EFFECT OF GLYCATED PROTEINS AND INHIBITORY COMPOUNDS INTERVENING WITH THE AGE-RAGE PATHWAY ON MACROPHAGES

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A thesis submitted in fulfilment of the requirements for the degree of Master of Science (Honours)

Centre for Complementary Medicine Research
College of Health & Science

University of Western Sydney

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Lastly, but most importantly, I thank my family and friends, for their moral support, encouragement and understanding during the pursuit of this research.
Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Parisa Younessi
Pharm. D.

Signature: Date: 28 July 2010
Conference Presentation and Publication


Poster presentation (invited for oral presentation).

Free registration award.

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Nomenclature

Definitions

Akt is also called protein kinase B (PKB) and plays an important role in cellular signaling.

EF hand is a helix loop helix structural domain found in a family of calcium binding proteins.

IC$_{50}$ is the concentration of an inhibitor within a biological process that inhibits half maximal of that biological process.
## General Abbreviations

<table>
<thead>
<tr>
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<th>Description</th>
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<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
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<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>AA</td>
<td>amyloid associated</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>AβA</td>
<td>amyloid beta aggregate</td>
</tr>
<tr>
<td>AβF</td>
<td>amyloid beta fibrillar</td>
</tr>
<tr>
<td>AβO</td>
<td>amyloid beta oligomer</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>AL</td>
<td>amyloid light chain</td>
</tr>
<tr>
<td>Apo E</td>
<td>Apo lipoprotein E</td>
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<td>Term</td>
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</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>C</td>
<td>Constant</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecules</td>
</tr>
<tr>
<td>CEA</td>
<td>chicken egg albumin</td>
</tr>
<tr>
<td>CML</td>
<td>N-carboxymethyl lysine</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
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<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CV</td>
<td>cell viability</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>DN-RAGE</td>
<td>dominant-negative RAGE</td>
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<td>DPI</td>
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</tr>
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<td>EDTA</td>
<td>ethylenediamine tetra- acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular Signal-Regulated Kinase</td>
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<tr>
<td>Es-RAGE</td>
<td>endogenous secretary RAGE</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<td>FPRL</td>
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XVI
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<tr>
<td>HMGB-1</td>
<td>high-mobility-group B 1</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular cell adhesion molecule</td>
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<td>Immunoglobulin</td>
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<td>Interleukin</td>
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<tr>
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<td>interferon-gamma</td>
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<tr>
<td>JNK</td>
<td>jun N-terminal kinase</td>
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<tr>
<td>LMW</td>
<td>low molecular weight</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
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<td>LRP</td>
<td>lipoprotein receptor related protein</td>
</tr>
<tr>
<td>MAPKs</td>
<td>mitogen-activated protein kinases</td>
</tr>
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<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
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<td>MS-AGE</td>
<td>methylglyoxal modified AGE</td>
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<td>mTNF-α</td>
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<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
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<td>NFTs</td>
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<td>NMDAR</td>
<td>N-methyl-D-aspartic acid receptor</td>
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<td>NO</td>
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<td>NtRAGE</td>
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<td>PBS</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptors</td>
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<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
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<tr>
<td>rHMGB1</td>
<td>recombinant HMGB-1</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RT</td>
<td>room temperature</td>
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<td>soluble RAGE</td>
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<td>tumor necrosis factor-alpha</td>
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Abstract

**Introduction:** Glycation is a specific type of protein modification in ageing. The reaction of reducing sugar with a primary amino group is the most common non-enzymatic modification of proteins. Subsequent rearrangement, oxidation and dehydration yield a heterogeneous group of mostly coloured and fluorescent compounds, termed “Maillard products” or “advanced glycation end products (AGEs)”’. AGEs are thought to play a role in the aetiology of various age-related diseases such as diabetes mellitus (DM) and Alzheimer’s disease (AD). AD is a multifactorial disease, in which the rate of synapse loss and neuronal cell death determines the onset and/or progression of dementia. Activation of microglia and astrocytes with subsequent oxidative stress and cytokine release may be an important progression factor for AD. It is also suggested that the receptor for advanced glycation end products (RAGE) ligands-AGEs interaction might be another cause of glial activation, cytokine and reactive oxygen species (ROS) release. Different antioxidants, receptor mediated compounds and ROS scavenging enzymes might be able to intervene with the AGE-RAGE signalling pathway and slow down the progression of AD. The aim of this study is to investigate the effect of different compounds of antioxidants, receptor mediated compounds and ROS scavenging enzymes on inflammation induced by glycated proteins and to explore the suggested AGE-RAGE signalling pathway by examining inhibition of different parts of the signalling network.

**Methods:** Bovine serum albumin (BSA)-AGE and chicken egg albumin (CEA)-AGE were produced by incubation of BSA or CEA with D-glucose in phosphate buffer saline (PBS) at pH 7.4 at 65°C for 4 weeks. The ability of each glycated modified protein for nitric oxide (NO) and tumor necrosis factor (TNF)-α production was tested in RAW 264.7 and J774 murine macrophage cell lines by Griess reagent and enzyme-linked immunosorbent assay (ELISA) methods. Production of hydrogen peroxide (H₂O₂) was measured in response to CEA-AGE and lipopolysaccharide (LPS) treatment by Amplex Red assay in RAW 264.7 murine macrophage cell line. In addition, the effect of different intervening compounds such as polyphenol antioxidants (apigenin, resveratrol and *Polygonum cuspidatum*), receptor mediated...
compounds (anti-RAGE antibodies and a RAGE antagonist), ROS scavenging enzymes (catalase) and NADPH oxidase inhibitors (acetovanillone and diphenyl iodonium chloride) on inflammation markers like NO and TNF-α was tested along with cell viability by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) or alamar blue assay.

**Results:** The results showed that glycated modified proteins specifically CEA-AGE can induce NO production in RAW 264.7 and J774 murine macrophage cell lines. CEA-AGE was more potent than BSA-AGE in induction of inflammatory markers and comparable with (LPS+INF-γ) induction. In addition, CEA-AGE and LPS stimulated H2O2 production in a similar concentration within 30 minutes in RAW 264.7 murine macrophage cell line. The IC50 value of apigenin was found to be 5 µM for TNF-α and 1 µM for NO reduction of glycated protein induced inflammation. TTP488 IC50 value was also found to be 1 µM for NO reduction, 8.5 µM for TNF-α reduction induced by glycated proteins, 2.5 mg/mL for hyaluronic acid with 1.7 × 10^6 kDa molecular weight and 3.25 µM for diphenyl iodonium chloride for NO reduction of glycated protein induced inflammation. Anti-RAGE antibodies reduced NO and the TNF-α inflammation marker at concentrations less than 10 µg/mL.

