α-Dicarbonyls and degradation of ascorbic acid induce glycation and interfere with neural and immune cell function

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Abstract

One of the hallmarks of ageing is the diminishing capability of cells and tissues to compensate for protein damage and accumulation. Glycation and the generation of advanced glycation endproducts (AGEs) represent a growing field in ageing research, as they have been associated to most age-related diseases. Neurodegenerative conditions as well as dysregulation of the immune system are etiologically connected to the accumulation of AGEs. The strongest glycation reactions are frequently observed after treatment with highly reactive α-dicarbonyls like methylglyoxal (MGO) or glyoxal (GO). Ascorbic acid (AA), commonly known as vitamin c, is a carbohydrate with high abundancy throughout the whole body. Its degradation is only partially reversible, forms reactive intermediates including dicarbonyls and could therefore contribute to generation of AGEs in the ageing brain. In this work, the role of ascorbic acid in glycation and its effects on neuronal differentiation were investigated. We could show that ascorbic acid and dehydroascorbic acid are able to induce glycation of PC12-cells. Furthermore, they interfere with neurite outgrowth and differentiation. To elucidate the effects of glycation on neuronal cell proteostasis, we investigated protein turnover of two major neuronal cell surface molecules, the neuronal cell adhesion molecule (NCAM) and the receptor for advanced glycation endproducts (RAGE) after glycation. Polysialylation as a specific PTM of NCAM was accessed, in order to differentiate between enzymatic and non-enzymatic glycosylation. We could show an increase in turnover of both NCAM and RAGE after MGO-induced glycation as well as an increased half-life of NCAM after polysialylation. The above stated changes in proteostasis might also take part in age-related impairment of innate and adaptive immunity. NK-cell cytotoxicity depends on cell to cell contact and the interaction of cell surface proteins. We could show a negative effect of α-dicarbonyl-induced glycation on NK-cell conjugate formation and target-lysis. Prolonged wound healing and decreased resilience against microbial pathogens represent additional effects of advanced age. Glycation of THP-1 macrophages with MGO led to increased levels of inflammatory cytokines and impaired phagocytic efficiency, indicating a negative effect of glycation on macrophage morphology and function.

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Referat

Eines der Hauptkennzeichen des Alterns ist die abnehmende Fähigkeit von Zellen und Geweben, Proteinschäden und -akkumulation auszugleichen. Glykierung und die Entstehung von advanced glycation endproducts (AGEs) stellen dabei ein wachsendes Feld der Alternsforschung dar. Sowohl neurodegenerative Erkrankungen, als auch eine zunehmende Dysregulation des Immunsystems hängen vermutlich mit der Akkumulation fehlerhafter Proteine und AGEs zusammen. Eine ausgeprägte Glykierung kann hierbei mithilfe reaktiver Dicarbonyle wie Methylglyoxal (MGO) oder Glyoxal (GO) induziert werden. Ascorbinsäure, besser bekannt als Vitamin C, ist ein im menschlichen Körper weit verbreitetes Kohlenhydrat. Ihr Abbau ist jedoch nur teilweise reversibel, bildet reaktive Dicarbonyle und könnte im alternden Gehirn zur Bildung von AGEs beitragen. In dieser Arbeit wurde ihre Rolle bei der Glykierung und ihre Auswirkungen auf die neuronale Differenzierung untersucht. Wir konnten zeigen, dass Ascorbinsäure in der Lage ist Glykierung von PC12-Zellen zu induzieren. Darüber hinaus verringerte sie das Neuritenwachstum und die Differenzierung. Die vorliegende Arbeit untersucht weiterhin den Proteinumsatz von zwei neuronalen Zelloberflächenmolekülen, dem neuronalen Zelladhäsionsmolekül (NCAM) und dem Rezeptor für advanced glycation endproducts (RAGE). Wir konnten eine Umsatzsteigerung sowohl von NCAM als auch von RAGE nach MGO-induzierter Glykierung nachweisen. Die oben beschriebenen Veränderungen der Proteostase könnten ebenfalls an altersassoziierten Schäden des angeborenen und adaptiven Immunsystems beteiligt sein. Die Cytotoxizität von NK-Zellen hängt stark vom Zell-zu-Zell Kontakt und der Interaktion von Oberflächenmolekülen ab. In dieser Arbeit wird ein negativer Effekt von Dicarbonyl-induzierter Glykierung auf Konjugatbildung und Cytotoxizität von NK-92-Zellen dargestellt. Neben den genannten Mechanismen stellen eine gestörte Wundheilung und eine verringerte Abwehr gegen Mikroben weitere Folgen von Immunoseneszenz dar. Die Inkubation von THP-1 Makrophagen mit MGO führte zu einer verstärkten Expression pro-inflammatorischer Interleukine und einer verminderten Phagozytose, was auf einen möglichen negativen Effekt von Glykierung auf die Morphologie und Funktion von Makrophagen hindeutet.

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Contents

Abstract II
Referat III
Abbreviations VI
1 Introduction 1
1.1 Glycation 1
1.1.1 Definition 1
1.1.2 Targets, mediating factors and effects of glycation 1
1.1.3 Reactive α-dicarbonyls as potent glycating agents 2
1.2 Ascorbic acid 2
1.2.1 Definition 2
1.2.2 Redox cycle 2
1.2.3 Degradation of dehydroascorbic acid 3
1.2.4 Transport and Distribution 4
1.3 Neuronal differentiation and plasticity 4
1.3.1 Neuronal plasticity 4
1.3.2 PC12-cells as a model for neuronal differentiation 5
1.4 Cell surface molecules of the immunoglobuline-superfamily 5
1.4.1 NCAM (CD56) 5
1.4.2 RAGE 6
1.5 The monocyte/macrophage system 8
1.5.1 Ontogeny, morphology and function 8
1.5.2 Immunosenescence and “inflammaging” as manifestations of immunological dysregulation 9
1.5.3 Glycation in the context of immunosenescence and “inflammaging” 10
1.6 Natural killer cells 11
1.6.1 Ontogeny, morphology and function 11
1.6.2 NK-92 cells 12
1.6.3 NK-cells and immunosenescence 12
1.7 Aim of work 14
2 Discussion 15
2.1 Ascorbic acid and dehydroascorbic acid induce glycation of PC12-cells 15
2.2 Ascorbic acid decreases cell viability of PC12-cells 16
2.3 Ascorbic acid increases NGF-mediated phosphorylation of ERK1/2 16
2.4 Ascorbic acid and dehydroascorbic acid interfere with neurite outgrowth of PC12-cells. .. 17

2.5 Glycation of NCAM and RAGE via methylglyoxal increases protein turnover in PC12-cells and PC12-polySia-cells 17
2.6 Methylglyoxal and Glyoxal induce glycation of NK-92 cells 18
2.7 Glycation interferes with conjugate-formation and cytotoxic function of NK-92 cells 19
2.8 Methylglyoxal induces glycation of THP-1 macrophages 19
2.9 Glycation of THP-1 macrophages increases pro-inflammatory cytokine expression while interfering with phagocytic activity 20
3 References ..... **Fehler! Textmarke nicht definiert.**
4 Theses 33
Publications 34

