bAEC versus control cells. However, we could not make a detailed analysis by high-resolution 2-dimensional gel electrophoresis,
because the major part of the 14C-MG-label is removed from the proteins in the urea buffer that is used for isoelectrofocusing of the proteins (our unpublished results).

The actual biological function of phosphorylated GLO1 — as well as the function of the NO-mediated modification of GLO1 — is not yet known. However, in both L929 cells and bAEC, the phosphorylation of GLO1 is not accompanied by a significant change in the MG-detoxification activity of GLO1, which suggests that phosphorylated GLO1 is not involved in this pathway. A possible role for the TNF-induced transient phosphorylation of GLO1 in bAEC could be the facilitation of a metabolic switch that may be required for inducing a particular signaling pathway. TNF induces multiple pathways at the same time, and the cellular response to TNF is highly cell-specific and is also determined by an intricate interplay among these pathways. Therefore, it could be that the duration of the phosphorylation of GLO1 plays a role in determining the biological response to TNF. However, it is clear that TNF induces methylglyoxal-modification of specific target proteins, which are cell-type specific. This suggests that methylglyoxal may indeed function as a signaling molecule, as other investigators have also proposed. This may be another signaling pathway by which TNF may contribute to the development and progression of diabetic vascular complications.

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Methylglyoxal suppresses TNF-α-induced NFκB Activation by inhibiting NFκB DNA Binding
1. Abstract

Methylglyoxal is a cytotoxic metabolite that is produced in vivo mainly from glycolysis. Increased production of methylglyoxal can be induced by tumor necrosis factor and occurs in a number of pathological conditions, including diabetes and neurodegenerative disorders. Methylglyoxal is highly reactive and can modify proteins, which results in the formation of advanced glycation end products. Yet, we, and others, have recently proposed a role for methylglyoxal as a signaling molecule. In this study, we show that methylglyoxal inhibits TNF-induced NF-κB activation and NF-κB-dependent reporter gene expression by inhibiting the DNA binding capacity of NF-κB p65. Methylglyoxal slightly delayed, but did not inhibit, TNF-induced degradation of IκBα and strongly inhibited TNF-induced NF-κB-dependent re-synthesis of IκBα. The TNF-induced nuclear translocation of NF-κB p65 was also delayed, but not inhibited, in the presence of methylglyoxal. TNF-induced phosphorylation of p65 was not affected by methylglyoxal. We show that the conserved Cys 38 residue, which is located in the DNA binding loop of NF-κB p65 and responsible for the redox regulation of the transcription factor, is involved in the methylglyoxal-mediated inhibition of p65 DNA binding. Furthermore, overexpression of p65 inhibited TNF-induced cell death; however, in the presence of exogenously added methylglyoxal, overexpression of p65 caused far greater TNF-induced cell death. These findings suggest that methylglyoxal provides another control mechanism for modulating the expression of NF-κB-responsive genes and that methylglyoxal may be responsible for tipping the balance towards TNF-induced cell death in cells with constitutive NF-κB activation.

2. Introduction

Methylglyoxal (MG) is a highly reactive and cytotoxic metabolite that is primarily produced during normal cellular metabolism through the elimination of phosphate from dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate, both intermediates of glycolysis. MG can also be synthesized by MG synthase. MG is normally detoxified to D-lactate by the glyoxalase system, which is comprised of glyoxalase I (GLO1), and II (reviewed in ). However, the full biological function of the glyoxalase pathway has not yet been elucidated. The work of Szent-Györgyi suggested that GLO1 and its substrate MG were involved in the regulation of cell division, but a direct mechanistic link has yet to be identified. For many years, MG has been known to be carcinostatic, but its direct use as an anticancer drug has been prevented by its rapid detoxification in vivo by the glyoxalase system. This characteristic provided the rationale for the development of GLO1 inhibitors as potential anti-cancer agents.

Increased expression of GLO1 is associated with several diseases, including diabetes, Alzheimer’s disease and several types of cancer. GLO1 is particularly overexpressed in the more aggressive and invasive forms of ovarian cancer and the Her-2/neu-positive breast cancers, which are refractory to various types of therapy and associated with
a poor prognosis. Furthermore, over expression of GLO1 is involved in the resistance of human leukemia cells to anti-tumor agent-induced apoptosis. Up to now, why the cytotoxic component MG is produced during normal cellular metabolism has been a mystery. But, we and others, have proposed a role for MG as a signaling molecule. Increased levels of methylglyoxal lead to the rapid modification of proteins to generate advanced glycation end products (AGEs). Formation of AGEs contributes to the development of pathological conditions in vivo, such as diabetes and cancer.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that plays a role in the pathophysiology of various diseases (reviewed in ). Furthermore, at low concentration, TNF can promote tumor growth and, at high concentration, TNF has potent anti-tumor and anti-malignant cell effects. In various cell types, TNF induces two signaling pathways: one leads to gene activation, which is primarily mediated through activation of NF-kB; and the other leads to cell death by either apoptosis or necrosis. The final outcome of TNF-induced signaling is dependent on the cell type and the cross-talk between the two pathways. For instance, NF-kB can act as an anti-apoptotic transcription factor that leads to the induction of anti-apoptotic proteins.

We have recently described how TNF-induced necrosis – characterized by oxidative stress – in the fibrosarcoma cell-line L929 is accompanied by increased intracellular concentrations of methylglyoxal. This – along with the TNF induced phosphorylation of GLO1 – leads to MG-modification of specific target molecules (MG-derived AGEs). Furthermore, exogenously added MG is strongly synergistic with TNF-induced cell death and can even sensitize resistant L929 clones to TNF-induced cell death (our unpublished results). It is well known that NF-kB is necessary and sufficient for the prevention of TNF-induced cell death. Therefore, the purpose of this present investigation was to examine whether the synergistic action of MG on TNF-induced cell death is (partially) mediated through an effect on NF-kB.

3. Materials and methods

3.1. Cell lines

L929 cells were cultured in Dulbecco’s modified Eagle’s medium with glutamax supplemented with heat-inactivated fetal calf serum (10%, v/v), penicillin (100 units/ml), and streptomycin (0.1 mg/ml), at 37 °C in a humidified incubator under an 8% CO₂ atmosphere. Human carcinoma (HeLa) cells were grown in Dulbecco’s modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambrex) at 37 °C in a humidified incubator under an 8% CO₂ atmosphere. Human umbilical vein endothelial cells (HUVEC) cells were grown in EGM-2 medium (Cambrex) according to manufacturer’s instructions, at 37 °C under a 5% CO₂ humidified atmosphere.
3.2. Reagents

Murine TNF (mTNF) was obtained from Roche (Roche Diagnostics, Mannheim, Germany). Methylglyoxal was obtained from Sigma. Antibodies against phosphorylated IkBa, IκBα, phosphorylated p65 and ubiquitin were from Cell Signaling Technology; anti-p65 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-coupled secondary antibodies from Amersham Biosciences; and Alexafluor 488-coupled secondary anti-mouse antibody from Molecular Probes.

3.3. Preparation of cell lysates

L929 cells were seeded 24 h prior to the experiment. After TNF incubations (1000 U/ml), the cells were rinsed three times with ice-cold PBS buffer, and total cell lysates for the NF-κB Transcription Factor Assay were prepared in Totex buffer (20 mM Hepes/KOH pH 7.9; 350 mM NaCl; 20% glycerol; 1% NP-40; 1 mM MgCl₂; 0.5 mM EDTA pH 8.0; 0.1 mM EGTA; 5 mM DTT; 10 mM NaF; 40 mM β-glycerophosphate; Protease inhibitor cocktail)²². Cell lysates for monitoring the phosphorylation, ubiquitination and protein levels of IkBa and p65 were prepared in a CHAPS-containing cytosol extraction buffer²³.

3.4. Measurement of the NF-κB DNA binding activity

The DNA binding of the NF-κB p65 subunit was measured with a colorimetric non-radioactive NF-κB p65 Transcription Factor ELISA Assay (Chemicon® International, CA, USA) according to the manufacturer’s instructions.