**Conclusion:** Different compounds such as antioxidants, RAGE antagonists, antibodies, ROS scavenging enzymes and NADPH oxidase inhibitors were tested for reduction of inflammation. Among these, polyphenol antioxidants (apigenin) and RAGE antagonists reduced inflammation markers the most in *in vitro* experiments. ROS scavenging enzymes and NADPH oxidase inhibitors were also able to reduce inflammation markers to some level, indicating that oxidative stress is involved in glycated protein induced signalling pathways. These findings show that the proposed signalling network may be a possible AGE-RAGE signalling network and that the inflammation inhibitors of antioxidants, receptor mediated compounds and ROS scavenging enzymes of glycated protein induced inflammation may be useful in slowing the progression of AD.
CHAPTER ONE

INTRODUCTION
1.1 Advanced glycation end products, their receptors and their biological role

1.1.1 Chemistry

The amino groups of proteins, particularly the side chain of lysine, arginine and histidine react non-enzymatically with reducing sugars. This post-translational modification called “non-enzymatic glycosylation”, “glycation” or a “Maillard reaction”, leads via reversible Schiff-base adducts to protein bound Amadori products (Kikuchi et al., 2003). By subsequent oxidation and dehydration, a broad range of heterogeneous fluorescent and brown products with nitrogen- and oxygen-containing heterocyclic compounds are formed, called “advanced glycation end products” or “AGEs”. AGEs formation is irreversible and causes a resistant protein deposition to protease (Münch et al. 2000). The Maillard reaction was first described by the chemist L. C. Maillard, who reported the formation of brown products upon heating a solution containing amino acid (aa) and sugar (Danehy et al., 1986) and is shown in Figure 1.1.

1.1.2 Pathological consequence of AGEs

Protein glycation in vivo modifies the structural properties of protein, and this leads to inflammation and oxidative stress. The pathological role of AGEs in diseases such as diabetes mellitus (DM) is not fully understood. In addition to the change of the protein structure, the receptor mediated mechanism of AGEs is of special interest (Cooper, 2004).

The pathological features of AGEs that are not receptor mediated can be observed in the progression of cataracts. Evidence suggests (Hennessey et al., 1990) that the glycation of lens protein is one of the causes of cataract and is observed in long-lived proteins such as collagen and eye crystalline (Scalbert and Birlouez-Aragon, 1993). However, the pivotal role of AGEs and the interaction with the receptor is not fully understood.
Figure 1.1 Schematic representation of the Maillard reaction (A) and the structure of arginine AGEs pyrimidine and pentosidine (B and C) (Reddy and Beyaz, 2006).
1.1.3 AGEs and ageing
Ageing can be characterised by progressive impairment of organs, tissue and cells. This is a progressive process and it is not categorised as a disease, unless it interferes with the normal function of the organs. DM and Alzheimer’s disease (AD) are the most prevalent ageing diseases and are examples of the tissue impairment by ageing (Puglielli, 2008). One of the characteristics of ageing is the acceleration of production of glycated proteins and accumulation of those in different tissues. Glycated proteins form aggregations that are insoluble and more resistant to degradation than non-glycated proteins (Rondeau et al., 2010).

1.1.4 AGEs and Alzheimer’s disease
AD is the most common type of dementia in elderly people (Malloy et al., 2007). Approximately four million people in the United States have AD, and this number is expected to increase by 2050. The prevalence estimation of AD among people aged 85 years or older was estimated to increase seven-fold from 1980 to 2050; however, the rise is slower in people from the age of 65 to 74 years old during that same period (Evans, 1990).

AD is characterised by initial mild memory impairment and this progresses to loss of mental and physical activities. The cognitive decline is associated with the widespread loss of synapses, neuronal cell death and the formation of amyloid plaques and neurofibrillary tangles, markers of AD. AGE modification and the resulting cross-linking of protein deposits were observed in both plaques and tangles (Cummings et al., 1996).

1.2 AGE receptor (RAGE)
Macrophages were first described to uptake AGEs via a specific receptor called RAGE (Gu et al., 2006). RAGE has been identified in monocytes, macrophages, microglia, astrocytes, neurons, smooth muscle cells and endothelial cells (Hoppmann et al., 2010).
Different AGE modified proteins such as AGEs and β-sheet fibrils like amyloid proteins and other ligand families, such as high-mobility-group B (HMGB or amphoterin) and S100/calgranulin, were identified as ligands of RAGE (Gu et al., 2006). Binding to different families of ligands is a unique characteristic of RAGE (Hofmann et al., 1999) and RAGE is referred to as pattern recognition receptor (PRR). The interaction between RAGE and AGEs is a complicated process that has been shown to be related to problems in different ageing related diseases. It is also known as scavenger receptor in microglia cells (Hopmann et al., 2010). Increased expression levels of RAGE were found in the optic nerve of AD patients in proximity to astrocytes (Alarcon et al., 2005). While there are many studies on AGEs, there is little information about the receptors. The current data show that glycated modified protein binding to RAGE triggers some components of different signalling pathways. However, the complete networks of those signalling pathways are still unclear (Tsoporis et al., 2007).

1.2.1 RAGE and mitogen activated protein kinases
RAGE was first described as a 35 kDa AGE-binding protein belonging to the immunoglobulin superfamily (Hofmann et al., 1999). The mitogen activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) were implicated as components of the RAGE signalling pathways (Shen et al., 2004). These MAPKs are induced by cytokines and stressors. Further down in the signal transduction cascade, transcription factors like nuclear factor-kappa B (NF-κB) are also activated. Thus, ligand-RAGE interaction activates NF-κB through these MAPKs signalling pathway. The cascade of signal transduction depends on the binding of AGEs to RAGE, as blocking RAGE with either an excess of soluble RAGE (s-RAGE) or with an anti-RAGE antibody prevents cellular activation (Liu et al., 2010).