Abbreviations

AA	ascorbic acid
ADCC	antibody dependent cytotoxicity
AGE	advanced glycation endproduct
BBB	blood-brain-barrier
BSA	bovine serum albumin
CD	cluster of differentiation
CEL	carboxyethyllysine
CLP	common lymphoid progenitor
CML	carboxymethyllysine
CRP	c-reactive protein
DHA	dehydroascorbic acid
DHAP	dihydroxyacetone phosphate
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GA3P	glycerinaldehyde 3-phosphate
GLUT	glucose-transporter
GO	glyoxal
GOLD	glyoxal-lysine dimer
GSH	glutathione
HIF	hypoxia inducible factor
HLA	human leucocyte antigen
IFN-γ	interferon-γ
IGF	insulin-like growth factor
Il-6	interleukin-6
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based activation motif
JAK/STAT	janus kinase/ signal transducer and activator of transcription proteins
KIR	killer-cell immunoglobulin-like receptor
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MG-H1	methylglyoxal-derived hydroimidazolone
MGO	methylglyoxal
MHC	major histocompatibility complex
MODIC	methylglyoxal-derived imidazolium crosslink

MOLD	methylglyoxal-lysine dimer
MTOC	microtubule-organizing centre
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor 'kappa light-chain-enhancer' of activated B-cells
NGF-β	nerve growth factor-β
PAMPs	pathogen-associated molecular patterns
PGE	prostaglandin
PI3K	phospoinositide 3-kinase
PLC	phospholipase C
polySia	polysialic acid
PTM	posttranslational modification
RAGE	receptor for advanced glycation endproducts
ROS	reactive oxygen species
RTCA	real-time cell analysis
SDA	semidehydroascorbic acid
STAT	signal transducers and activators of transcription
ST8Sia	α2,8-sialyltransferase
SVCT	sodium dependent vitamin c transporter
TGF	transforming growth factor
TIM-4	t-cell immunoglobulin mucin receptor-4
TNF-α	tumor-necrose factor-α
TLR	toll-like receptor
TrkA	tropomyosin kinase receptor A
α2,3-DKG	2,3-diketogulonic acid
3-DG	3-deoxyglucosone

1 Introduction

1.1 Glycation

1.1.1 Definition

Glycation, the well-known browning-reaction first described by Louis C. Maillard in 1912 is an undirected and non-enzymatic process which gained growing attention in clinical and ageing research over the last decades. The term describes a condensation reaction between the carbonyl group of a reducing carbohydrate (e.g. glucose, ribose or fructose) and a free amino group, which can originate from proteins, lipids or nucleic acids (*1*). The next step is the reversible formation of a Schiff-Base which can further rearrange, building a so-called Amadori-product. Irreversible modification of this Amadori-product ultimately results in the formation of advanced-glycation endproducts (AGEs). AGEs represent a large group of heterogenous molecules with distinct chemical and functional properties (*2*). Many have been identified up to date, this work however will concentrate on those generated in and around neurons, glial cells and cells of the innate immune system, highlighting AGEs connected to ascorbic acid degradation products. Ascorbic acid (AA) shares structural similarities to many common carbohydrates like glucose or fructose, which arises the question whether AA degradation may contribute to glycation *in vivo* in situations of impaired AA homeostasis. Previous research could display AA as a potent inducing agent of AGEs. Many AGEs generated through this pathway like carboxymethyllysine (CML) and Pentosidine belong to the more common ones and are well studied by now (*3, 4*).

1.1.2 Targets, mediating factors and effects of glycation

Not only the substrates, but also the surrounding milieu heavily influences the process of glycation. The redox state represents an important factor, since further oxidation of AGEs and dicarbonyl-intermediates as well as generation of reactive oxygen species account for many detrimental effects of the Maillard reaction in ageing tissues (*5*). Typical target molecules of glycation are long-lived proteins and peptides with free amino groups from lysine or arginine residues (*6*). Especially extracellular proteins seem to be susceptible to non-enzymatic modification. Laminin and type IV collagen, both highly abundant components of the extracellular matrix in neuronal tissue show altered structure after glycation, interfering with cellular adhesion and neurite outgrowth (*7–9*). It is tempting to speculate that this might be associated with a different extracellular enzymatic and non-enzymatic defensive system. Cells usually have the ability to prevent or alleviate glycation and oxidation via antioxidative agents including ascorbic acid, glutathione and a-tocopherol, multiple reductases as well as glyoxalases I and II (*10, 11*). On an extracellular level, different defense mechanisms might not be sufficient to

prevent long lived compounds like extracellular matrix proteins from receiving oxidative stress (*12*). Glycated proteins show different properties when compared to their non-glycated counterparts. They can be altered in structure, function and polarity, ultimately becoming defective (*2*). Cross-linking, accumulation and further oxidation of dysfunctional peptides are connected to the generation of neurodegenerative diseases like Alzheimers disease (*13, 14*). All of the mentioned processes have been shown to impair differentiation, neurite outgrowth, synaptogenesis and neuronal survival (*15, 16*).

1.1.3 Reactive α-dicarbonyls as potent glycating agents

Non-enzymatic carbohydrate-degradation leads to the generation of reactive dicarbonyl compounds. Among those MGO, 3-deoxyglucosone (3-DG) and GO represent some of the better investigated intermediates in the context of glycation. Although being less abundant in the extracellular and intracellular space than their predecessors, these reagents express a much higher glycating potential and require fast enzymatic decomposition (*17, 18*). The degradation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3AP) as intermediates of glycolysis and carbohydrate metabolism has been identified as the main source of MGO in humans (*19, 20*). Reactive dicarbonyls have been shown to modify both lysine and arginine residues. Many AGEs generated through this mechanism have been identified up to date, including MG-H1, CEL, CML, argpyrimidine, MOLD and MODIC (*21, 22*). As shown by Smuda et al., α-dicarbonyls are also generated by the degradation of ascorbic acid under physiological conditions. Interestingly, MGO has been identified as a possible byproduct of ascorbic acid degradation. (*4*).

1.2 Ascorbic acid

1.2.1 Definition

Ascorbic acid is a reducing 6-carbon backbone carbohydrate with a wide variety of functions in the human body. Being described first in 1928 by Albert Szent-Györgyi as “hexuronic acid” after isolation from adrenal gland and later identified by the working group of C. G. King as vitamin C, it quickly gained interest in medicine and food chemistry (*23, 24*). Most of the functions of AA arise from its ability to donate two electrons and two protons. Its water-solubility, the high abundancy throughout the whole body as well as a variety of possible transport pathways enable it to act as one of the most important antioxidants (*25*).

1.2.2 Redox cycle

As described above, AA can reversibly be oxidized and thereby donate two electrons and protons. The first oxidation step results in the formation of semidehydroascorbic acid (SDA), often also described as ascorbyl radical (*26*). Two molecules of SDA can

disproportionate into AA and dehydroascorbic acid (DHA). Further oxidation of SDA offers a second electron and leads to formation of DHA. Up to this point, the oxidation chain is reversible. DHA has a relatively short lifetime under physiological conditions and needs to be reduced quickly to prevent further irreversible degradation (*27, 28*). Similar to its precursor SDA, recycling of DHA is accomplished by enzymatic and non-enzymatic pathways. DHA can be reduced non-enzymatically via redox-coupling with GSH, resulting in the formation of AA and glutathione-disulfide (*29–31*). DHA can also be recycled by a variety of enzymes showing DHA-reductase activity. Most of these reductases rely on the presence of GSH, underlining its importance for maintaining the redox cycle of AA (*31–33*). One of the major advantages of this redox system lie in its thermodynamical properties. AA is described to be positioned at the “bottom of the pecking order” for oxidizing radicals due to its very low reduction potential (*30*). Together with the lipophilic vitamin E and glutathione it forms a potent antioxidative system (*34*). In aquaeous solutions at pH 7 and in the presence of oxygen AA has been described to auto-oxidize. However this process seems to be strongly dependent on the presence of catalytic metals (*35*).