3.5. NF-κB-dependent reporter gene expression assay

For the reporter assays, we used L929sA cells that are stably transfected with a synthetic reporter construct (IL6-κB)₃ - 50IL6P-luc + and the selection vector pPGKBGeobpA (encoding a [neo]-β-galactosidase fusion protein conferring resistance to G418 as well as constitutive β-galactosidase enzymatic activity). These cells and constructs were described previously by²⁴. The (IL6-κB)₃-50IL6P-luc + plasmid contains a concatenated trimer of the IL6-κB motif atgtGGGATTTTCCCatg (capitals indicating the IL6-κB core sequence) in front of a minimal IL6 promoter in the pGL3 reporter vector (Promega Biotec, Madison, WI), which encodes a luciferase reporter gene. Luciferase assays were carried out according to the manufacturers instructions (Promega Biotec). Cells were lysed in a buffer containing 25mM Tris phosphate (pH 7.8), 2mM DTT, 2mM cyclohexanediaminetetraacetic acid, 10% glycerol and 1% Triton X-100 for 15 min at room temperature. From the cell extracts, 20 µl was transferred into a 96-well plate together with 50 µl of the luciferase assay reagent (20 mM Tricine, 1.07 mM [MgCO₃]₂Mg(OH)₂·5H₂O, 2.67mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzymeA, 470 µM D-luciferin, 530 µM ATP). This mixture was then analysed in a luminescence microplate counter (TopCount; Packard, Meriden, Conn.) for luciferase
activity and corrected for β-galactosidase activity (GalactoLight kit; Tropix, Bedford, MA).

3.6. Western blotting

Proteins were separated by SDS-PAGE (12%) and transferred to a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the respective antibodies, followed by ECL-based detection (Amersham Pharmacia Biotech).

3.7. Confocal immunofluorescence microscopy

L929 cells were plated on glass coverslips 48 h prior to the experiment. After incubation with TNF (and/or with 750 µM MG) for the respective time periods, the cells were washed three times with PBS and then fixed with 100% ice-cold methanol for 10 min at -20 °C. The cells were then rinsed three times with PBS and incubated with a primary monoclonal antibody against p65 NF-κB (1:100) for 1 h at room temperature. The cells were washed three times with PBS and incubated with an anti-mouse Alexa-488-conjugated secondary antibody for 1 h and again washed three times with PBS. The coverslips were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA). Images were taken with a Biorad laser scanning confocal microscope.

3.8. Plasmids

The pcDNA-p65 wild type plasmid was obtained from Dr. R. Hay. This plasmid was used as a template to construct the C38A mutant. The forward 5’-GCGCTTCCGCTACAAGGCCGAGGGCGCTCCGCGGGGCGCTCCGCGGG-3’ and reverse 5’-CCCGCGGAGCGCCCCTCGGCCTTGTAGCGGAAGCGC-3’ primers were used to mutate the Cys38 to an Ala residue using a site-directed mutagenesis kit (Stratagene). The mutation was confirmed by sequencing.

3.9. Transient transfection experiments

Subconfluent monolayers of HeLa cells (6-well plates) were used for transient transfections using the lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. Cells were transfected with pcDNA-p65 WT and with pcDNAp65 C38A mutant. Mock-transfected cells were used as control. 24 h after transfection, the cells were treated for 1 h with 1000 U/ml TNFα and/or methylglyoxal concentrations, as indicated. After treatment, the cells were washed three times with phosphate-buffered saline and lysed on ice in Totex buffer.

3.10. Measurement of TNF-induced cell death by flow cytometry

Cell death in L929-derived cell lines has been described previously 23. Briefly, Cell death was induced by the addition of TNF (100 U/ml) to the cell suspension. Cell death
was measured by quantifying PI-positive cells by FACS (FACS Calibur, Becton Dickinson, San Jose, CA). Routinely, 3000 cells were analysed. Cell death is expressed as the percentage of PI-positive cells in the total cell population.

4. Results

4.1. Methylglyoxal suppresses TNF-α-induced NF-κB activation

To determine whether MG had an effect on TNF-induced NF-κB activation, we measured the DNA binding of the NF-κB p65 subunit. L929 cells were incubated with TNF (1000 U/ml) in the presence of increasing concentrations of MG for 1 h, a time point at which there is no detectable cell death. At the end of the incubation, total cell lysates were prepared in Totex buffer (see Section 3). The DNA binding of the NF-κB p65 subunit was determined by the colorimetric non-radioactive NF-κB p65 Transcription Factor Assay in 96-well format (Chemicon®). In this assay, a double-stranded biotinylated oligonucleotide, containing the consensus sequence for NF-κB binding, is incubated with total cellular extracts. This capture probe binds only the active form of NF-κB p65. The mixture is then incubated on a streptavidin-coated plate. The amount of p65 bound to the oligonucleotide is then detected with a specific polyclonal anti-NF-κB p65 primary antibody and a HRP-conjugated secondary antibody. A representative experiment of three independent experiments is shown in Fig. 1. As is clear from this figure, increasing concentrations of MG strongly inhibit the TNF-induced DNA binding of the NF-κB p65 subunit in L929 cells. Maximum inhibition (80%) of p65 DNA binding is observed at 750 µM MG. The rather modest induction of NF-kB activation by TNF alone is due to the time point chosen, namely 1 h of TNF treatment. This is a time point when there is already a considerable TNF-induced NF-κB-dependent resynthesis of the NF-κB inhibitor IκBα (see Fig. 3). Analysis of the protein levels of p65 by Western blotting showed that they are not affected by MG (data not shown). This excluded the possibility that the reduced DNA binding in the presence of MG could be caused by a reduction in the amount of the p65 protein.

TNF induces NF-κB activation in many cell types. Therefore, we examined whether the inhibition of the TNF-induced NF-κB activation by methylglyoxal was cell-type specific or if it was a general phenomenon. HUVEC cells as well as HeLa cells were treated with TNF (1000 U/ml) for 1h in the presence of increasing concentrations of MG. As shown in Fig. 1B and C, respectively, inhibition of the TNF-induced p65 DNA-binding was already observed at 100 µM MG and maximum inhibition (±45%) was obtained at 750 µM MG. In all three cell lines, no further inhibition of the TNF-induced p65 DNA binding was observed with MG concentration higher than 750 µM, which indicates that the MG-mediated inhibition can be saturated.
4.2. Methylglyoxal inhibits TNF-induced NF-κB-dependent reporter gene expression

Because NF-κB DNA-binding does not always correlate with NF-κB-dependent gene transcription, we investigated whether methylglyoxal also had an effect on TNF-induced NF-κB-dependent reporter gene expression. For this purpose, we used L929sA cells that were stably transfected with the NF-κB-driven reporter gene construct (IL6-kB)₅-50IL6P-luc + that contains multiple NF-kB responsive elements coupled to a minimal IL-6 promoter in front of the luciferase reporter gene. These cells have been previously described and also contain an internal control plasmid pPGKβGalA for the constitutive expression of the (neo)²-β-galactosidase fusion protein. Cells were stimulated with TNF for 3 h in the presence of increasing concentrations of methylglyoxal. Cell lysates were assayed for corresponding reporter gene activity and the expression of the internal control protein. MG (at all concentrations used) had no effect on the expression of the internal control protein (data not shown). The TNF-induced NF-κB-regulated reporter gene expression is shown in Fig. 2. The average of three independent experiments is shown. It is clear that MG also inhibited the TNF-induced NF-κB-dependent reporter
Methylglyoxal inhibits NFκB DNA Binding