1.2.2 RAGE isoforms
The RAGE receptor was first described in 1992 (Schlueter et al. 2003). RAGE is a multi-ligand cell surface receptor from the immunoglobulin (Ig) superfamily,
composed of 404 aa (Srikanth et al., 2009). It contains an extracellular part with 320 aa, a single transmembrane domain with 21 aa and a short cytoplasmic domain with 40 aa (Kalea et al., 2009). The extracellular part is composed of a variable (V) domain and two constant (C) domains (Talts et al., 1999). It has 94 aa in an Ig-like V-type domain, 98 aa in an Ig-like C2-type 1 domain and 91 aa in an Ig-like C2-type 2 domain. Alternative splicing plays a major role in the production of different RAGE isoforms (Srikanth et al., 2011). Alternative splicing is a mRNA splicing mechanism that occurs prior to mRNA translation. During alternative RNA splicing, exons or introns may be retained or removed in different combinations, resulting in the production of different proteins with unique characteristics (Lopez, 1998). Over 20 different splice variants of human RAGE have been identified to date (Sparvero et al., 2009). Generally, there are four main RAGE isoforms: full length (FL)-RAGE, N-terminal truncated (Nt)-RAGE and endogenous secretory (Es)-RAGE or s-RAGE (Figure 1.2). s-RAGE is a product of recombinant technology as well as of cleavage of cell surface RAGE by extracellular metalloproteinases (Hanford et al., 2004). Neither s-RAGE nor Es-RAGE has the cytoplasmic and transmembrane domains of FL-RAGE (Devangelio et al., 2007; Ghidoni et al., 2008). The Nt-RAGE lacks the V-type domain of FL-RAGE. Moreover, it cannot act as a FL-RAGE and different ligands cannot bind to Nt-RAGE (Ding and Keller, 2004). RAGE ligands such as amphoterin, S100B and Aβ oligomer bind to the V domain, however, some ligands such as Aβ also bind to the C domain (Leclerc et al., 2009). Moreover, there are two N-glycosylation sites in and close to the AGE binding domain. Osawa et al. (2007) showed that a G82S mutation in the second N-glycosylation motif increased the AGEs affinity in COS-7 cells. As the blood sugar level is high in diabetic patients, translational modification of RAGE by de novo N-glycosylation and N-glycosylation sites could be a potential reason for RAGE activation and deactivation (Osawa et al., 2007). In addition, s-RAGE can suppress the RAGE signalling and it was shown that administration of s-RAGE in animal models for cancer and multiple sclerosis can suppress tumor growth and autoimmune response (Ostendorp et al., 2006). The s-RAGE plasma level is lower in patients with cognitive impairment, non-alcoholic fatty liver disease and type 2 diabetic patients when compared with control subjects (Devangelio et al., 2007; Benussi et al., 2008).
1.2.3 \textbf{s-RAGE: a marker and a treatment}

s-RAGE is the extracellular ligand-binding domain of RAGE. It is present in the human plasma and it has the potential to function as a decoy for the RAGE signalling pathway by binding to circulating plasma AGEs. It has been suggested that s-RAGE can be a biomarker for RAGE-mediated disease development, especially vascular diseases (Basta, 2008).

There is controversy about some studies on the s-RAGE plasma level and its relationship to disease development. Some studies show low s-RAGE plasma level and high N-carboxymethyllysine (CML) levels along with abundant natural AGEs in diabetic patients with severe complications (Grossin et al., 2008). In contrast, the s-RAGE plasma level is reported to be lower in patients with arthritis and hypertension than in healthy subjects (Chen et al., 2009). Yamagishi et al. (2006) reported the positive relationship between plasma level of AGEs and s-RAGE in a non-diabetic
population. Interestingly, one study reported an elevated plasma level of s-RAGE in septic patients. That study is the only report of high s-RAGE plasma level in acute and non-chronic diseases (Bopp et al., 2008). It was proposed that the s-RAGE plasma level is negatively correlated with body mass index (BMI), as overweight people with a high BMI have a low s-RAGE plasma level (Norata et al., 2009). Remarkably, a high s-RAGE level positively correlates with an increase in inflammatory markers such as tumor necrosis factor (TNF)-α and monocyte chemo attractant protein-1 (MCP-1) in patients with type-2 diabetes (Nakamura et al., 2008).

The decoy characteristic of s-RAGE is not completely known whether it functions as a negative feedback as a biomarker or whether it has a protective effect by increasing the AGEs level in plasma in different diseases. Recombinant s-RAGE administration was tested in a DM animal model with atherosclerotic lesions and it was found to suppress the development of atherosclerotic lesions in the apo E null mice diabetic model (Schmidt, 1998). In another study, administration of s-RAGE to diabetic C57/BJ6 and RAGE-transgenic mice with diabetic symptoms inhibited blood-retinal barrier breakdown and leukostasis, which are the signs of retinopathy in diabetic patients (Kaji et al., 2007). These studies suggested an anti-ageing characteristic of s-RAGE in ageing related diseases.

1.2.4 RAGE: a transporter of amyloid beta in AD

Deposition of amyloid beta (Aβ) as a senile plaque in the brain and the soluble oligomer form of Aβ have been identified as one of the major features of AD (White et al., 2005). Salminen et al. (2009) reported that “full-length RAGE can be expressed in astrocytes, microglia and neurons, but also endothelial cells can show a high level of RAGE expression in the brain”. It is known that the blood brain barrier (BBB) is important for Aβ brain balance and that the BBB regulates the transport of Aβ through two receptors: the low-density lipoprotein receptor related protein 1 (LRP1) and RAGE. The RAGE protein mediates the influx of amyloid protein from plasma to the brain, whereas, the LRP protein mediates the efflux of amyloid protein.
through the BBB (Salminen et al., 2009). Deane et al. (2004) suggested that brain cerebrospinal fluid (CSF) is separated from blood by tight junction between endothelial cells. Therefore, Aβ peptide movement through the BBB needs a receptor, such as RAGE, to transfer Aβ from plasma to the CSF through endocytosis (Deane et al., 2004). Generally, the efflux that is mediated by LRP1 is greater than the influx by RAGE. In AD, changes in RAGE expression might create an imbalance between the influx and efflux rate of Aβ peptide through the BBB (Wegiel et al., 2004).

1.3 RAGE ligands

1.3.1 AGEs
AGEs bind to a wide number of cell surface receptors other than RAGE, such as scavenger receptor types I and II, oligosaccharyl transferase (OST-48) and 80K-H phosphoprotein (Miura et al., 2004). The diversity in biological AGE structures makes their \textit{in vivo} characterisation difficult. Heterogeneity of AGE binding was first demonstrated by competitive binding assays involving radio-labelled methylglyoxal modified (MS)-AGE and bovine serum albumin (BSA)-AGE (Nakano et al., 2006). A cross-linked formation and polymerisation of AGEs was known to contribute to the nature of these macromolecules, reducing their solubility, increasing heat denaturation times and increasing protease resistance \textit{in vivo} (Sell and Monnier, 1989; Vlassara et al., 1992;). Activities of AGEs are facilitated through three general mechanisms: intracellular signalling pathway activation following receptor-ligand binding, structural modification of biological structures via the Maillard reaction, and oxidative effect of AGE molecules (Guglielmo et al., 2010).