1.2.3 Degradation of dehydroascorbic acid

If not reduced, DHA will undergo further irreversible degradation pathways. These steps do not require enzymatic catalysation and happen under physiological circumstances (*21*). Spontaneous decay of DHA has been evaluated by Bode et al.. They describe a half-life time of about 100 min at pH 7 and room temperature (23 °C). Higher pH values related to faster decay, while an acidic environment stabilized DHA. Fastest degradation was detected at temperatures of 45 °C, indicating that this process could be favoured at a physiological body temperature (*27*). Koshshiishi et al. investigated the rate of DHA degradation in rat plasma. They could show a rapid decay as well as rising levels of AA and 2,3-diketogulonic acid (2,3-DKG) as the main products of AA metabolism (*28*). The first step of irreversible DHA degradation is a non-enzymatic lactone-ring opening hydrolysis, which occurs under the above-mentioned circumstances and forms 2,3-DKG. This intermediate acts as the starting point for the majority of further fragmentation (*36*). The degradation chain of 2,3-DKG may be secluded in several distinct pathways. Among those β-dicarbonyl cleavage and oxidative α-dicarbonyl fragmentation seem to be the most important in vivo. Both result in different intermediates and induce the generation of a variety of AGEs. Interestingly, there has been evidence on the existence of ascorbic-acid-specific amide-AGEs, namely N6-xylonyl lysine, N6-lyxonyl lysine and N6-threonyl lysine. Detection of those amide-AGEs in vivo could lead to a better understanding of the role of DHA degradation in the generation of AGEs (*4*).

1.2.4 Transport and Distribution

The concentration of AA in tissues, extracellular space and cell types can differ strongly, depending on the specific demand for antioxidative compounds or other functions of AA. While plasma levels usually do not exceed 50-80 µM, much higher concentrations of up to 10 mM have been found in neurons from brain and adrenal gland (*37, 38*). To accomplish accumulation of AA in certain tissues, several transport mechanisms must allow for concentration gradients. An important role for regulation of AA transport plays the "switch" between its reduced and oxidized form, since both use distinct transport mechanisms (*39*). The reduced form is brought across cell membranes via sodium dependent SVCT1/2 transporters. They have initially been identified by Tsukaguchi in 1999. Both transporters are specific for L-ascorbic acid (*40*). SVCT1 is mostly expressed in epithelial cells, i.e. in kidney, liver and intestines. SVCT2 expression on the other hand is mainly located in cells of the meninges and the choroid plexus as well as neuroendocrine cells of the adrenal gland. It shows higher affinity than SVCT1 and facilitates the transport of AA into cerebrospinal fluid and in metabolically demanding cells (*41, 42*). Since most of AA exists in its reduced, monoanionic form in vivo, the above stated transport system supposedly helds responsible for the majority of AA transport into the cytoplasm (*39*). However, DHA can also be carried through membranes using glucose transporters GLUT1, -3 and -4 (*43, 44*). In contrast to SVCT1/2 they act bidirectional and thus cannot build up a concentration gradient of DHA (*42*). On an intracellular level, DHA quickly gets reduced via enzymatic or non-enzymatic pathways. The extracellular space lacks many of the antioxidant mechanisms of cytoplasm, which could result in insufficient recycling of DHA and thereby contribute to an increased degradation (*45, 12*). The existing data on DHA concentration in human plasma describes values between 2 to 13 µM in healthy subjects (*46, 47*). The blood brain barrier (BBB) is described to play a major role in regulating and maintaining AA concentration gradients between plasma and the brain interstitium. Agus et al. showed that GLUT1 is the main route of vitamin c transport across the BBB. They also discovered a competitive inhibition of this transport mechanism via high D-glucose concentrations, pointing towards a possible connection between hyperglycemia and altered DHA metabolism in brain (*48*). Supporting data from following research *in vivo* described a reduction of DHA transport across the BBB via GLUT1 by about 84% in hyperglycaemic rats compared to healthy individuals (*46*).

1.3 Neuronal differentiation and plasticity

1.3.1 Neuronal plasticity

Neuronal plasticity in general can be understood as the ability of neurons on a smaller scale or of the brain in general to undergo morphological changes following external or

internal stimuli. This complex mechanism is an important part not only of the developing and adult brain, but also of regenerating damage caused by inflammatory or neurodegenerative pathologies (*49*). In order to investigate neuronal differentiation as the basis for synaptic plasticity, multiple cell models have been established up to date (*50–52*).

1.3.2 PC12-cells as a model for neuronal differentiation

PC12-cells represent a single clonal cell line that was established in 1976 by the working group of Greene and Tischler. They originate from rat pheochromocytoma and exhibit characteristics of chromaffine neuron-like cells. Remarkably, it is possible to induce differentiation and neurite outgrowth of these cells through stimulation with nerve growth factor (NGF-β). PC12-cells show a slow proliferation with an estimated doubling time of 92h and tend to adhere to extracellular matrix proteins like collagen, laminin or fibronectin (*53, 54*). One should take into accout similarities between brain and adrenal gland in AA distribution and metabolism. Both have been shown to accumulate AA in high concentrations of up to 10mM (*38*). Taken together, these characteristics predestine PC12-cells as a suitable model for neuronal differentiation- and neurite formation studies in the context of AA metabolism. Differentiation of PC12-cells leads to activation of intracellular signaling cascades, followed by decreased proliferation, increased adhesion and ultimately neurite outgrowth (*53*). After binding of NGF-β to its high-affinity receptor TrkA, it induces signaling via the ras-raf-ERK pathway (*55*). Duration of ERK1/2-phosphorylation has hereby been identified as a regulating factor. Stimulation of EGF leads to transient ERK1/2-phosphorylation, while stimulation with NGF-β induces sustained activation of ERK1/2 (*56*). Aside from the ras-raf-ERK pathway, other signaling cascades have been identified in differentiating PC12-cells. Activation of PI3K is triggered after binding of NGF-β. This pathway was postulated to act not only as a distict signaling cascade, but also as a necessary cofactor in sustained ERK-activation (*57*). Furthermore, NGF-β has been shown to induce neurite outgrowth via the stimulation of a G-protein coupled receptor. This mechanism seems to favour rearrangement of microtubules, which is necessary for the morphological changes during neurite outgrowth (*58*).