Methylglyoxal inhibits NFκB DNA Binding

4.3. Methylglyoxal delays TNF-induced degradation of IκBα and inhibits TNF-induced NF-κB-dependent re-synthesis of IκBα

TNF-induced NF-κB activation in L929 cells is mediated through the classical pathway, which involves the phosphorylation, the subsequent ubiquitination and proteasomal degradation of its inhibitor IκBα, and the subsequent liberation of the NF-κB dimers (p65/p50 dimers) 27. Following degradation of IκBα, the p65/p50 heterodimer translocates to the nucleus, where it induces the transcription of its target genes. One of these target genes is its own inhibitor IκBα, and the re-synthesis of IκBα results in an auto-regulatory loop that culminates in the re-inhibition of NF-κB and translocation to the cytoplasm by newly synthesized IκBα 28,29. MG is highly reactive to Lys, Arg and Cys residues 30. Thus, a likely mechanism for the MG-mediated inhibition of TNF-induced NF-κB activation could be the inhibition of IκBα degradation via interference with the ubiquitination of Lys residues. Therefore, we investigated whether MG had an effect on the TNF-induced IκBα degradation. Also monitoring the TNF induced re-synthesis of IκBα allowed us to measure the NF-κB dependent transcription of one of its endogeneous target genes in the presence of MG. IκBα levels can be easily monitored by Western blotting, using a specific anti-IκBα antibody, as shown in Fig. 3. TNF treatment of L929 cells leads to a
rapid and drastic degradation of IκBα after only 10 min. Resynthesis of IκBα is already evident starting at 30 min of TNF treatment (Fig. 3). In the presence of 100 µM MG, the TNF-induced degradation of IκBα was not affected, but the NF-κB dependent re-synthesis of IκBα was already slightly inhibited. However, in the presence of 750 µM MG, the degradation of IκBα was slowed down, but not inhibited, because at 1 h of TNF treatment IκBα was fully degraded. At this time point, the NF-κB-dependent re-synthesis of IκBα was strongly inhibited in the presence of MG (Fig. 3). Even at 1.5 h of TNF treatment, NF-κB-dependent re-synthesis of IκBα was still inhibited (data not shown), indicating a sustained action of MG. Note, that MG treatment alone had no effect on the IκBα levels. Furthermore, phosphorylation studies of IκBα showed that MG did not affect the TNF-induced phosphorylation of IκBα (data not shown), nor was the ubiquitination of IκBα considerably affected by MG. This was investigated by immunoprecipitation of IκBα followed by detection with an anti-ubiquitin antibody (data not shown). A plausible explanation for the delayed TNF-induced degradation of IκBα in the presence of higher concentrations of MG could be partial stabilization of IκBα. A similar stabilization of the IκBα protein has also been observed in the presence of nitric oxide.

In conclusion, the above data indicate that, at higher concentrations, MG can delay the TNF-induced degradation of IκBα and can strongly inhibit the NF-κB-dependent re-synthesis of its inhibitor IκBα. At lower concentrations (100 µM), MG has no effect on the TNF-induced degradation of IκBα, but the NF-κB-dependent re-synthesis of IκBα is already inhibited. This indicates that the transcriptional activity of NF-κB in the TNF-induced NF-κB activation pathway is the primary target for methylglyoxal.

4.4. Methylglyoxal delays the TNF-induced nuclear translocation of NF-κB

Inhibition of NF-κB transcriptional activity could also result from inhibition of its nuclear translocation. This is conceivable as the nuclear localization signal (NLS) in p65 contains several Arg and Lys residues that could be potential target residues for MG-modification.
Methylglyoxal inhibits NFκB DNA Binding

Therefore, we investigated whether MG treatment (750 µM) affected the nuclear translocation of the NF-κB p65. L929 cells were incubated with TNF for the indicated time points in the absence and presence of 750 µM MG. Samples were prepared for immunocytochemistry using anti-p65 antibody and Alexa-488-conjugated anti-mouse IgG. Representative confocal images are shown. The transmission images are shown in the left panels, the immunofluorescence of the NF-κB p65 subunit is shown in the middle panels, and the overlay is shown in the right panels. The scale bar is 10 mm.

Fig. 4 - Methylglyoxal delays, but does not inhibit, the TNF-induced nuclear translocation of NF-κB p65. L929 cells were incubated with TNF for the indicated time points in the absence and presence of 750 µM MG. Samples were prepared for immunocytochemistry using anti-p65 antibody and Alexa-488-conjugated anti-mouse IgG. Representative confocal images are shown. The transmission images are shown in the left panels, the immunofluorescence of the NF-κB p65 subunit is shown in the middle panels, and the overlay is shown in the right panels. The scale bar is 10 mm.

Therefore, we investigated whether MG treatment (750 µM) affected the nuclear translocation of the NF-κB p65. Immuno-cytochemical analysis of the p65 subunit was performed by laser confocal immunofluorescence microscopy. As shown in Fig. 4, p65 is primarily located in the cytoplasm in untreated L929 cells as is evident from the presence of large dark voids in the confocal image, which correspond to the nuclei in the transmission image. In TNF-treated cells, p65 is concentrated mainly in the nucleus after only 10 min of TNF treatment (this translocation lasted for at least 30 min). After 1 h of TNF treatment, p65 was already more concentrated in the cytoplasm and the nuclei had already started to become void of p65. This cytoplasmic re-translocation of p65 is due to the NF-κB-dependent re-synthesis of its inhibitor IκBα. However, in the presence of MG the TNF-induced translocation of p65 was delayed, but not inhibited. The p65 remained largely cytoplasmic in the first 20 min of TNF treatment. After only 30 min of TNF treatment, p65 began to concentrate in the nucleus, and, after 1 h of TNF treatment, p65 was fully concentrated in the nucleus. This indicates that the cytoplasmic re-
translocation of p65 is also inhibited by the presence of MG. This is most likely due to the lack of NF-κB-dependent re-synthesis of IκBα, which is responsible for the cytoplasmic re-translocation of NF-κB. Significantly, MG’s strong inhibition of the DNA-binding activity of the p65 subunit was determined at 1 h of TNF treatment - that is, when the p65 subunit was fully concentrated in the nucleus in the presence of MG. Taken together, these results indicate that the MG-mediated inhibition of the TNF-induced NF-κB DNA-binding is not due to inhibition of its nuclear translocation. Furthermore, these results suggest that the inhibitory effect of MG on the TNF-induced NF-κB DNA binding and the NF-κB-dependent transcription is rather executed at the level of the transcription factor itself. This is not so unlikely, as it has been reported that certain NF-κB inhibitors, such as for example NO, suppress NF-κB activation by directly blocking the binding of NF-κB to the DNA.

4.5. Methylglyoxal does not affect the TNF-induced phosphorylation of p65

Optimal NF-κB activation is regulated by phosphorylation of the NF-κB proteins themselves (reviewed in ). TNF induces phosphorylation of p65, which is required for its transcriptional activity, but not for DNA-binding. Phosphorylation on Ser536, which is located in the transactivation domain, is mediated by IKKβ. Because p65 was fully clustered in the nucleus upon TNF stimulation in the presence of MG, we also wanted to investigate whether MG had an effect on the TNF-induced transactivation of p65. The TNF-induced phosphorylation of p65 in L929 cells in the presence and absence of methylglyoxal was analysed by Western blotting using phospho-specific antibodies against p65. As shown in Fig. 5, TNF induces a clear phosphorylation on Ser 536. However, in the presence of 750 µM MG, this TNF-induced phosphorylation of p65 is not affected, indicating that MG did not interfere with the TNF-induced transactivation of p65. Along the same line, TNF-induced phosphorylation on Ser 276, which is located in the Rel homology domain of p65, was not affected in the presence of 750 µM MG (data not shown). These data further suggested that the inhibitory effect of MG on the p65 DNA

![Fig. 5 - Methylglyoxal does not affect the TNF-induced phosphorylation of p65. L929 cells were incubated with TNF (1000 U/ml) for the indicated time points in the absence and presence of 750 µM MG. Cytosolic extracts were analysed by Western blotting using an antibody that specifically recognizes p65 phosphorylated on Ser 536. The same blot was then re-probed with an anti-p65 antibody.](image)
binding and NF-κB-dependent reporter gene expression must be executed at the level of p65 itself.