The ability of various Aβ ligands to bind RAGE is now known to involve specific residues. Whether these β-sheet fibrils bind common or separate V domain binding sites to BSA-AGEs and HMGB-1 ligands has been investigated in competitive I125-Aβ (1–40) binding assays (Yan et al., 1996). The conclusion that Aβ binds the V
domain of RAGE was further suggested by atomic force microscopy and modelling studies (Chaney et al., 2005).

The observation that monomeric and fibrillar forms of Aβ-peptide bound RAGE with various affinities prompted the question of whether the receptor recognised a sequence motif or a tertiary/quaternary structural motif involving β-sheet structures (Cummings et al., 1996; Yan et al., 2000). To address that question, RAGE binding studies were performed utilising non-amyloidogenic erabutoxin, β-sheet protein (protein has β sheet structure), and non-crosslinked β fibril collagen and elastin molecules as ligands. These structures did not interact with the receptor, suggesting that RAGE recognises crosslinked β-structured amyloids only (Yan et al., 2000). The necessity for these quaternary amyloid structures in RAGE binding was further confirmed by the ability of serum amyloid A(SAA) 1 (forming amyloidogenic fibrils) and not SAA 2 (does not form fibrillar structures) to bind and initiate cellular stress through RAGE (Malle et al., 2009). Recently, the oligomeric Aβ was found to influence RAGE binding. This study reports that Aβ aggregates specifically interact with the C1 domain, while monomeric and oligomeric Aβ bind to the V domain (Sturchler et al., 2008).

Further, RAGE mediated signal transduction is a significant pathway promoting Aβ outcomes, but it is not the only effect. A multitude of Aβ cell surface binding sites, other than RAGE, have been identified. These include alpha-5 and beta-1 integrin, N-methyl D-aspartate receptor (NMDA-R), nicotinic receptors, formyl-peptide receptor-like-1 (FPRL1) and certain adhesion and tyrosine kinase receptors (Boland et al., 1996; Paresce et al., 1996; Matter et al., 1998; Balleza-Tapia and Pena, 2009).

The mechanistic elements of RAGE-amyloid ligation remain unknown. However, the consequences of this interaction are well documented and include progressive rheumatoid arthritis, neoplasia, systemic amyloidosis and AD (Yan et al., 1996; Yan et al., 2000; Chen et al., 2007; Malle et al., 2009).
1.3.2 Calgranulins

Calgranulins are known as S100 proteins on the basis of their solubility in 100% saturated ammonium sulfate (Moore, 1965; Donato, 2001). This family of 21 Ca\(^{2+}\) modulated proteins is exclusively expressed by vertebrates in both intracellular and extracellular spaces (Donato, 2007). Calgranulins are characterised by a highly conserved EF-hand tertiary structure composed of two α-helices and a Ca\(^{2+}\) binding loop that collectively form a helix-loop-helix motif. The first loop in the amino-terminus EF hand structure is long and disordered in comparison to the second carboxyl-terminus EF hand loop, which is canonical in arrangement. These differences result in low and high Ca\(^{2+}\) binding affinities respectively (Donato, 1986; Donato, 2007). Although the calcium binding regions are highly conserved within the S100 family, the hinge region joining the two EF hands is highly variable (Santamaria-Kisiel et al., 2006). S100 proteins are acidic, low molecular weight species often within the range of 9–14 kDa. The human genes encoding 13 members of this family are found on chromosome 1 (Schafer et al. 1995; Donato, 2001). The majority of in vivo S100 proteins exist as non-covalently bound homophilic oligomers with the exception of S100G, which is monomeric (Skelton et al., 1994; Donato, 2001). Extracellular heterodimers of S100A12/S100B, S100A8/S100A9 and S100B/S100A6 are common, with the A8/A9 dimer being the preferred intracellular form for S100A8 and S100A9 (Hunter and Chazin, 1998; Donato, 2001). Monomers are in an anti-parallel orientation to each other to form non-covalent structures that upon binding of Ca\(^{2+}\), Zn\(^{2+}\) or Cu\(^{2+}\), undergo significant conformational changes to expose a hydrophobic cleft (Santamaria-Kisiel et al., 2006; Moroz et al., 2009). This hydrophobic region is one mechanism by which oligomeric S100 quaternary structures affect intracellular target molecules (Leclerc et al., 2009). It is, however, postulated that other unidentified aspects of calgranulin structure are also involved (Donato, 2007).

S100 molecules are implicated in a broad array of physiological processes that include cell proliferation, differentiation and cytoskeletal organisation, membrane trafficking, protein phosphorylation, gene expression and metabolism (Hofmann et al., 1999; Leclerc et al., 2007; Leclerc et al., 2009). Calgranulins are expressed in a
cell specific manner (Leclerc et al., 2009). Whether that is the reason for certain S100 proteins to be associated with diseases affecting particular tissues or whether various calgranulins have unique and/or specific biological targets requires considerable clarification.

Based upon the high degree of structural homology within the functional Ca\(^{2+}\) binding domain of this family, it is not unreasonable to suggest that S100s may bind a number of common targets. It was thought likely that a number of calgranulins bind RAGE based on initial observations that S100B and S100A12 are RAGE ligands (Shaw et al., 2003; Bianchi et al., 2007; Yang et al., 2007). Subsequent studies have shown that S100A1, A4, A6, A7, A8/A9 dimers, A11, A13 and S100P potentially promote physiological outcomes, in part, through RAGE (Donato, 2007; Leclerc et al., 2009).