1.4 Cell surface molecules of the immunoglobuline-superfamily

1.4.1 NCAM (CD56)

The neural cell adhesion molecule (NCAM) is a glycoprotein localized at the cell surface. It belongs to the immunoglobuline superfamily showing structural similarities to the receptor for advanced glycation endproducts (RAGE) (*59*). As a cellular adhesion molecule it mediates neurite outgrowth and adhesion in brain via multiple signaling

pathways, indicating its major role in neuronal regeneration and plasticity. NCAM is abundant both in the embryonic and adult nervous system, although its expression pattern changes throughout development (*60, 61*). Alterations in NCAM expression have been correlated to age-related neurodegenerative conditions like Alzheimers disease (*62–64*). Several isoforms of NCAM, mainly generated by alternative splicing, have been identified. The best investigated isoforms are called NCAM 120, NCAM 140 and NCAM 180 in respect to their molecular weight (*65, 66*). NCAM is involved in multiple signaling pathways and acts as a major player in mediation of neurite outgrowth (*65*). As a member of the immunoglobulin superfamily, NCAM enables heterophilic as well as homophilic binding, mainly regulated by its N-terminal Ig-like domains. Homophilic binding is followed by a calcium-independent mechanism which starts an PLC-mediated intracellular signaling cascade. This induces a calcium influx into the neuron and ultimately leads to neurite outgrowth. Another pathway necessary for neurite outgrowth is the activation of fyn-FAK by stimulation of NCAM-140 (*67*). One major characteristic of NCAM is its possibility to be posttranslationally modified through a process called polysialylation (*68*). Polysialic acid (polySia) is a homopolymer of sialic acid molecules which adds a negative charge to cell NCAM and enables for hydration. This unique structural change and its consequences for interaction between cells is held responsible for the inhibiting effect of polysialylated NCAM on cellular adhesion. The glycosylation site for this molecule resides at the N-glycans at the 5^{th} and 6^{th} site in the 5^{th} Ig-like domain of NCAM. Polysialylation is catalyzed by ST8SiaII and ST8SiaIV (*69*). It is specific and restricted to NCAM. Expression patterns of polySia-NCAM change depending on age and localization, indicating its role in neuronal plasticity and development. PolySia can thereby (in a simplified matter) be a switch, enabling neurons to change between a stable state and a state favouring plasticity, since it has been shown to increase neuronal migration and differentiation neurite outgrowth (*70*). Polysialylation of NCAM is increased in neurons during regeneration after axonal damage. This upregulation however decreases during ageing together with the regeneration capacity (*71, 72*).

1.4.2 RAGE

RAGE, the receptor for advanced glycation endproducts, was first identified in 1992 by Neeper et al. as a member of the immunoglobuline-type receptor superfamily. Due to its structure it acts as a receptor for a broad palette of ligands, among which AGEs were the first to be described (*59*). To this day, many physiological and pathophysiological properties of this multiligand receptor have been demonstrated, indicating a major role in cell proliferation, differentiation and survival, but also in inflammatory responses,

apoptosis and oxidative stress (*73–75*). RAGE shows structural similarities to NCAM. It consists of an extracellular domain, a transmembrane spanning chain and a cytoplasmic tail. The extracellular domain itself can be secluded into three distinct regions, two consistent C-type domains and one variable V-type domain. While the V-type region of the extracellular region helds responsible for ligand binding, the cytoplasmic tail has been shown to act as the starting point for further intracellular signaling (*76, 59*). However, the above-mentioned structure only accounts for the full-length, non-modified isoform of RAGE. Plenty of other, truncated isoforms have been identified up to date, most of them being generated by alternative splicing and proteolytic cleavage. To those belong human RAGE secreted (hRAGEsec), endogenous secretory RAGE (esRAGE) and soluble RAGE (sRAGE) (*77–80*). RAGE expression has been discovered in most human tissues and cell types, among those are fibroblasts, endothelial cells of the vascular system, microglia, monocytes and macrophages as well as lymphocytes and neurons (*81, 82*). Further investigations have also shown changes in RAGE expression in brain during embryonic and adult development, indicating a differential role of the receptor depending on age (*83, 84*). Growing research in the field of RAGE has revealed a wide variety of physiological functions. After being discovered in 1992, RAGE had been assumed to play a major role in scavenging and inducing degradation of AGEs (*59*). Further investigations however implicated that RAGE functions more as a signal transducting receptor rather than a scavenging one, inducing multiple pathways depending on localization, surrounding circumstances and RAGE-isoform (*75, 84*). Although initially described as a receptor for advanced glycation endproducts, it is important to mention that due to its structure, RAGE can bind a plethora of diverse ligands, most of which accumulate in tissues during ageing or inflammatory processes (*83, 85, 86*). The complexity of RAGE-mediated effects arises not only from its multiple ligands, but also from a variety of intracellular signaling cascades: p42/44 and p38 MAPK, PI3K, JAK/STAT kinase as well as multiple other protein kinases have been identified as downstream pathways of RAGE-activation (*74*). Furthermore it increases expression of NF-kB, a transcription factor known for mediating pro-inflammatory effects which is suspected to take part in pathological changes induced by Amyloid-β in Alzheimers disease. Binding of extracellular AGEs as well as Amyloid-β to RAGE in PC12-cells has been shown to induce apoptosis via NF-kB mediated signaling (*87*). Interestingly, activation of NF-kB leads to a positive feedback-loop, which upregulates expression of RAGE. Thus, dysregulated binding of accumulating ligands possibly induces a vicious cycle, amplifying pathological changes (*86, 88*). Since downstream signaling via MAPK has been shown to regulate multiple processes like proliferation, differentiation, cell survival or inflammation and apoptosis, RAGE-activation most probably also serves a

variety of physiological and pathophysiological purposes. Thus, the understanding of RAGE should be approached in the context of cell type, surrounding milieu, age, and available ligands (*84, 75*). Furthermore, signaling of RAGE has been shown to be heavily influenced by the surrounding redox state. Increased levels of ROS in the context of neurodegenerative diseases or inflammation may interfere with the mentioned pathways, highlighting the major role of a functioning antioxidant system (*89*). Taken together, existing data suggest both positive and negative effects of RAGE-mediated signaling. However, dysregulation via increased oxidative stress or pro-inflammatory mediators as well as increased binding of accumulating RAGE-ligands seems to favour the negative effects of ageing and neurodegeneration (*74*). Posttranslational modifications like glycation might also influence RAGE function and turnover, ultimately leading to impaired homeostasis and dysregulated signaling (*75*).

1.5 The monocyte/macrophage system

1.5.1 Ontogeny, morphology and function

The monocyte/macrophage system displays an important compartment of the innate immune system, as it includes cells with phagocytic and antigen-presenting as well as secretory behaviour (*90*). Monocytes originate from mutilineage precursors, the common myeloid progenitor cells (CMPs). Independently from origin, multiple subsets with distinct characteristics can be set apart, with CD14 and CD16 representing the defining surface molecules. The $CD14^{++}$/ $CD16^{-}$ subset is commonly called the “classical” subset, while the “non-classical” one is defined by $CD14^{++/}$ $CD16^{++}$ (*91, 92*). Interestingly, most of the known major subtypes have been shown to represent differential stages of the same cell line, indicating a shift of monocyte function and characteristics throughout its lifecycle. Classical monocytes ($CD14^{++}$/ $CD16^{-}$) localize mostly in bone marrow. They differentiate through intermediate subtypes ($CD14^{++}$/ $CD16^{+}$) to non-classical monocytes ($CD14^{++}$/ $CD16^{++}$), which circulate in peripheral blood (*92*). When confronted with pro-inflammatory stimuli, patrolling monocytes are able to infiltrate tissues neighboring blood-vessels. Migrating monocytes are then called macrophages (*93*). Depending on external chemokines, microenvironment and microbial pathogens, macrophages can alter their phenotype. This “macrophage polarization” allows for a adapted reaction to the surrounding inflammation or tissue wound (*94*). Polarization towards the pro-inflammatory M1-type is triggered by external ligands like LPS, TNF-α or IFN-γ. M1-macrophages primarily act as phagocytic and cytokine-releasing cells, taking up antigens from infected cells, cell debris and microbial patterns. They also release pro-inflammatory cytokines like TNF-α and Il-6, attracting other phagocytic and antigen-presenting cells (*95*). Especially in later phases of wound healing or inflammation,

regenerative processes need to be favored. To accomplish this, macrophage polarization is shifted towards the 2-type. M2-macrophages are associated with tissue-remodelling and release a different set of anti-inflammatory growth factors including TGF-β, Il-10 and IGF (*96, 97*).