4.6. Involvement of Cys 38 in NF-κB p65 for inactivation by methylglyoxal

Members of the NF-κB family, including p65, are redoxregulated transcription factors. This redox regulation is mediated by the redox state of a cysteine residue located in the N-terminal conserved region that is responsible for DNA binding in all NF-κB family members and corresponds to C38 in p65. NF-κB must be in a reduced state to bind DNA in vitro. The C38 residue is located within a polypeptide loop used to make many of the specific contacts with the κB motif DNA and contacts a phosphate in the DNA backbone. Furthermore, inhibition of the NF-κB p50 DNA binding activity by nitric oxide (NO) is mediated through NO modification of this conserved Cys 62 residue, and the DNA binding of a p50 C62S mutant is much more resistant to inhibition by NO. MG can react with Cys residues to form a hemithioacetal and furthermore, MG has been shown to be involved in the regulation of the yeast transcription factor Yap1 via modification of Cys residues. This prompted us to investigate whether the conserved residue Cys 38 in p65 was involved in the methylglyoxal-mediated inhibition of the NF-κB DNA binding. To this end, we made use of the fact that transient transfection with NF-κB p65 by itself is sufficient to induce NF-κB DNA binding activity, and TNF stimulation of these transfected cells did not further increase the p65 DNA binding activity (data not shown). NF-κB p65 WT and a C38A mutant were transiently overexpressed in HeLa cells. Twenty-four hours after transfection, the cells were treated with increasing concentrations of MG (as indicated in Fig. 6) for 1 h. Total cell lysates were prepared in Totex buffer, and the DNA binding activity was determined with a quantitative transcription factor assay as described above. The expression levels of the ectopically expressed p65 WT and mutant protein were analysed by Western blotting and were found to be comparable and not affected by MG treatment (Fig. 6A). Mock-transfected cells were used to determine the DNA binding activity of endogeneous p65, which was negligible compared to the activity of the ectopically expressed proteins (Fig. 6B). Three independent experiments were performed and a representative experiment is shown in Fig. 6B. The p65 C38A mutant protein had already reduced DNA-binding compared to the WT p65, but DNA binding was still sufficient to measure the effect of MG. MG treatment of HeLa cells transfected with p65 WT resulted in a drastic inhibition of its DNA binding activity, with at least 50%–60% inhibition at 100 µM MG and 90% at 750 µM MG. However, the p65 C38A mutant was considerably more resistant to inhibition of its DNA binding activity by MG as compared to the WT protein (Fig. 6B). The relative inhibition (mean of three independent experiments) of DNA binding activity of the WT and mutant protein is shown in Fig. 6C. The DNA binding of the p65 C38A mutant was only 20% to maximum 25% inhibited by 100 µM MG. The mutant protein was thus 50%–60% more resistant to inhibition by MG (100 µM) as compared to the WT protein (Fig. 6C). Also, at higher concentrations of MG, the mutant protein was still 50% more resistant to inhibition by MG.
Fig. 6 - Reduced sensitivity of the p65 C38A mutant towards inhibition of DNA binding activity by methylglyoxal. (A) Western blot with anti-p65 antibody. Transient expression level of p65 WT and p65 C38A mutant in HeLa cells. Expression levels of endogeneous p65 are also shown. a: untreated, b: 100 µM MG, c: 250 µM MG, d: 750 µM MG. Note that the expression level of the p65 proteins was not affected by MG treatment. An anti-actin antibody was used as control for equal protein load. (B) Inhibition of DNA binding activity by MG of the p65 WT and the p65 C38A mutant. The absorbance values at 450 nm are shown of one representative experiment. The DNA binding activity of endogeneous p65 in mock transfected cells is negligible compared to that of the ectopically expressed proteins. (C) Relative inhibition of DNA binding activity by MG of the p65 WT and the p65 C38A mutant. The mean of three independent experiments is shown.
In conclusion, these data indicate that Cys 38 in NF-κB p65 is involved in the MG-mediated inhibition of the DNA binding activity. Furthermore, these data suggest that MG may directly modify Cys 38 in NF-κB p65 and that MG-modification of the transcription factor is a very site-specific and targeted process.

4.7. Overexpression of NF-κB p65 increases the synergistic effect of methylglyoxal on TNF-induced cell death

Since NF-κB activation inhibits TNF-induced cell death, we wanted to further explore the role of NF-κB activation in the synergistic action of methylglyoxal on TNF-induced cell death. For this, we used overexpression of NF-κB p65, which results automatically in NF-κB activation. Because there is a large clonal variability in sensitivity to TNF-induced cell death in L929 cells, we used an inducible expression system so that TNF sensitivity can be compared in the same clone upon induced expression of p65, circumventing the problem of clonal variability. Murine fibrosarcoma L929 cells were transfected with pSP64Mx NF-κB p65. In this expression vector, p65 is under the control of the murine Mx promoter, which is inducible with interferon α (IFNα). After transfection, G418-resistant clones were screened and retained when they showed low-level leak expression in the non-induced condition and strong expression in the induced condition. Four clones for p65 and four mock clones were selected for use in further experiments. For induction, cells were incubated with 500 IU/ml IFNα for 16 h prior to TNF treatment. Levels of ectopically expressed p65 protein in the non-induced and induced conditions and the endogenous levels of p65 in the mock clones are shown in Fig. 7A. Compared to the mock clones, the p65-expressing clones had a leaky expression in the non-induced condition, a phenomenon that we had previously observed with this inducible expression system in L929 cells. As expected, overexpression of p65 resulted in its increased DNA binding activity in untreated cells as measured by the non-radioactive NF-κB p65 Transcription Factor Assay (data not shown). TNF-treatment of these clones did not strongly activate NF-κB, because basal NF-κB activity was already very high in these clones.

TNF-induced cell death in the clones was measured as a function of time by flow cytometry using the uptake of propidium iodide as a measure for cell death. The experiments were repeated three times and produced similar results each time. Fig. 7B depicts a representative experiment showing the percentage of cell death for the various clones after 4 h of TNF treatment (100 U/ml) under the non-induced and induced condition and in the presence and absence of MG. The percentage of cell death in all control conditions was negligible and is therefore not shown. As it is clear from Fig. 7B, all p65 clones - in the induced as well as in the non-induced condition - are considerably more resistant to TNF-induced cell death compared to the mock clones. The low sensitivity for TNF-induced cell death of these clones in the non-induced condition is most probably due to constitutive NF-κB activity caused by leaky expression of p65. Induced overexpression of p65 in these clones caused a further decrease (average of 40%) in TNF-induced cell death. These data agree with the longknown fact that NF-κB
Fig. 7 - Overexpression of NF-κB p65 sensitizes for the synergistic action of methylglyoxal on TNF-induced cell death. (A) Expression levels of ectopically expressed p65 (WT clones) and endogenously expressed p65 (Mock clones) in the induced (i) and the non-induced (ni) condition. Note that all p65 WT clones (WT) have leak expression in the non-induced condition, as compared to the expression level of endogenous p65 in the mock clones (Mo). Equal amounts of protein were loaded for all clones. (B) Percentage of TNF-induced cell death expressed as the percentage of PI-positive cells after 4 h of TNF treatment (100 U/ml) of the different clones in the non-induced (ni) and the induced (i) condition and in the absence and presence of MG. (C) Synergistic effect of methylglyoxal on TNF-induced cell death of the different clones in the non-induced and the induced condition for the same experiment as shown in (B). The synergistic effect is expressed as the fold-increase in percent of TNF-induced cell death without MG for the same condition.
activation leads to inhibition of TNF-induced cell death\textsuperscript{20,47,48}. As we have previously shown\textsuperscript{23}, IFNa treatment of mock clones had no significant effect on TNF-induced cell death, which excluded the possibility that inhibition of TNF-induced cell death in the p65 clones was solely due to IFNa treatment.

The synergistic effect of MG (750 µM) on TNF-induced cell death in the various clones in the induced and non-induced conditions is presented in Fig. 7C as the fold increase in % of TNF-induced cell death in the presence of MG over the % of TNF-induced cell death without MG for the same condition. The degree of synergism of MG on TNF-induced cell death in the mock clones is comparable to that of parental L929 cells, which we have described previously\textsuperscript{12}. However, upon overexpression of NF-κB p65, the cells became extremely sensitized for TNF-induced cell death in the presence of MG. The average fold increase in TNF-induced cell death was 4.6 in the p65 clones in the induced condition, compared to an average of 1.5 in the mock clones. Furthermore, the fold increase in TNF-induced cell death caused by MG was not considerably different in the induced and non-induced conditions in the mock clones, while the synergistic effect of MG on TNF-induced cell death in the p65 clones was even more pronounced upon induced overexpression of p65.