S100B was reported to be expressed in brain tissue by astrocytes at levels greater than any other cell type (Donato, 2001; Santamaria-Kisiel et al., 2006). Of all the S100 family, S100B is the only member with an encoding gene located on chromosome 21 (Donato, 2001). The prevalence of S100B in Down’s syndrome (chromosome 21 trisomy) and in a number of neurodegenerative disorders has promoted the hypothesis that S100B-RAGE plays either a neuro-protective role or contributes to neurological pathogenesis. It is known that outcomes of S100B/RAGE interaction are ligand- and RAGE concentration-dependent. The duality of S100B activity being both neurotrophic and degenerative was demonstrated with reports of concentrations above 500 nM resulting in neuronal apoptosis (Ahlemeyer et al., 2000; Donato, 2007). The specific cellular outcomes of calgranulins and the involvement of RAGE in the transduction of intracellular signals is complex. In brief, S100A4 has been clearly shown to progress tumor metastasis and pathophysiology as seen in the tumor-like behaviour of rheumatoid arthritis synovium (Shaw et al., 2003; Helfman et al., 2005; Senolt et al., 2006). S100A6 similarly has a role in tumor development through direct ligation with the tetramerisation domain (residues 325–355) of p53, and neuronal apoptosis in a RAGE-dependent manner (Leclerc et al., 2007; van Dieck et al., 2009). S100A8 and A9 are often referred to as neutrophil cytosolic proteins because of their prevalence.
in neutrophils, monocytes/macrophages and activated glial cells, and by their role in 
leukocyte adhesion and migration (McCormick et al., 2005; Donato, 2007). Like 
S100B, S100A8 and A9 are secreted from leukocytes at sites of inflammation in 
response to pro-inflammatory stimuli, through a positive feedback mechanism 
involving RAGE and heparan sulphate proteoglycans (Donato, 2007). These 
calgranulins are, therefore, often associated with chronic inflammatory disorders 
such as diabetes and tumor metastasis, making them accurate blood-borne markers of 
ongoing inflammation (Foell et al. 2007). The S100A8/A9 heterodimers promote a 
number of physiological changes in homeostasis that generally increases vascular 
permeability, platelet aggregation and the recruitment of leukocytes (Roth et al., 
2003; Leclerc et al., 2009). NF-κB activation similarly follows S100A8/A9 up-
regulation, resulting in gene transcription of pro-inflammatory cytokines and cell 
adhesion receptors such as vascular cell adhesion molecules (VCAM) (Roth et al., 
2003; Ehlermann et al., 2006; Donato, R. 2007). S100A12 is expressed in 
granulocytes and keratinocytes, exerting physiological outcomes such as leukocyte 
activation and infiltration at sites of inflammation (Roth et al., 2003; Donato, 2007). 
RAGE mediates S100A12 activity through NF-κB activation, particularly in 
monocytes and endothelium following exposure to TNF-α and endotoxin (Rouleau et 
al., 2003; Wittkowski et al., 2007). Such stimuli promote S100A12 expression as can 
be seen in the synovial fluid of rheumatoid arthritis patients (Chen et al., 2009). As 
with S100A8/A9 proteins, RAGE is unlikely to be the sole receptor of S100A12 
biological outcomes, with S100A12 activating mast cells not expressing RAGE 
(Yang et al., 2007).

The S100 family of molecules represents a complex of molecules with autocrine and 
paracrine effects on neurons, microglia, astrocytes, monocytes, endothelial cells, 
vascular smooth muscle cells and numerous tumor cells (Reeves et al., 1994; Bianchi 
et al., 2007; Donato, 2007; Ponath et al., 2007). RAGE was shown to be a clear 
mediator in a number of these cell types for some, but not all, calgranulins. What is 
clear from the current literature is that RAGE plays a significant role in calgranulin 
signal transduction. This role is present in a number of pathologies involving pro-
inflammatory responses, and represents a novel opportunity for intervention in a
number of diseases. A better understanding of the nature of interaction between RAGE and calgranulins requires more investigation.

1.3.3 High-Mobility Group Box Chromosomal Protein 1

High-mobility group-1 box proteins (HMGB-1) are a family of multi-functional, non-histone proteins that bind to DNA in a sequence independent manner (Bianchi et al., 2007). Within the HMGB-1 set of molecules are three sub-groups defined by common functional motifs: HMG-1/2 (HMG box), HMG-14/17 (nucleosome binding domain) and HMG-I/Y (AT-hook) (Bianchi and Manfredi, 2007). Despite these distinguishing characteristics, all members of HMGB-1 possess an ability to modify the conformation of DNA, enhancing the efficiency of gene transcription and chromatin plasticity (Bianchi and Manfredi, 2007). The ability of these molecules to interact with DNA is mediated by two homologous HMG box domains made up of α-helical secondary structures (Read et al., 1993). The original name, amphoterin, reflects the contiguous organisation of two dipolar regions in HMGB-1, consisting of a basic 185 aa domain and a short 30 residue anionic region (Rauvala and Rouhiainen, 2007).

First identified in the 1970s, the role of this family of proteins was believed to be limited to intra-nuclear functions such as gene transcription, replication and repair, and nucleosome stabilisation (Fiuza et al., 2003; Kokkola et al., 2005). Recently, however, a number of extra-nuclear roles have been identified for HMGB-1. These involves pro-inflammatory responses, central nervous system development, mediation of endotoxicity and modulation of hemostasis and thrombosis (Huttunen et al. 2002; Fiuza et al., 2003; Kokkola et al., 2005; Bianchi and Manfredi, 2007; Manfredi et al., 2009).

Early on, it was reported that B16 melanoma, neuroblastoma and immune cells such as monocytes secreted HMGB-1 in response to early pro-inflammatory signals, including lipopolysaccharide (LPS), TNF-α and interleukin 1 (IL-1) (Parkkinen et al., 1993; Rauvala and Rouhiainen, 2007). However, the mechanism by which this occurred was unknown. Normal cytokine secretion occurs through classical
endoplasmic reticulum-Golgi exocytosis, in which protein trafficking is determined by a leader sequence (Rubartelli et al., 1990). As with a number of pro-inflammatory molecules, HMGB-1 imparts a positive influence at non-toxic levels, recruiting macrophages to sites of injury and promoting monocyte tissue infiltration and senescence (Ulloa and Messmer, 2006; Hock et al., 2007).

High levels of circulating HMGB-1 are a late-stage marker of endotoxicity, immediately followed by increased TNF-α and IL-1 expression (Wang et al., 1999; Hoesel and Ward, 2004). Inhibitors of both TNF-α and IL-1 has little impact on endotoxicity survival in mice (Wang et al., 1999). Knockout mice for TNF-α and IL-1, however, demonstrate a significant increase in endotoxemic tolerance (Wang et al., 1999; Hoesel and Ward, 2004). This clinical finding was explained by the observation that a rapid release of TNF-α and IL-1 promoted some stage secretion of HMGB-1, which in turn imparted symptoms of endotoxicity. This was confirmed by injection of rHMGB-1 into double negative TNF-α and IL-1 mice, which then developed endotoxemia with 60% mortality (Wang et al., 1999).