1.5.2 Immunosenescence and "inflammaging" as manifestations of immunological dysregulation

The innate and adaptive immune system have been shown to undergo multiple alterations during ageing. Immunosenescence as a term for phenotypical changes in ageing immune cells heavily affects monocytes and macrophages in their morphology and function (*98*). On the contrary, increasing age has been connected to a process called "inflammaging". Here, a constitutively over-active state of pro-inflammatory processes and higher base-levels of mediators like TNF-α, Il-6 and Il-1 lead to auto-inflammation and age-related degenerative processes (*99*). Immunosenescence and inflammaging can thereby be seen as two sides of one coin and an expression of the dysregulated immune system in the elderly. Although most knowledge of age-related alterations has been collected using rodents as subjects, a growing pool of data indicates a major influence of immunosenescence on human phagocytic cells as well. A plethora of affected mechanisms have been identified up to date, including expression of surface receptors like TLRs, transmembrane and intracellular signaling pathways as well as phagocytic behaviour and secretion of immunomodulatory molecules (*100*). When investigating age-related changes of phagocytic cells, one has to differentiate between peripheral monocytic cells and tissue-associated macrophages. Nyugen et al. were able to demonstrate an increase of $CD16^+$ cells in aged subjects, accompanied by a decrease of $CD16^-$. The overall number of monocytes however did not seem to be deficient. Furthermore, the authors discovered a decrease in TLR1 expression as well as ERK1/2-phosphorylation in monocytes from aged individuals. These changes however were restricted to $CD14^{++/+}$/ $CD16^+$ cells. Taking into account the relative shift towards a $CD16^+$-phenotype, these findings highlight the functional differences between monocyte subsets. Nyugen et al. propose an impaired production of pro-inflammatory cytokines as a result of TLR1/ ERK1/2-mediated pathway (*101*). Although other TLRs are associated with pro-inflammatory responses to binding of PAMPs, little or no increase of cytokine-secretion could be found for the other receptors like TLR4, -5, -7 during ageing (*102, 100*). Existing data on the expression of MHCII molecules on peripheral monocytes of elderly subjects have mostly been inconsistent. While some researchers proposed that there is no significant change in HLA class II expression, others could detect a decrease in its expression on monocyte surfaces (*103–105*). Activated macrophages from aged individuals have been shown to produce increased levels of prostaglandin E2 (*106*).

PGE2 represents an anti-inflammatory chemokine which suppresses secretion of pro-inflammatory mediators like Il-12 while simultaneously increasing release of anti-inflammatory Il-10. Higher levels of PGE2 in plasma or wounds of aged individuals could therefore potentially prolonge early-phase wound healing. Interestingly, Chambers et al. were able to show a block of antigen-specific cutaneous immune-reaction due to increased PGE2 secretion by monocytes in wounds caused by injection with air or saline. Their findings hint that non-pathogen related injury could possibly hamper antigen-specific immune responses. This reaction was mediated via an increase in p38 MAPK signaling (*107*). Furthermore, efferocytosis as a mechanism for cleavage of apoptotic cell debris could play a role in age-related impairments in wound healing. De Maeyer et al. detected a decrease in expression of TIM-4 (t-cell immunoglobulin mucin receptor-4) in aged subjects, accompanied by a suppressed phagocytosis of apoptotic neutrophils. Similar to the above mentioned increase in PGE2 secretion, this process was mediated by activation of p38 MAPK (*108*). Elderly people show alterations in their microbiome which are connected to elevated levels of pro-inflammatory markers Il-6; Il-8 ; and CRP (*109*). These changes in pathogen-abundancy might influence macrophage function by activating or inhibiting TLRs and thereby cytokine secretion. In vivo data by Agius et al. displayed a reduced secretion of TNF-α, Il-6 and IFN-γ after contact of skin-tissue with Candida albicans. In vitro experiments could not recreate that effect, indicating that the microenvironment might play an important role in age-related changes (*110, 100*). Existing data on release of pro-inflammatory cytokines from tissue macrophages after stimulation via LPS have been contradictory, suggesting that it depends on multiple factors like the surrounding tissue, ontogeny as well as the microenvironment (*100, 111, 102, 112*).

1.5.3 Glycation in the context of immunosenescence and "inflammaging"

As the generation of advanced glycation endproducts is tied to age-related changes in the proteome, its influence on immunosenescence and inflammaging is progressively gaining scientific interest. Liu et al. investigated the effect of AGEs on macrophage polarization. They found an increase of RAGE- and TLR4-expression after coincubation of isolated primary macrophages with glycated BSA. Pro-inflammatory pathways were consecutively induced, leading to higher phosphorylation of STAT-1 as well as higher levels of intracellular ROS. Ultimately, AGE-exposed macrophages would differentiate towards the M1-phenotype and produce Il-6, Il12 and TNF-α (*113*). This shift towards the M1-phenotype might be supported by an upregulation in HIF-a/PDK-4, which has been found in murine macrophages after exposure to AGEs (*114*). Not only AGEs, but also high levels of glucose might influence macrophage activation. Incubation of murine

macrophages with glucose led to M1-activation. Therefore, not only exposure to external AGEs, but also glycation of cellular components possibly interferes with macrophage activity (*115*). Although current data indicate pro-inflammatory properties of macrophages in tissues with higher concentrations of AGEs, phagocytic efficiency might be impaired. Xie et al. could show an ARL8-mediated decrease in autolysosomal activity of THP-1 cells after exposure to AGEs, hinting towards a hampered lysis of pathogens (*116*). Taken together, there is growing evidence on a negative effect of glycation and exposure to AGEs on immunoregulatory processes. The microenvironment of macrophages in atherosclerotic or wound healing tissue might have a major role in affecting their function.

1.6 Natural killer cells

1.6.1 Ontogeny, morphology and function

NK-cells represent an integral part of the human immune system which originate from the the clp-cell line. Although being structurally related to B- and T- lymphocytes, their characteristics and functions make them part of the innate immune system (*117*). NK-cells do not express antigen-specific receptors like their counterparts from the adaptive immune system. Their main tasks as a group of cytotoxic lymphocyte-like cells is the elimination of virus-infected or malignant cells as well as the production of immune-modulating cytokines. NK-cell function is regulated by a complex set of receptors and ligands, which ultimately lead to either activation or inhibition of its cytotoxic activity and thereby the fate of its effector target (*118, 119*). About 10-15% of peripheral lymphocytes are NK-cells (*120*). They comprise of different subsets with specific expression patterns and immunological functions. Two of the major characterizing surface molecules are CD56 and CD16 with CD56bright/CD16dim/- as well as CD56dim/CD16+ being the most abundant and best investigated NK-cell subpopulations (*119*). It is noteworthy that CD56 displays the cluster of differentiation nomenclature for NCAM, the neural cell adhesion molecule (*121*). Each of the stated subtypes diverge in regard to their localisation as well as their immunological function. CD56-bright cells have been shown to act as cytokine releasing cells mostly localized in secondary lymphatic tissues, while CD56-dim cells primarily localized in peripheral blood favor cytotoxic behavior (*122*). In general, two distinct courses are possible upon binding of NK-cells to their target cells. If the activating signal outweighs the inhibitory one, NK-cells will release lytic granules which mediate lysis of the effector cell (*123*). Cell to cell contact and interaction between NK-cells and their target cells are restricted to smaller areas located on the cell surface, which are commonly called immunological synapses (*124*). Longer lasting contact between the NK-cell and its target has been proposed to activate recognition receptors, followed by