L929 clones that overexpressed the p65 C38A mutant protein were also generated. These clones could still be sensitized by MG for TNF-induced cell death to an extent similar to that of the L929 clones overexpressing the p65 WT protein (data not shown). Similar to the WT p65 clones, untreated cells expressing the p65 C38A protein also had a high basal NF-κB activity as measured by DNA-binding, because mutant p65 still binds DNA, but to a lesser extent than the WT protein (see Fig. 6B). High basal NF-κB activity (either in p65WTclones or p65 C38A clones) leads to induction of its target genes - a number of anti-apoptotic genes (among others) - before the cells are even treated with TNF. This makes them more resistant to TNF-induced cell death, but at the same time these cells are much more sensitized by MG for TNF-induced cell death. These results indicate that the sensitizing effect of MG on TNF-induced cell death in cells with constitutive NF-κB activity cannot be explained solely by inhibition of the p65 DNA-binding activity, but that MG may also act on NF-κB target proteins that play a role in conferring resistance to TNF-induced cell death. Yet, the nature of these target proteins remains to be determined.

In summary, these data indicate that more NF-κB activation leads to stronger synergistic action of MG on TNF-induced cell death. This is particularly interesting for anti-tumor therapy, as many tumors have constitutive NF-κB activity that confers resistance to chemotherapy- or radiotherapy-induced cell death\textsuperscript{27}. Therefore, agents that could increase the intracellular concentration of MG, such as inhibitors of the non-phosphorylated form of GLO1, may increase the therapeutic effectiveness when used in combination with TNF-, chemo- or radio-therapies.
5. Discussion

In the current study, we show that methylglyoxal inhibits TNF-induced NF-κB DNA binding and NF-κB-dependent reporter gene expression in a concentration-dependent manner. Furthermore, we provide evidence that this MG mediated inhibition of TNF-induced NF-κB DNA binding is not due to inhibition of the degradation of its inhibitor IκBα. At low concentrations, MG had no effect on the TNF-induced degradation of IκBα, but the NF-κB-dependent re-synthesis of IκBα was already inhibited. However, at higher concentrations, MG caused a delayed degradation of IκBα, but the degradation was not inhibited because no residual amount of IκBα remained after 1 h of TNF treatment. At this time point, the TNF-induced NF-κB-dependent re-synthesis of IκBα was strongly inhibited in the presence of higher concentrations of MG. A conceivable explanation would be that MG had an inhibitory effect on IKKβ, the catalytic subunit of the kinase that is responsible for the phosphorylation of IκBα, because the TNF-induced activation of IKKβ can be inhibited by NO through S-nitrosylation of Cys residue 179 of IKKβ. Therefore, it would be plausible that MG could also modify this residue and interfere with the phosphorylation of IκBα. However, this explanation is not in line with our data, because the phosphorylation of IκBα was not affected by the higher concentration of MG, nor was the TNF-induced phosphorylation of p65 on Ser 536 affected, which is also mediated by IKKβ. A possible explanation for the delayed degradation of IκBα could be stabilization of the IκBα protein, an effect that has also been observed in the presence of nitric oxide. The delayed degradation of IκBα by MG was also reflected by a delayed translocation of NF-κB to the nucleus. Significantly, the DNA-binding activity of the NF-κB p65 subunit was strongly inhibited in the presence of MG, even when the NF-κB p65 subunit was fully clustered in the nucleus upon TNF stimulation. Furthermore, we show that the conserved Cys 38 residue that is located in the DNA binding loop of p65 is involved in the MG-mediated inhibition of the DNA binding activity of p65. The p65 C38A mutant protein was 50% more resistant, compared to the WT protein, to inhibition by MG. The fact that the C38A mutant could still be partially inhibited by MG might be due to MG-modification of Arg or Lys residues, which are located in the DNA-binding loop and involved in the specific contacts with DNA. The DNA binding region containing the Cys 38 residue is conserved in all NF-κB family members and makes many of the specific contacts with the NF-κB motif DNA. The Cys 38 residue contacts a phosphate in the DNA backbone and is responsible for the redox regulation of NF-κB. This conserved Cys residue needs to be in the reduced state to bind DNA. For instance, it has been shown that NO can also inhibit the DNA binding activity of NF-κB p50 by NO-modification of the Cys 62 residue. Furthermore, thioredoxin regulates the DNA-binding activity of NF-κB by reduction of a disulphide bond involving this conserved Cys residue. Because methylglyoxal can react with Cys residues in proteins to form a hemithioacetal, it is quite conceivable that MG directly modifies the Cys 38 residue in NF-κB p65, thereby inhibiting the contact with DNA. Our results are also in line with the recently described inhibitory effects of Plumbagin on the TNF-induced NF-κB activation. Plumbagin is a potential...
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anti-cancer agent (derived from the medicinal plant Plumbago zeylanica) that inhibits the TNF-induced NF-κB p65 binding to DNA. This inhibition is also mediated through the conserved Cys 38 residue in the DNA binding loop of p65.

Our data are also in line with recent data obtained from yeast, where it has been shown that MG directly modifies Cys residues in the oxidative-stress responsive transcription factors Yap1 and Pap1 in S. cerevisiae and S. pombe, respectively. Yap1 is the functional homologue of mammalian AP-1, whose DNA-binding activity also depends on the redox regulation of a conserved Cys residue. Direct MG modification of Yap1 is sufficient for translocation to the nucleus and activation of its target genes, thus indicating that MG regulates the transcription factor positively. Furthermore, in retinal Müller cells, increased concentrations of methylglyoxal, caused by hyperglycemia, have been linked to increased expression of angiopoietin-2. This is caused by MG modification of the co-repressor mSin3A. All these data point to a general role for methylglyoxal in the regulation of transcription factors.

It is worth noting that the promoter region of human glyoxalase 1 contains consensus sites for NF-κB, as well as for AP-1, suggesting that MG may play a role in the redox regulation of these transcription factors in both physiological and pathophysiological processes. Furthermore, the concentrations of MG needed to inhibit the DNA binding of NF-κB p65 could be in the physiological range, as it has been reported that intracellular concentrations of MG in normal growing cells can be up to 300 µM. In addition, in plasma of diabetic patients, MG concentrations of up to 400 µM have been reported. In L929 cells, partial (20%) inhibition of p65 DNA binding was observed at 100 µM MG. Maximum inhibition (±80%) in these cells is observed at 750 µM, while in HUVEC and HeLa cells no more than ±45% inhibition could be obtained at high concentrations of MG. Thus, the sensitivity of NF-κB p65 to MG seems to be strongly cell-type dependent and may be dependent on the expression levels of glyoxalase 1, the enzyme responsible for detoxification of MG. For the moment, it remains to be determined whether MG-modified p65 occurs in vivo. Currently, the detection of this is hampered by a lack of the proper tools such as antibodies that specifically recognize MG-modified p65 on residue Cys 38.

More and more, NF-κB is being considered an important target for cancer therapy, as its activation is one of the major obstacles on the road to tumor cell death (reviewed in ). For instance, it has been shown that tumors with constitutive NF-κB activity usually show increased resistance to radiation and chemotherapy. Inhibition of NF-κB not only leads to enhanced apoptosis but also to increased sensitivity to radiation and chemotherapy. Furthermore, in this paper we show that increased NF-κB activation, induced by overexpressing p65, leads to stronger synergistic action of MG on TNF-induced cell death. Our data suggest that the sensitizing effect of MG on TNF-induced cell death in cells with constitutive NF-κB activity cannot be explained solely by inhibition of the p65 DNA-binding activity, but that MG may also act on NF-κB target proteins that play a role in conferring resistance to TNF-induced cell death. In addition, our data may
explain why overexpression of GLO1 is involved in resistance to chemotherapy-induced tumor cell death. Overexpression of GLO1 leads to excessive detoxification of MG, and thus to concentrations that are too low to modify NFκB and inhibit its DNA binding activity. Therefore, agents that could increase the intracellular concentration of MG, such as inhibitors of GLO1, may increase the therapeutic effectiveness when used in combination with TNF-, chemo- or radio-therapies.

In conclusion, the data presented in this report indicate that the glycolytic metabolite methylglyoxal provides another control mechanism for modulating the expression of NF-κB responsive genes. Furthermore, MG may be responsible for tipping the balance towards TNF-induced cell death by suppressing NF-κB activation and/or by acting on NF-kB target proteins. This may be one of the molecular mechanisms responsible for the deteriorating effect of methylglyoxal and its role in the pathophysiology of several diseases.