The ability of HMGB-1 to bind RAGE in a saturable, dose-dependent manner was first reported by Hori et al. (1995). The region of HMGB-1 interacting with RAGE was refined to a COOH-terminal motif consisting of residues 150–183 (Huttunen et al., 2002; Rauvala and Rouhiainen, 2007). Interestingly, this region of HMGB-1 shares sequence similarity with residues 3–39 of S100A12, residues that constitute the calgranulin EF-hand motif (Huttunen et al., 2002). In endothelial cells, RAGE activation by HMGB-1 resulted in a cell adhesion phenotype characterised by increased expression of intercellular cell adhesion molecule (ICAM) and VCAM, and activation of β1 and β2 integrins (Fiuza et al., 2003; Chavakis et al., 2007). Activation in both endothelial cells and monocytes results in the secretion of interleukin-8 (IL-8), MCP-1, TNF-α secretion, RAGE up-regulation and the production of fibrinolysis modulators (Fiuza et al., 2003). Activation of these pro-inflammatory elements defines HMGB-1 as an agonist of diseases exacerbated by chronic inflammation.
Early studies showed co-localisation of RAGE with HMGB-1 in developing rat brain, suggesting a homeostatic role for RAGE distinct from chronic disease (Huttunen et al., 2000). Tissue surveys confirmed the expression of RAGE during mammalian development, in particular in the central nervous system (Brett et al., 1993; Hori et al., 1995). HMGB-1 culture flasks promote neurite outgrowth of cultured rat cortical neurons, with the presence of s-RAGE or anti-RAGE antibodies along with inhibiting neuronal development (Hori et al., 1995; Huttunen et al., 1999). Cell motility is also, in part, influenced by RAGE-HMGB-1. A study utilising neuroblastoma cells transfected with a cytosolic domain deleted (DN)-RAGE construct observed a complete loss of cell migration, otherwise apparent with wild type RAGE (Huttunen and Rauvala, 2004). This observation supported a role for HMGB-1-RAGE in cytoskeletal reorganisation affecting cellular outgrowth, cytokinesis and cellular differentiation.

The understanding to which RAGE mediates HMGB-1 bioactivity is still somewhat unclear. A number of alternative cell surface receptors exist for HMG proteins, including syndecan-1, toll-like receptors (TL2 and TL4), receptor-type tyrosine phosphatise β/ζ, and sulfoglucuronyl carbohydrates found on a number of cell surface adhesion molecules (Salmivirta et al., 1992; Chou et al. 2004; Park et al., 2004; Rauvala and Rouhiainen, 2007). It is, however, clear that activation of RAGE by HMGB-1 in neuronal, tumor, endothelial and leukocyte cells leads to the activation of transcription factors NF-κβ, Sp1 and cAMP response element binding (CREB) (Huttunen et al., 1999; Taguchi et al., 2000; Fiuza et al., 2003; Rauvala and Rouhiainen, 2007; Urbonaviciute et al., 2008).

The common role between HMGB-1 and RAGE represents a significant and complex element in both disease and health. How HMGB-1-RAGE facilitates these polar opposites of physiology through a myriad of intracellular signalling events still requires significant investigation.
1.3.4 Amyloid beta

The term amyloid was first used in 1854 by Virchow (Bhat et al., 1998). Today, amyloids are characterised as fibrillar structures often composed of amyloid light chain (AL) or amyloid associated (AA) protein with crosslinked β-structures, an affinity for Congo red and green birefringence (Westermark et al., 2005). A number of in vivo amyloid fibril precursors have been identified and associated with local and systemic pathologies, generally termed amyloidoses (Pepys, 2001; Westermark et al., 2005). Endogenous Aβ is a 39–43 aa peptide that makes up the majority of extracellular fibrillar aggregates known as amyloid plaques (Balleza-Tapia and Pena, 2009). Aβ exists as a monomer, insoluble (AβF) and soluble (AβO) oligomeric fibrillar structures, and aggregate (AβA) forms (Sturchler et al., 2008).

RAGE directly interacts with amyloid fibrillar structures (Yan et al., 1996). Early studies showed that this was found in both brain endothelium and cortical neurons, and that synthetic Aβ-peptide (1–40) (Aβ (1–40)), bound RAGE with nanomolar affinity (Yan et al., 1996; Ito et al., 2005). This interaction is inhibited in a dose-dependent manner by anti-RAGE IgG or excess s-RAGE in both in vivo and in vitro cell binding assays (Yan et al., 1996). The ability of various synthetic Aβ ligands to bind RAGE is now known to involve residues 25–35 of the peptide, with scrambled Aβ (25–35) having no binding activity (Cummings et al., 1996; Yan et al., 1996).

1.4 RAGE activation and biological consequences: inflammation, oxidative stress, cell survival and proliferation

RAGE is found on the surface of different kinds of cells such as lymphocytes, leukocytes, macrophages/microglia/monocytes, astrocytes, neurons, smooth muscle cells and endothelial cells (Chavakis et al., 2004).

RAGE is shown to influence cell survival, cell proliferation, oxidative stress and inflammatory responses. Likewise, AGEs effect on proliferation and cell death was reported in some studies, this effect was suppressed by blockade of RAGE in T lymphocytes (Moser et al., 2003; Peterszegi et al., 2006). This shows that AGEs have effects on cell proliferation and cell survival through RAGE. Moreover, several
studies demonstrated a role of AGEs in over production of intracellular reactive oxygen species (ROS), impairments in proteasomal activities, inflammatory responses and cell insensitivity to insulin in DM. In contrast, AGEs can induce nitric oxide (NO) production in retinal neurons and N11 (microglia-like) cell line (Kobayashi et al., 2005; Berbaum et al., 2008). Both H$_2$O$_2$ and NO are part of the redox signalling and are markers of oxidative stress. In addition, RAGE activation resulted in the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Higai et al., 2006). The product of this enzyme activation is superoxide ion ($O_2^-$), another ROS. In contrast, interaction of ligands with RAGE induces production of cytokines followed by up-regulating multiple signalling pathways. Ligand-induced RAGE activation is shown to drive NF-$\kappa$B expression, followed by up-regulation of inflammatory markers and adhesion molecules and, consequently, inflammatory cell recruitment to the site of inflammation (Chavakis et al., 2004). In addition, migration of monocytes was reported in AD patients and Aβ-transgenic mice. This migration may play an important role in the RAGE-mediated inflammatory responses in the brain of AD patients (Wegiel et al., 2004).

Furthermore, C-reactive protein (CRP) is a key marker of inflammation in cardiovascular diseases and it is a mediator for developing atherosclerosis. CRP increases the expression of cell adhesion molecules (CAMs), chemokines, RAGE and the production of ROS. Mahajan et al. (2010) justified that CRP up-regulates RAGE expression and it was demonstrated that this up-regulation can be reduced by MAPKs inhibitors; therefore it was suggested that the p38, ERK and JNK signalling pathways are involved in CRP-induced RAGE expression (Mahajan et al., 2010).