adhesion. Important signaling molecules mediating this process are LFA-1 and MAC1, both members of the integrin-family (*125, 126*). Consecutive steps are characterized by actin-cytoskeleton reorganisation as well as directional transport of effector molecules and lytic granules along microtubules to the MTOC and consecutively to the immunological synapse (*127*). Lytic granules share structural similarities to lysosomes while also containing secretory components like granzyme A and B, perforin, FAS-ligand (*118*). Multiple receptors associated with active NK-cells have been discovered until now, the best investigated being CD16, NKG2C/CD94 as well as NKp30, NKp44 and NKp46, NKG2D, 2B4 or CD2 (*123*). Binding to these receptors leads to the activation of multiple signaling cascades. Depending on each signaling cascade, either cytotoxic or secretory functions can be executed. Activation of immunoreceptor tyrosine-based activating motifs (ITAMs) displays one of the major activating pathways, leading to tyrosine residue phosphorylation and activation of src homology 2 domain containing kinases (*123*). However, if the NK-cell is confronted with a healthy target sufficiently expressing MHC-I molecules, a different signaling cascade is initiated (*128*). KIR and NKG2A represent two of the major inhibitory receptors. Ligand-binding leads to the activation of ITIMs and consecutively to the activation of src homology 2 domain containing phosphatases, which antagonise the above mentioned signaling cascades (*123*).

1.6.2 NK-92 cells

NK-92 cells are a natural killer cell line established by Gong et al. in 1994. They represent human, IL-2 dependent NK cells which have frequently been used for immunological studies over the course of the last 30 years due to their high cytotoxic potential (*129*). These cells express most of the activating molecules of healthy NK-cells while also expressing less inhibitory factors, enabling them to induce cytotoxicity on many malignant tissues. Considering the expression pattern of surface molecules, NK-92 cells share more similarities with the CD56-bright NK-cell subtype than with the CD56-dim one (*130*). Furthermore, NK-92 cells lack expression of CD16, excluding antibody-dependent cell-mediated cytotoxicity (ADCC) from its palette of cytotoxic mechanisms. This circumstance however might be of minor relevance, as Solana et al. proposed an independency of CD16-expression and thereby ADCC from age (*117*).

1.6.3 NK-cells and immunosenescence

Morphological changes in the ageing body include immunological senescence, a term which is frequently used to describe the decreasing capability to form a proper immunological response to infection, autoinflammatory processes as well as cancer (*131*). NK cells are part of this process due to changes in cell numbers and expression patterns (*132, 98*). The overall cell number of NK-cells in the peripheral blood has been

shown to increase with age. Furthermore there is a relative increase of CD56-dim cells compared to CD56-bright cells, indicating a shift from immature NK-cells towards mature successors. This shift is accompanied by an increase in CD57-expression (*98, 133*) According to the working group of Solana et al., these changes occur due to age-related impairment of hematopoietic stem-cells in primary lymphoid tissue (*117*). In contrast, cytotoxicity and cytokine production by NK-cells are impaired in the elderly (*134*). The expression of activating and inhibitory NK-cell surface receptors also undergoes changes during ageing. While MHC-I associated inhibitory receptor KIR is upregulated, this shift towards an inhibitory signal is proposedly compensated by decrease in CD94/NKG2A expression (*135*). On the contrary, expression of the activating receptors NKp30 and NKp46 was found to be decreased in NK-cells of elderly subjects (*136*). Taken together, the existing data indicate an overall impairment of the cytotoxic and secretory function of single NK-cells due to immunological senescence. These defects are presumably compensated by an increase of mature cytotoxic CD56-dim cells as well as a preserved sensitivity towards stimulation via chemokines (*117*).

1.7 Aim of work

Post-translational modifications like glycation and enzymatic glycosylation play a central role in regulating proteostasis. Especially neurodegenerative diseases like Alzheimer's disease show high levels of AGEs and accumulation of dysfunctional peptides. Many compounds responsible for glycation have been identified up to date, among which the antioxidative carbohydrate ascorbic acid inherits an ambivalent status. Dysregulation of ascorbic acid recycling and increased ascorbic acid degradation under conditions with increased oxidative stress could favour generation of AGEs and have a negative impact on neurons. This work investigates the role of ascorbic acid induced glycation on survival, differentiation and neurite outgrowth of PC12-cells as a model for neuronal plasticity. Enzymatic glycosylation in the form of N-linked polysialylation represents a selective posttranslational modification of the neural cell adhesion molecule NCAM, which is crucial for neuronal differentiation and plasticity in the developing and adult brain. RAGE, the receptor for advanced glycation endproducts is an important modulator of AGE-induced inflammation. Together with NCAM it belongs to the superfamily of immunoglobuline-like receptors, as both molecules share structural similarities and mediate overlapping intracellular signaling cascades regulating cell survival, differentiation and apoptosis. Studying alterations in NCAM and RAGE half-life could lead to better understanding of age-related changes in neuronal differentiation in the ageing brain. Therefore, this work aims to elucidate the differential effects of non-enzymatic glycation and enzymatic polysialylation on the turnover of NCAM and RAGE in PC12-cells. Due to its unspecific nature and wide variety of substrates, glycation affects not only long-lived neuronal cells, but also cells from the innate and adaptive immune system. Reactive dicarbonyls have increasingly been identified as driving factors especially in tissues with high oxidative stress, indicating that even short-time exposure might already cause persistent changes in protein structure and function. Investigating the effects of dicarbonyl-induced glycation on NK-cells and macrophages might shed light on age-related changes in immunoregulatory processes. It might furthermore highlight glycation as an important factor in cell to cell contact and secretion of inflammatory cytokines.

2 Discussion

2.1 Ascorbic acid and dehydroascorbic acid induce glycation of PC12-cells

As one of the major antioxidants of the human body, ascorbic acid takes a central role amidst cell metabolism, differentiation and defense against glycoxidation. Existing data confirm the multiple functions of ascorbic acid. Most of these processes demand ascorbic acid in its reduced form, as most of its functions arise from its antioxidative properties (*137*). Degradation of ascorbic acid beyond the threshold of reversible oxidation generates reactive metabolites responsible for glycation. MGO as well as 3-deoxythreosone have been identified as possible intermediates following degradation of ascorbic acid. Both molecules have already been displayed as potent glycating agents (*3*). Strong evidence suggests glycation and the generation of AGEs as participants in the generation of neurodegenerative diseases (*138*). It is not implausable to assume that degradation of ascorbic acid may contribute to this pathomechanism. In this study we investigated the impact of ascorbic acid on glycation of PC12-cells. To accomplish this, PC12-cells were incubated with different concentrations of ascorbic acid and its oxidation product DHA in low-serum conditions for four hours. Generation of the AGE carboxymethyl-lysine was qualified via western blot analysis of cell lysates. AA and DHA induced glycation, however DHA showed much higher glycation potential. This observation seems logical, since DHA is one step ahead in the same degradation chain and further decomposition should happen at a faster rate. It should be highlighted that the short incubation time of 4h was sufficient to induce glycation, although the concentrations of DHA (0.1 mM and 0.3 mM) exceeded physiological extracellular values of 0.01 mM in plasma. However, high concentrations of ascorbic acid in human brain (up to 10 mM) together with increased oxidative stress might also elevate concentrations of DHA to a higher level *in vivo* (*139, 140*). A possible amelioration of the cellular glycation process due to serum proteins in cell culture media could not entirely be avoided. To minimize this effect we used low-serum conditions for the incubation period. The difference between AA and DHA concerning their glycation-potential underlines the importance of AA recycling. Taking into account the short half-life of DHA under physiological conditions (*27*), together with our findings of increased glycation capacity, it is tempting to speculate that conditions with increased oxidative stress, like Alzheimer's disease, might also be connected to a higher glycation potential of AA and its degradation products. Thus, further investigations regarding this mechanism are necessary.