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8. Unpublished results

8.1. Validation of our results in endothelial cells

In the previous paper we showed that NF-κB DNA binding can be inhibited by methylglyoxal in L929 and that this inhibition is probably due to direct modification of NF-κB. Although we already validated our results in ‘human umbilical vein endothelial cells’ (HUVEC), we also wanted to verify these results in aortic endothelial cells as these cells are primary targets in the development of diabetic vascular complications. For the assays we made use of primary bovine aortic endothelial cells (bAEC) and GM7373 (a bovine aortic endothelial cell-line) and used the same protocols as described in the paper. bAEC and GM7373 were cultured in minimum essential medium (MEM), supplemented with fetal calf serum (10% v/v), penicillin (100 units/ml), streptomycin (0,1 mg/ml) and non-essential amino acids at 37°C in a humidified incubator under an 5% CO₂ atmosphere.

First we measured TNF induced NF-κB DNA binding after incubating bAEC with increasing concentrations of methylglyoxal (Figure 1). bAEC were grown until confluency and then TNF treated with or without methylglyoxal. As expected, the TNF induced p65 DNA binding was drastically reduced upon methylglyoxal treatment. Low concentrations of methylglyoxal did not influence DNA binding but higher concentrations (starting from 100µM on) diminished p65 DNA binding. Note that these concentrations are probably within physiological range for diabetes since concentrations of methylglyoxal up to 400µM are found in diabetic patients.

![Fig. 1 - Methylglyoxal inhibits TNF-induced NF-κB p65 DNA binding. bAEC were incubated with TNF (1000 U/ml) for 1 h in the presence of increasing concentrations of MG. Control cells were treated with MG alone. Cellular extracts were prepared as described in materials and methods. The DNA-binding activity of NF-κB was determined by a colorimetric NF-κB p65 Transcription Factor Assay (Chemicon®). Absorbance was measured at 450 nm. The figure shows a representative experiment. White bars: control treated cells; black bars: TNF-treated cells.](image-url)
Additionally we also transfected GM7373 cells with the synthetic, NF-κB responsive reporter construct which is described in material and methods. GM7373 were transfected by means of lipofectin (Invitrogen) according to the manufacturer’s instructions. One day after transfection we treated these cells for 3H with TNF and increasing concentrations of methylglyoxal. As is evident from Figure 2, TNF treatment of control cells resulted in a nearly twofold increase in expression of the NF-κB responsive reporter. However, upon methylglyoxal treatment, this TNF induced increase was completely abolished, similar to L929 cells.

While L929 cells die from necrosis following TNF treatment 2, endothelial cell die from apoptosis 3. Thus, although signaling to NF-κB activation in L929 is not inhibited by methylglyoxal, it could not be excluded that methylglyoxal inhibits TNF induced NF-κB activation in endothelial cells, since TNF induces another pathway in these cells. Therefore TNF induced IkBα breakdown and NF-κB dependent re-synthesis was examined in bAEC. As shown in Figure 3, IkB was rapidly degraded in control cells after TNF treatment, and resynthesis was evident starting from 60 minutes after TNF treatment. However, in the presence of 100µM or 750µM methylglyoxal, IkBα breakdown was delayed but not inhibited. This demonstrates that the TNF induced NF-κB activation is not inhibited in endothelial cells upon methylglyoxal treatment. In addition, after incubation with 100µM methylglyoxal, IkBα resynthesis is also not inhibited but instead delayed, like we observed in L929 cells. However, after treatment with 750µM methylglyoxal, re-synthesis of IkBα could not be detected within the 90 minutes following TNF treatment. This points to a
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strong and persistent inhibiting effect of methylglyoxal. Yet, inhibited IκBα re-synthesis could also be due to a delayed transport of NF-κB to the nucleus, a phenomenon which we also observed in L929. Normally after 90 minutes of TNF treatment, NF-κB is fully clustered to the nucleus (Figure 4). At the same timepoint, treatment with 750µM methylglyoxal decreased transport to the nucleus but did not inhibit it. These data show that upon treatment with 750µM methylglyoxal, IκBα resynthesis is inhibited, even when NF-κB is present in the nucleus, pointing to a strong inhibition of NF-κB DNA binding.

Fig. 3 - Effects of methylglyoxal on the TNF-induced degradation of IκBα and NF-κB-dependent re-synthesis of IκBα. bAEC were incubated with TNF for the indicated time points in the absence and presence of 100 and 750 µM MG. Total cell extracts were probed for IκBα by Western blotting.

Fig. 4 - Methylglyoxal delays, but does not inhibit, the TNF-induced nuclear translocation of NF-κB p65. bAEC were incubated with TNF 90 minutes in the absence and presence of 100 or 750 µM MG. Samples were prepared for immunocytochemistry using anti-p65 antibody. Representative images are shown. The immunofluorescence of the NF-κB p65 subunit is shown in the left panels, and the overlay with the nucleus is shown in the right panels.
Together, these results suggest that methylglyoxal induces a similar inhibition of p65 NF-κB DNA binding in endothelial cells as in L929 and that the inhibition is thus not cell-line specific.

8.2. References


General Discussion
1. The diabetic endothelium

Diabetes is becoming one of the world’s most important diseases and this has much to do with our modern lifestyle characterised by inactivity, high blood pressure, (over-) consumption of foods rich in fat... As mentioned before, there are mainly two types of diabetes. Type 1 diabetes is characterised by a destruction of the insulin producing pancreatic beta-cells. In type 2 diabetics, the beta cells (initially) produce enough insulin, but the body has become resistant to the action of the hormone. Insulin is important for muscle cells and adipocytes, since these cells take up glucose from the blood in response to insulin. So when insulin production is impaired or when fat and muscle cells become resistant to the hormone, glucose uptake is diminished and the sugar remains in the blood (called hyperglycaemia). This hyperglycaemia affects first of all endothelial cells, because they delineate the blood vessel and are directly exposed to high blood sugar levels. Normally, endothelial cells prevent undesirable leakage of metabolites and nutrients from the blood vessel into the underlying tissue by forming a tight barrier. However, their function is not limited to this barrier function as they also play an important role in blood clotting, inflammation and in regulating blood flow through the secretion of vasoconstrictors like endothelin (ET) and vasodilators like nitric oxide (NO). The production of both of these molecules is induced by insulin. Since these two molecules have opposing effects, the production of them needs to be balanced to guarantee normal blood flow. Any disturbance of this balance may lead to endothelial dysfunction and eventually to the development of vascular complications. When endothelial cells, in addition to fat and muscle cells, become resistant to the action of insulin, the balance between ET and NO is disturbed as NO production is impaired resulting in vascular occlusion. As a consequence, blood sugar levels rise even more as less glucose is transported to fat and muscle cells. Besides insulin resistance, hyperglycaemia itself may also underlie endothelial dysfunction since hyperglycaemia also inhibits production of NO and also gives rise to the formation of AGEs, which are known to perturb normal cellular function.

However, diabetes is not only associated with hyperglycaemia and insulin resistance but also with a low grade inflammation, which may also contribute to endothelial dysfunction. For instance, it is known that in endothelial cells the pro-inflammatory cytokine TNF induces insulin resistance, vascular leakage and apoptosis. Also, TNF is a powerful activator of the transcription factor NF-kB which induces the expression of cellular adhesion molecules (CAMs) attracting inflammatory cells. In a previous study it was reported that in a fibrosarcoma cell-line (L929), TNF induces the formation of a specific methylglyoxal derived AGE, directly or indirectly mediated by phosphorylated glyoxalase. Glyoxalase normally detoxifies the cytotoxic metabolite methylglyoxal, which is the main precursor of AGEs. Since both TNF and AGEs contribute to endothelial dysfunction, we wanted to investigate whether TNF could also induce the formation of methylglyoxal derived AGEs in endothelial cells. Additionally, we also wanted to investigate the role of phosphorylated glyoxalase in this process.
2. TNF induced methylglyoxal modification of specific proteins

In part 3 of this thesis, we investigated the relationship between TNF and the formation of AGEs in primary bovine aortic endothelial cells (bAEC). First of all, we found that TNF induces a rapid, but transient phosphorylation of glyoxalase1. Similar as in L929, this phosphorylation could be blocked by H89, an inhibitor of PKA. These findings suggested that in endothelial cells, a similar mechanism could be active as in L929. Indeed, a first set of experiments confirmed this hypothesis. Through immunoblotting techniques, we demonstrated that TNF stimulation of bAEC cells leads to the formation of a TNF specific methylglyoxal derived argpyrimidine AGE-epitope, similar as in L929 cells. In a second set of experiments, through labelling our cells with $^{14}$C methylglyoxal, we observed the formation of a second TNF specific methylglyoxal modified protein with an apparent weight of 120kDa. In the same set of experiments, TNF also induced methylglyoxal modification of a third protein with an apparent weight of 45 kDa. Yet, this modification was already slightly present in unstimulated cells. We failed however to indentify these three proteins, because we could not separate and visualise these proteins on 2 dimensional (2D) gel electrophoresis. First of all, the mab6b antibody that recognises argpyrimidine epitopes does not recognise any epitope on 2D gels, and secondly the lysis buffer used in 2D gel electrophoresis removes most of the $^{14}$C methylglyoxal label from proteins (our own observations).