Donato et al. (2009) highlighted in a review article that S100B, an endogenous RAGE ligand, has protective and neurotrophic effects during brain development in low concentrations, while it has a toxic effect in higher concentrations through production of ROS in a RAGE-dependent manner in neurons. They also suggested that S100B-RAGE interaction stimulates pro-inflammatory responses and ROS production in monocytes/microglia/macrophages (Donato et al., 2009).

In addition, cyclooxygenase (COX) is an enzyme responsible for formation of prostanoids and increasing evidence points to the role of these enzymes in
inflammation (Scheuren et al., 2002). Additionally, S100B/RAGE interaction up-regulates COX-2 expression in BV-2 microglia (Bianchi et al., 2007). The up-regulation of the COX-2 expression was observed in AGE-BSA-induced human osteoarthritis (OA) chondrocytes (Nah et al., 2008). This up-regulation was also reported in cultured THP-1 monocytes and human peripheral blood monocytes stimulated by AGEs and S100B (Shanmugam et al., 2003). Those data support the overlapping of COX-2 and the RAGE signalling pathway.

It was shown that RAGE−/− mice in comparison with wild type (Wt) have a lower level of TNF-α and IL-6 plasma concentrations in response to HMGB1 stimulation (Kokkola et al., 2005). In contrast, a high level of circulating TNF-α was reported as a crucial mediator in the RAGE/AGE and NF-κB signalling pathway (Zhang et al., 2009).

Finally, prostaglandin (PG) F2α comprises a class of biologically active products of the arachidonic acid pathway and is considered a marker of oxidative stress (Dimopoulos et al., 2008). The relevance of urinary 8-iso-PGF2α with s-RAGE in diabetic patients was considered (Saenger et al., 2007). In addition, it was reported that PGF2α is high and has an inverse correlation with the s-RAGE plasma level (Devangelio et al., 2007; Dimopoulos et al., 2008). Furthermore, the overproduction of intracellular ROS was observed in THP1 monocytes after treatment with glycoxidised albumin (Rondeau et al., 2008). Moreover, human umbilical vein endothelial cells (HUVECs) induced by TNF-α have demonstrated a high level of RAGE expression. This expression was reduced by N-acetyl-L-cysteine (NAC), a ROS scavenger (Mukherjee et al. 2005). E-selectin is a cell adhesion molecule that is expressed in activated endothelial cells in inflammatory conditions and by AGEs (Belch et al., 1997; Basta et al., 2002). It is reported that E-selectin was unregulated in human saphenous vein endothelial cells in response to heterogeneous AGEs (Basta et al., 2002). It was also observed that E-selectin expression induced by glycated human albumin serum (HAS) has been reduced by NADPH oxidase inhibitors and scavengers of ROS, NAC in human saphenous vein endothelial cells. However, it was reported that this E-selectin expression could not be abolished by an anti-RAGE antibody in this study (Higai et al., 2006). This information suggests that the
RAGE/AGE interaction stimulates pro-inflammatory responses through the NF-κB signalling pathway in an oxidative stress dependent manner.

1.5 RAGE and signalling pathways

NF-κB and MAPKs such as ERK1/2, JNK, Akt and p38 were implicated to be involved in the RAGE signalling pathway (Ishihara et al., 2003; Sun et al., 2003; Ito et al., 2005; Origlia et al., 2008).

It was shown that S100A8 and S100A9 induced human prostate cancer cells can activate NF-κB transcription in vivo (Hermani et al., 2006). Moreover, Sun et al. (2003) showed that phosphorylated p38 is not associated with Aβ plaques and neurofibrillary tangles (NFTs) in subregions of the AD hippocampus. This therefore suggested that the p38/MAPKs signalling pathway is not crucial for Aβ neurotoxicity which is a RAGE ligand (Sun et al., 2003). Moreover, p38 MAPK phosphorylation increased in cultured neurons treated with a nontoxic concentration of Aβ42, a soluble amyloid beta peptide. This effect was reduced by an anti-RAGE antibody (Origlia et al., 2008).

The COX-2 enzyme is implicated in the pathogenesis of several inflammatory diseases. An increase in the expression level of COX-2 which was induced by S100B, another RAGE ligand, was reported in human peripheral blood monocytes from healthy donors (Shanmugam et al., 2003). Moreover, involvement of NF-κB in the expression of COX-2 induced by IL-1β in mesenchymal cells of human amnion has been shown (Yan et al., 2002). These studies implied that the role of COX-2 in the inflammatory response is RAGE-ligand dependent through the NF-κB signalling pathway. These studies are controversial and more investigations are needed into the COX-2 role in the RAGE signalling pathway.

In addition, proliferation of the mouse microglial cell line Ra2 was stimulated through increasing the expression of macrophage colony-stimulating factor (M-CSF) in Aβ treated cell line. This stimulation was blocked by an Akt inhibitor (Cobb, 1999; Ito et al., 2006). Furthermore, an electro-mobility shift assay (EMSA) showed that the M-CSF promoter region with a putative NF-κB binding site was associated
with Aβ-induced M-CSF expression in the Ra2 cells treated with Aβ (Ito et al., 2005). This suggests that Aβ, a RAGE ligand, might act through the RAGE and Akt/NF-κB signalling pathway.

Figure 1.3 Schematic of proposed AGE-RAGE signalling pathway (Fukunaga and Miyamoto, 1998; Shen et al., 2004; Donato et al., 2009).

1.6 Potential therapeutic approaches according to the AGEs hypothesis
A number of therapeutic approaches were proposed for ageing related diseases with relevance to glycated proteins. These approaches include AGE inhibitors, AGE breakers, AGE signalling blocking, anti-RAGE antibodies, RAGE antagonists and the use of antioxidants such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Peyroux and Sternberg, 2006; Sabbagh et al., 2008). Anti-RAGE antibody treatment in uremic apoE−/− mice showed a decrease in the development of atherosclerotic lesions (Bro et al., 2008). Moreover, vitamin C treatment of patients with renal failure (diabetic nephropathy and other types of renal failure) showed a decrease in AGEs plasma level (Ferretti et al., 2008). Oxidative stress and ROS
production, based on the AGEs hypothesis may potentially benefit from antioxidant treatment, especially AD patients.

1.7 Inflammation induced by glycated proteins, interferon gamma and lipopolysaccharide

Inflammation is a defence mechanism by the immune system to injuries, damaged cells, pathogens such as bacteria, viruses and any non-familiar compound. This is a mechanism to remove the pathogen/non-familiar compound or heal the injured organs. Inflammation is a sign to recruit white blood cells such as leukocytes and macrophages. These cells produce cytokines, a messenger among cells (Johnston et al., 2007). In addition, LPS, the outer membrane of gram negative bacteria, elicits the production of a number of cytokines (Duenas et al., 2006). Cytokines can bind to specific receptors and trigger the complex cytokine network signalling. The excessive elevated level of cytokines was observed in AD patients.