2.2 Ascorbic acid decreases cell viability of PC12-cells

AA, although being an antioxidant, has been shown to exhibit pro-oxidant behaviour *in vitro* under certain circumstances. Higher concentrations as well as the presence of catalytic metals like copper and iron favour this mechanism. The generation of reactive oxygen species in the presence of reduced catalytic metals is described in the literature as fenton reaction. AA will non-enzymatically reduce present Fe^{3+} (or other catalytic metals like copper Cu^{2+}), which can then generate hydroxyl radical via the fenton reaction. Oxidized Fe^{3+} will again be reduced by AA and thereby rejoin the reaction. Thus, increase in free catalytic metals might cause increased oxidative stress in and around cells (*42*). Neurodegenerative diseases contain higher levels of transition metals, possibly contributing to generation of reactive oxygen species and thereby increased oxidation and protein damage (*141*). In our experiments we were able to show a concentration-dependent decrease in viability of PC12-cells after application of AA. Treatment with DHA however did not have this effect, indicating that the reducing nature of AA and not glycation could be connected to its effect on viability. Previous research could show that AA can act as a pro-oxidant *in vitro* (*142*), which is supported by our data. If this mechanism is applicable to the state *in vivo*, remains to be investigated.

2.3 Ascorbic acid increases NGF-mediated phosphorylation of ERK1/2

PC12-cells represent chromaffine neuron-like cells with the ability to differentiate and form neurites after stimulation with neurotropic factors. Neurite outgrowth is affected by activation of multiple signaling cascades, with ras-raf-MEK being a major pathway. Binding of NGF-β to its receptor will thereby induce sustained activation of ERK1/2 and ultimately lead to decrease of proliferation, increased adhesion and neurite outgrowth. It is important to note that phosphorylation of ERK1/2 is an intersection of multiple signaling cascades (*55*). It has been connected to a wide variety of functions in the cell, including proliferation, differentiation, cell survival, but also pro-inflammatory pathways and apoptosis. Therefore, it needs to be tightly regulated in order to execute specific functions like neurite outgrowth (*56, 143*). This regulation can happen via duration of activation and co-activation of parallel cascades. We investigated the influence of AA on the downstream activation of ERK1/2 after binding of NGF-β. PC12-cells were incubated with AA for four hours in low-serum conditions and phosphorylation of ERK1/2 was accessed via western blot analysis of cell lysates. Interestingly, administration of AA did increase the NGF-mediated phosphorylation of ERK1/2. We could not observe this effect after administration of DHA or additional growth factors like EGF or FGF. Furthermore, application of AA without NGF-β did not induce ERK1/2-phosphorylation. As stated earlier, administration of AA strongly interfered with cell viability. If these effects arise solely from generation of ROS via fenton reaction or if it could at least partially be

associated to the altered activation of ERK1/2, needs further investigation. Nevertheless alterations in ERK1/2-phosphorylation can act as a “switch” between a differentiation- or an apoptosis-favouring state. This highlights the vulnerability of ERK1/2-phosphorylation and may indicate a negative influence of high AA concentrations on neuronal differentiation.

2.4 Ascorbic acid and dehydroascorbic acid interfere with neurite outgrowth of PC12-cells.

Neuronal differentiation can be accessed by studying not only intracellular signaling cascades, but also morphological effects on neurons like neurite outgrowth. PC12-cells will tend to form neurites after stimulation with NGF-β (*53*). We investigated the effect of AA and DHA on neurite outgrowth as a marker of differentiation and neuronal plasticity. Cells were incubated with different concentrations of both agents in low-serum media for four hours and then seeded on collagen IV-coated plates. NGF-β induced neurite outgrowth was measured using an impedance-based method, the RTCA. We could show that both AA and DHA decreased neurite outgrowth. Administration of AA caused higher damage to neurite outgrowth compared to DHA. We showed that while DHA induced glycation of PC12-cell proteins, it did not impair cell viability and had no impact on ERK1/2-phosphorylation. Thus, one could speculate that interference with neurite outgrowth might be connected to glycation and generation of AGEs. AA also induced glycation on a smaller scale, on top of that it interfered with ERK1/2-phosphorylation and decreased cell viability. Taken together, these findings suggest that AA and DHA influence differentiation of PC12-cells partly via different mechanisms. Neither of them showed any beneficial effects on cell viability, adhesion or neurite outgrowth.

2.5 Glycation of NCAM and RAGE via methylglyoxal increases protein turnover in PC12-cells and PC12-polySia-cells

Enzymatic polysialylation and non-enzymatic glycation display posttranslational processes that influence proteostasis in the context of age-related neurodegenerative diseases (*70*). We investigated the impact of both modifications on protein turnover of two major cell surface receptors in PC12-cells, RAGE and NCAM. PC12-cells and a ST8SiaIV-expressing clone (PC12-polySia-cells) were incubated with MGO for four hours. MGO is a physiological by-product of glycolysis and represents a reactive α-dicarbonyl with high potency to induce glycation. It is also generated via degradation of AA (*3*). Concentrations of MGO differ between tissues and cell types and rise under circumstances of higher glucose metabolism (*5*). The MGO concentrations which were used in our experiments exceed those normally found in neurons. This method was used to compensate for the short incubation time of four hours. After incubation, protein

synthesis was inhibited using cycloheximide. Protein turnover was measured before and after incubation using western blot analysis. We found that glycation of PC12-cells via MGO increased turnover of RAGE. Thus, faster degradation of RAGE might have an influence on RAGE-mediated signaling. Furthermore, glycation of PC12-cells via MGO increased the turnover of NCAM in PC12-cells and PC12-polySia-cells, however there was no significant difference between both clones. NCAM acts as a mediator of neuronal differentiation, thus alterations in NCAM half-life could have an impact on NCAM mediated signaling. Bennmann et al. investigated the effect of MGO-induced glycation on PC12-cells and found a strong decrease of neurite outgrowth (*15*). Whether this effect is partly influenced by decreased NCAM-turnover needs further research. Additionally, glycation via MGO reduced the half-life of polySia in PC12-polySia-cells. PolySia-modification of NCAM displays a major change in structure and function. As stated earlier, polysialylation of NCAM is associated with axon regeneration and a state favouring neuronal plasticity (*70*). A loss of regeneration capacity during ageing might be connected to changes in NCAM expression and decrease of NCAM-polysialylation. The present work supports the theory that glycation in the ageing brain could contribute to this mechanism.