Next, we wanted to further investigate the role of phosphorylated glyoxalase1 in the formation of methylglyoxal derived AGEs, by inhibiting phosphorylation of glyoxalase1 with PKA inhibitor H89. Unlike in L929 cells, we could not block methylglyoxal modification on the $^{14}$C methylglyoxal labelled protein by means of H89. This indicated that phosphorylated glyoxalase1 is probably not involved in the methylglyoxal modification of this specific protein. These findings also show that, although the two models, L929 and endothelial cells, share a same feature, i.e. TNF-induced MG-AGE formation, the mechanism that underlies this event is different. To date, the exact reason for this discrepancy is not known and we can only speculate on this subject. First of all, L929 and endothelial cells have different responses to TNF, namely necrotic cell death in L929, and gene activation in endothelial cells. Also, the TNF induced modifications of glyoxalase1 are also different. In L929, TNF induces prolonged phosphorylation of glyoxalase1 (more than 1.5h), while in endothelial cells, the phosphorylation is only transient (<20 min). Lastly, the molecular weight of the methylglyoxal modified proteins identified is different in L929 and endothelial cells, indicating that methylglyoxal modification is cell type specific. Although phosphorylated glyoxalase1 is probably not involved in the formation of the $^{14}$C methylglyoxal labelled protein, we cannot exclude the possibility that phosphorylated glyoxalase1 is involved in the formation of other MG-AGEs that we could not detect with our experimental settings.

It thus largely remains a mystery how exactly TNF induces specific MG-AGE formation in endothelial cells. Yet, there might be a common element in TNF induced AGE formation in L929 and endothelial cells. In L929, TNF is unable to induce specific AGEs
when treated with the glycolytic inhibitor 2-deoxyglucose, suggesting an important role for glycolysis in specific AGE formation. In this thesis, we showed that, in endothelial cells, TNF stimulated glycolysis by increasing glucose uptake by about 40% and increasing levels of fructose-2,6-bisphosphate (F2,6BP) by 40-50%. F2,6BP is a powerful activator of the glycolytic PFK1 which is a key enzyme in the glycolysis. Since methylglyoxal is a sideproduct of the glycolysis, it is very likely that increased glycolytic flux also increases methylglyoxal formation. Although we did not succeed in measuring methylglyoxal concentrations directly, we did see an increase in the formation of D-Lactate, in which methylglyoxal is detoxified by the glyoxalase system. This increase in D-Lactate formation could not be attributed to changes in the glyoxalase1 activity, since the latter was not changed upon TNF treatment. Therefore, we assume that the increase in D-Lactate production simply reflects an increased flux of methylglyoxal through the glyoxalase system and thus reflects increased intracellular levels of methylglyoxal. Similarly, it has been shown that in red blood cells, increased flux to D-lactate can be attributed to increased methylglyoxal formation. Taken together, these results indicate that TNF increases concentrations of methylglyoxal and that this increase is not due to inhibition of glyoxalase1. Instead, the increase in methylglyoxal production is probably due to increased glycolytic flux.

With the mab6b antibody (raised against in vitro methylglyoxal-modified keyhole limpet hemocyanin), we clearly saw the formation of one specific AGE upon TNF treatment. However, hyperglycaemia itself also induced AGE formation on that protein. Since both TNF and hyperglycaemia increase methylglyoxal formation, this hinted us to the fact that the TNF induced AGE formation is probably due to increased methylglyoxal production. However, when we labelled our cells with $^{14}$C-MG we again observed that TNF induces MG-AGE formation on other specific proteins, but this time, hyperglycaemia alone did almost not affect MG-AGE formation. This finding demonstrates that in this case, AGE formation is TNF specific and probably does not depend on increased MG formation. To date, we do not know how TNF induces the formation of specific MG-AGEs and we can only speculate on this subject. A first possibility is that the specific AGE reflects increased translation and consequently increased transcription of the targeted protein. In that case, the protein we identified could already be modified by methylglyoxal under control conditions, and TNF stimulation would just make the modified protein more apparent. Yet, treatment with cycloheximide (CHX), an inhibitor of translation, did not inhibit the TNF induced formation of this specific AGE (data not shown). Similar to L929 cells, it is also possible that the AGE formation occurs enzymatically, but in that case glyoxalase1 is probably not involved. A last possibility is that TNF induces a conformational change in the protein, exposing a site which is sensitive for methylglyoxal modification.

Question remains however how TNF is able to increase glycolytic flux and glucose uptake. Almeida and co-workers showed that nitric oxide (NO) is able to induce an increase in F2,6BP levels in astrocytes. They argued that NO mediated inhibition of
cytochrome c oxidase, leads to activation of AMPK, which is known to activate glycolysis when AMP/ATP levels are high \(^{18,19}\). AMPK in turn activates phosphofructokinase2 (PFK2) which catalyses the formation of F2,6BP from fructose-6-phosphate. A similar mechanism has been proposed in endothelial cells in which nitric oxide inhibits cytochrome c oxidase, leading to oxidative stress and activation of AMPK \(^{20}\). NO plays crucial roles in endothelial physiology and is produced by endothelial NO synthase (eNOS) which is shown to be activated by TNF \(^{21}\). It is thus tempting to speculate that TNF increases NO production in endothelial cells, thereby inhibiting cytochrome c oxidase, leading to oxidative stress and activation of AMPK. AMPK can then subsequently activate PFK2, resulting in increased formation of F2,6BP and activation of glycolysis through stimulation of PFK1. This view is partially supported by our finding that glucose uptake is also increased. After all, it has been demonstrated that AMPK enhances glucose transport in cells that only express glucose transporter 1 (GLUT1) \(^{22}\), which is also the main glucose transporter in endothelial cells.

An interesting observation was that TNF is able to increase the glycolytic flux regardless of the glycaemic state. So even under hyperglycaemic condition, in which glycolysis is already upregulated, TNF is able to increase glycolytic flux even more. This is probably because hyperglycaemia increases glycolytic flux by increasing the workload and that in contrast, TNF induces an enzymatic activation.