2.6 Methylglyoxal and Glyoxal induce glycation of NK-92 cells

Although being relatively short-lived and mobile cells compared to longer-living, tissue-bound neurons, NK-cells are not completely defiant against microenvironmental influences. Plasma levels of reactive α-dicarbonyls have been shown to be elevated under hyperglycemic circumstances (*144*). We incubated NK-92 cells with 0.3; 0.6; 1 and 2 mM of MGO or GO for four hours under low serum conditions. As established in earlier experiments, we used western blot analysis to test for glycation. Both MGO and GO induced the generation of AGEs in concentration-dependent manner. One has to take into account possible differences in the spectrum of AGEs induced by both substrates. Experimental differentiation between AGEs remains difficult due to a limited availability of valid antibodies. Elevated levels of dicarbonyls in human plasma might therefore be associated with increased glycation of peripheral NK-cells. Whether there is an imbalance between extracellular or intracellular target glycation remains unclear. It would seem plausible due to differing concentrations of MGO spanning from 2-4 nM intracellular to 100 nM extracellular, as stated by Rabbani and Thornalley. (*19*). Interestingly, the effects of both agents on metabolic activity and thereby cell survival were relatively low. We investigated these by performing a MTT metabolic activity assay. Incubation with GO did not interfere with cell viability, while treatment with MGO

decreased viability at higher concentrations of 1 and 2 mM. This underlines the importance of rapid MGO decomposing by the glyoxalase system.

2.7 Glycation interferes with conjugate-formation and cytotoxic function of NK-92 cells

NK-cell function highly depends on proper receptor-ligand binding as well as intact intracellular signaling. Building of NK-cell/target-cell conjugates resembles one step in the generation of immunological synapses (*118*). We incubated NK-92 cells with MGO or GO for four hours under low serum conditions and measured conjugate forming with K562-target cells using flow cytometry. Incubation of NK-92 cells with either of both α-dicarbonyls led to a decrease in conjugates, however MGO had an overall bigger impact than GO. Glycation of NK-cell surface molecules might therefore play a role in age-related impairments of cytotoxic function on the level of the immunological synapse. The exact mechanism needs to be further elucidated. Ageing has already been associated with alterations of NK-cell surface receptor expression (*136*). Considering our previous research on NCAM and RAGE proteostasis after MGO-induced glycation, a change in cell surface protein turnover might contribute to this mechanism. To investigate downstream events of NK-cell/target-cell binding, we analyzed cytotoxicity after glycation of NK-cells. Again, NK-92 cells were treated with MGO and GO as stated above. Cytotoxicity on K562-cells was measured via a flow-cytometry-based assay. Similar to conjugate formation, cytotoxicity was impaired after incubation with either MGO or GO, with MGO showing stronger effects. Since cell viability was not hampered after incubation with GO and only after incubation with higher concentrations of MGO, the mechanism for this decrease in NK-cell function seems to be at least partially separate from metabolic activity. As both GO and MGO generate a different, though overlapping set of AGEs, the specific targets and mechanisms might also differ (*21, 22*).

2.8 Methylglyoxal induces glycation of THP-1 macrophages

Macrophages represent stationary phagocytic cells residing in tissues, which sets them apart from circulating immune cells. Due to their extended lifespan they might be exposed to prolonged changes in their corresponding microenvironment (*145*). Tissues from aged individuals have been shown to display increased levels of carbohydrates and reactive intermediates, which arises the question if macrophages might be subject to glycation during ageing (*5, 146, 147*). We incubated THP-1 cells with MGO in a concentration dependent manner (0.5 and 1 mM) for 24h and measured the generation of AGEs using western blot analysis. Treatment with MGO led to glycation of THP-1 macrophages. As shown by immunofluorescence staining, these effects were mainly located at the cell surface. Our findings hint towards a difference between intracellular

and extracellular metabolism of reactive α-dicarbonyls. As phagocytic activity depends on the function of cell surface receptors, an increased susceptibility to glycation might also influence ligand binding.

2.9 Glycation of THP-1 macrophages increases pro-inflammatory cytokine expression while interfering with phagocytic activity

Macrophages possess the ability to change the expression patterns of cell surface molecules as well as signaling pathways in order to polarize towards specific effector functions. Current publications reveal an increasing complexity of these macrophage subsets (*90, 148*). Depending on the surrounding microenvironment and stimulation by chemokines, cytokines or pathogen associated molecular patterns, the macrophage will differentiate towards pro-inflammatory or anti-inflammatory phenotype (*97*). We investigated the effect of direct glycation on this differentiation as opposed to the exposure to already glycated proteins. To accomplish this THP-1 cells were incubated with 1 mM MGO or medium containing 10% AGE-BSA for 24h. Expression of cytokines was measured using rt-PCR. We could find a significant increase in the expression of pro-inflammatory markers Il-1β, Il-8 and TNF-α after treatment with MGO. Treatment with AGE-BSA however did not show any effect on interleukine-expression. Our results suggest a dysregulation of macrophage polarization, leading to a pro-inflammatory reaction. These effects most likely arise from the glycation of cell surface molecules, as shown by the immunofluorescence assay we performed earlier. Whether there are specific receptors that are vulnerable to these changes remains subject of further research. In order to evaluate changes in macrophage function, we analyzed the phagocytic activity of THP-1 cells after incubation with MGO 1mM or AGE-BSA for 24h. We found a decrease in phagocytic activity after treatment with MGO. Incubation with AGE-BSA did not have any effect. In our experiments we were not able to show a major influence of external glycated BSA on THP-1 phagocytic activity or expression of pro-inflammatory cytokines. In contrast to our experiments, Liu et al. displayed an increase in iNOS, cytoplasmic RAGE, TLR4, STAT1, and pro-inflammatory interleukines after incubation of primary macrophages with glycated BSA for 48h (*113*). Possible explanations for these variances might be found in the different exposure times, a different set of surface molecules between primary and THP-1 macrophages as well as structural dissimilarities of BSA-AGE subtypes depending on the dicarbonyl-substrate. Although the process of glycation had been discovered over a century ago (*1*), its effects on proteostasis and the ageing cell are still subject to ongoing research. The results of this thesis show negative effects of glycation on immune cell function and neuronal differentiation and might contribute to a better understanding of age-related diseases.

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4 Theses

1. Ascorbic acid and dehydroascorbic acid induce glycation of PC12-cells.
2. Glycation of PC12-cells by dehydroascorbic acid reduces neurite outgrowth.
3. Ascorbic acid increases NGF-β mediated phosphorylation of ERK1/2.
4. Ascorbic acid reduces viability of PC12-cells in a concentration dependent manner.
5. Polysialylation of NCAM in PC12-polySia cells decreases NCAM turnover.
6. Glycation of PC12-cells by methylglyoxal increases turnover of NCAM and RAGE.
7. Glycation of NK-92 cells leads to reduced conjugate-formation and impaired cytotoxic function.
8. Glycation of THP-1 cells increases expression of pro-inflammatory cytokines and interferes with phagocytic function.

Publications

Ascorbic acid leads to glycation and interferes with neurite outgrowth

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Experimental gerontology, 2018 https://doi.org/10.1016/j.exger.2018.08.005

Advanced glycation endproducts and polysialylation affect the turnover of the neural cell adhesion molecule (NCAM) and the receptor for advanced glycation endproducts (RAGE)

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Glycation interferes with natural killer cell function

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Glycation of macrophages induces expression of pro-inflammatory cytokines and reduces phagocytic efficiency

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