### 3. NF-κB : a novel MG-AGE?

In part 4 of this thesis we showed that methylglyoxal is a powerful modulator of TNF induced NF-κB activation. Our results indicated that methylglyoxal inhibits NF-κB DNA binding, possibly by a direct modification of the conserved cysteine38 residue. This research was mainly done in L929 cells, a fibrosarcoma cell-line, but we provided evidence that this model is applicable in other cell-lines, including (primary) endothelial cells. Furthermore we showed that this methylglyoxal mediated inhibition of NF-κB DNA binding and NF-κB dependent reporter gene expression is not due to inhibition of the degradation of its inhibitor IkBα, nor to inhibited transport to the nucleus of NF-κB, nor to inhibited transactivation of p65. However, TNF induced IkBα breakdown in the presence of methylglyoxal, is delayed which is also reflected in a delayed transport of NF-κB to the nucleus. This could be explained by stabilisation of the IkBα protein which is also seen in the presence of NO \(^{23}\). Furthermore, we showed that the conserved Cys 38 residue, which is located at the DNA binding loop of p65, is involved in the methylglyoxal induced inhibition of the NF-κB DNA binding capacity. After all, the p65 C38A mutant was 50% more resistant to inhibition by methylglyoxal compared to the WT protein. Methylglyoxal is known to interact with cysteine residues. For instance it reacts with glutathione forming a hemithio-acetal, the substrate of the glyoxalase system. It seems thus reasonable to speculate that methylglyoxal modifies this Cys 38 residue directly. This is in line with other papers in which was suggested that methylglyoxal modifies cysteine residues in transcription factors YAP1 and Pap1 in S.cerevisiae and S. pombe \(^{24,25}\).
NF-κB activation in endothelial cells is very much like a double edged sword: on the one hand it induces a ‘pro-inflammatory’ response and on the other hand a ‘protective’ response. The ‘pro-inflammatory’ response is associated with NF-κB induced transcription of i.e. chemokines and adhesion molecules (like ICAM and VCAM) that attract and activate leukocytes resulting in a local inflammation \(^{11,26}\). This type of response is for instance activated when there is a viral or bacterial infection. Normally, endothelial cells are fairly resistant to this type of response \(^1\), and in fact one does not want to inhibit this process since it warrants protection against infections. However, persistent NF-κB activation during massive or chronic inflammation (like in diabetes) may eventually lead to endothelial cell death \(^{27}\) and thus vascular leakage. The ultimate goal of endothelial cells is of course survival, and therefore these cells induce the transcription of several protective genes like A20, Bcl2 and BcL-X\(_L\) \(^{28,29}\). These genes are NF-κB responsive \(^{30-32}\) and at the same time inhibit the action of NF-κB in endothelial cells \(^{29,33}\). This process is referred to as the ‘protective’ response.

Methylglyoxal mediated inhibition of NF-κB DNA binding in endothelial cells could thus be beneficial during chronic inflammation, since this may decrease ICAM and VCAM expression leading to decreased inflammation. However, it has been shown that inhibition of NF-κB renders endothelial cells susceptible for TNF induced apoptosis due to decreased transcription of anti-apoptotic genes \(^{34-36}\). Therefore, methylglyoxal mediated inhibition of NF-κB DNA could sensitise endothelial cells towards TNF induced cell death. This view is supported by the fact that methylglyoxal alone is also able to induce apoptosis in several cell-lines \(^{37,38}\).

In an attempt to demonstrate this sensitising effect of methylglyoxal, we overexpressed WT p65 and the C38A mutant in L929 cells. In a previous study, it was reported that methylglyoxal is synergistic with TNF induced cell death in these cells \(^{12}\). And although these cells die necrotically, inhibition of NF-κB or treatment with a protein synthesis inhibitor enhances cell death in these cells \(^{39-41}\). If our hypothesis was true and methylglyoxal sensitises cells to TNF induced cell death by inhibiting NF-κB DNA binding, then cells overexpressing the C38A mutant should be more resistant to the sensitising effect of methylglyoxal in TNF induced cell death than cells that overexpress the WT protein. However upon overexpression of the WT or the C38A mutant protein, L929 were extremely sensitive to TNF induced cell death in the presence of methylglyoxal. It is known that overexpression of NF-κB automatically leads to NF-κB activation and thus NF-κB dependent transcription. Thus, even before cells are treated with TNF, the cells already transcribed, amongst others, several anti-apoptotic genes. This should make these cells resistant to TNF induced cell death. Yet, we noticed that these cells were extremely sensitive to methylglyoxal, indicating that methylglyoxal may also act on other proteins induced by NF-κB, rendering the cells sensitive to methylglyoxal upon TNF treatment.
4. Model of TNF induced endothelial apoptosis and dysfunction

Besides TNF, methylglyoxal is also known to induce apoptosis in several mammalian cells including endothelial cells, and several groups have pointed to a role of p38\textsuperscript{37,38,42}. Our results suggest that TNF is able to increase methylglyoxal levels very rapidly (within minutes) suggesting a possible interplay between TNF and methylglyoxal in the apoptotic signalling. In that regard, we propose the following model (see Figure). Binding of TNF to its receptor increases glycolytic flux and glucose uptake. As discussed above, this could be due to TNF stimulated production of NO by eNOS and inhibition of cytochrome c oxidase, leading to ROS and activation of AMPK. AMPK in turn increases glucose uptake and activates the F2,6BP producing enzyme PFK2. As methylglyoxal is a sideproduct of the glycolysis, increased flux through this pathway is probably responsible for the accumulation of methylglyoxal. This cytotoxic metabolite then signals to cell death via the p38 kinase pathway and also gives rise to the formation of AGEs, which are known to induce endothelial dysfunction\textsuperscript{3}. While increased concentrations of methylglyoxal alone are sufficient to modify some proteins\textsuperscript{13}, we have shown here that TNF can also induce the formation of specific AGEs. One of the proteins modified by methylglyoxal is possibly NF-κB as we have shown here. Methylglyoxal modification of NF-κB inhibits its DNA binding capacity, possibly resulting in a decreased transcription of anti-apoptotic genes and sensitisation to TNF induced cell death. In this view, methylglyoxal may amplify the apoptotic effect of TNF by inhibiting NF-κB DNA binding and producing AGEs.

As mentioned in the introduction (page 11), hyperglycaemia activates four pathways underlying endothelial dysfunction, including the formation of AGEs and activation of the PKC, the polyol and the hexosamine pathway. These pathways are all initiated by oxidative stress and subsequent inhibition of the glycolytic enzyme GAPDH. Since TNF triggers oxidative stress in endothelial cells\textsuperscript{9,43} and since TNF is also able to increase glycolytic flux as we have shown here, it is tempting to speculate that TNF probably also activates these four pathways, although this needs further investigation. Moreover, we showed that TNF is able to activate the glycolysis even under hyperglycaemic conditions. This indicates that TNF could amplify the deleterious effects of hyperglycaemia.

5. Conclusions

Here we show that TNF increases glycolytic flux in endothelial cells, leading to accumulation of methylglyoxal and the formation of specific AGEs. Moreover, we also showed that TNF is able to induce MG-AGE modifications on specific proteins. Our results indicate that TNF increases the glycolytic flux even under hyperglycaemic conditions, suggesting that TNF could worsen hyperglycaemic damage, by mimicking the effects of hyperglycaemia on the glycolysis. Furthermore, we demonstrated that NF-κB can be considered as a new AGE, and that AGE modification of this protein results in impaired DNA binding, which could enhance the apoptotic effect of TNF. Especially since NF-κB induces the transcription of several anti-apoptotic genes. These data point to a new
General Discussion

Hypothetical mechanism of TNF induced MG-AGE formation and possible cell death. Binding of TNF to its receptor activates endothelial NO synthase (eNOS). NO then possibly inhibits cytochrome c oxidase leading to mitochondrial stress and ROS formation. ROS in turn activates AMPK which increases glucose uptake and activates PFK2. PFK2 mediates the formation of F2,6BP which is a powerful activator of the glycolytic PFK1. Increased glucose uptake and increased glycolytic flux might additionally lead to increased oxidative stress in the mitochondria. Besides activating AMPK, ROS also inhibits the glycolytic GAPDH, resulting in accumulation of all upstream intermediates, including GAP and DHAP, two precursors of methylglyoxal. Increased methylglyoxal levels then lead to AGE formation, which has diverse effects on cellular function. How TNF contributes to the formation of specific MG-AGEs is not clear at the moment. One of the AGEs formed by methylglyoxal is possibly NF-κB, and methylglyoxal modification of this protein leads to decreased DNA binding and gene expression. NF-κB transcribes several anti-apoptotic genes and inhibition of its DNA binding may thus sensitize endothelial cells to cell death. The glycolysis is in blue color, the TNF induced pathway in green, the methylglyoxal induced pathways in red and the oxidative stress induced pathway in brown.
role for TNF and methylglyoxal in the development of vascular complications. Moreover, our results provide a novel link between inflammation and hyperglycaemia and link TNF induced endothelial dysfunction with the formation of specific MG-AGEs.
6. References


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Linking TNF-\alpha-induced dysfunction and formation of methylglyoxal derived AGEs in endothelial cells

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