Among those, INF-\(\gamma\), which was originally called macrophage-activating factor, is of potential interest (Reyes-Gibby et al., 2008). Macrophages are sensitive to INF-\(\gamma\). In addition, a non-lethal dose of LPS was shown to be toxic in combination with INF-\(\gamma\) in mice. The involvement of cytokines with LPS in Shwartzman-like lethal inflammatory response, was observed in rabbits and mice (Heremans et al., 1987; Heremans et al., 1990).

It is also reported that glycated proteins trigger the inflammation. There is evidence that this protein modification results in an elevated level of cytokines (Hein and Franke, 2002).

1.8 Inhibition of glycated protein induced inflammation by different antioxidants

Inflammation is known as a potential cause of neurodegenerative diseases such as AD and Parkinson’s. Several studies show neuronal cell death to be regulated by inflammation. Microglia, resident macrophages in the brain, can produce
inflammation markers such as NO and TNF-α. Such inflammation has a deleterious effect on neurons and results in neurodegenerative diseases (Ekdahl et al., 2009). Reduction of inflammation might help to decrease the level of induced damages. Polyphenols are a group of compounds with defined structures and are abundant in fruit and vegetables and are known as antioxidants and anti-inflammatory compounds (Lee et al., 2005).

The use of polyphenols as anti-inflammatories has been studied extensively (Nichols and Katiyar, 2010). The flavone structure is known as the anti-inflammatory structure in a wide range of plant extracts. Resveratrol, however belongs to a different structural family of plant extracts and has a stilbene structure (Jeong et al., 2009). This study investigates the anti-inflammatory effect of polyphenols, such as resveratrol, apigenin and Polygonum cuspidatum (see Figure 1.4) on the induction of inflammation markers induced by glycated proteins.
Figure 1.4  Chemical structures of resveratrol (A) and apigenin (C) along with their core structures and related chemical groups (B and D, respectively) (Li et al., 2005).
1.9 Inhibition of AGE-induced inflammation by receptor mediated compounds

Protein glycation is a protein modification and it is observed in ageing. Glycated proteins can be taken up via the RAGE receptor (Chavakis et al., 2004). There are various ways to intervene at the receptor level in the AGE-RAGE signalling pathway, such as blockade of the receptor by antagonist and antibody (Gilliam-Davis et al., 2007).

The RAGE receptor was implicated to be involved in AD and diabetic vascular diseases (Natarajan and Nadler, 2004). Blockade of the RAGE-ligand interaction by soluble RAGE, RAGE antibody and RAGE antagonist was considered in the reduction of plaque formation (Maillard-Lefebvre et al., 2009). RAGE-ligand interaction triggers the AGE-RAGE signalling pathway and leads to further inflammation and oxidative stress (Devangelio et al., 2007). Any intervention along this signalling pathway could reduce inflammation and, consequently, the induced damages.

In this study, several RAGE blockers have been tested, including TTP488 (previously called PF-04494700). In addition, naturally occurring biopolymers such as hyaluronic acid (HA) have been shown to have RAGE antagonising activity (Neumann et al., 1999). HA injection has also been introduced as a treatment for osteoarthritis (Sabbagh et al., 2008).

In addition to chemicals and polymers acting as antagonists for RAGE, antibodies against the RAGE receptor can be an alternative way of antagonising the receptor. Studies of such antibodies have not yet been reported; therefore, anti-RAGE antibodies, TTP488 and HA effects on AGEs induced inflammation are investigated here.

1.10 Inhibition of AGE-induced inflammation by catalase, NADPH oxidase inhibitors

The physiological role of NO signalling as an extracellular signalling molecule is now widely appreciated (Neill et al., 2002). H₂O₂ is another messenger with biochemical characteristics, is similar to NO and is predominantly involved in pro-
inflammatory signalling (Orozco-Cardenas et al., 2001; Orozco-Cardenas and Ryan, 2002). H$_2$O$_2$ can act as a messenger and transmit pro-inflammatory signals between adjacent cells (Sobey and Miller, 2005). H$_2$O$_2$ can act as a mediator molecule and it can act as a toxic substance. In higher concentrations, it is toxic whereas in lower concentrations, it is a messenger (Park et al., 2006). The special role of H$_2$O$_2$ in inflammation by some types of cells such as T-cells has been extensively investigated (Song et al., 2007). H$_2$O$_2$ is a ROS and it is hypothesised that it acts as a messenger in gene regulation and signal transduction pathways (Xing et al., 2008). It is also reported that concentration of H$_2$O$_2$ in leukocytes increases from 1 to 100 mM during phagocytosis (Ikeda and Taira, 2002). Various activities within the cells are related to H$_2$O$_2$ production.

To study the involvement of H$_2$O$_2$ in the glycated protein mediated signalling pathway, different parts of the expected signal transduction will be abolished by inhibitors. In addition, the effect of the H$_2$O$_2$ scavenging enzyme and NADPH oxidase inhibitors on inflammation markers such as NO and the synergistic effect of H$_2$O$_2$ and glycated protein on cell viability will be investigated (Figure 1.5).

NADPH oxidase

\[
\begin{align*}
\text{Acetovanillone/DPI} &\quad \rightarrow \quad \text{ROS production} \\
\text{GSH} &\quad \rightarrow \quad \text{GSSG} \\
\text{Glutathion peroxidise (GPX)} &\quad \rightarrow \\
\text{ONOO}^- &\quad \rightarrow \quad \text{NO} \\
\text{O}_2^- &\quad \rightarrow \quad \text{SOD} \\
\text{H}_2\text{O}_2 &\quad \rightarrow \quad \text{H}_2\text{O} \\
\end{align*}
\]

Catalase

**Figure 1.5** Schematic of the chemical reactions involved in antioxidant defence mechanisms (Guglielmotto et al., 2010). DPI: diphenyl iodonium chloride, GSH: reduced glutathione, GSSG: oxidized glutathione, GPX: Glutathion peroxidise, ONOO$: peroxynitrite, NADPH: nicotinamide adenine dinucleotide phosphate, ROS: reactive oxygen species, SOD: superoxide dismutase.
1.11 AIMS

The overall aim of this study is to investigate the proposed signalling pathway (see Figure 1.3) by testing the effect of different compounds such as antioxidants, receptor mediated compounds, hydrogen peroxide scavenging enzymes and NADPH oxidase inhibitors on inflammation that is induced by glycated proteins.

AIM 1 To compare the production of inflammation markers, such as NO, induced by BSA-AGE, CEA-AGE and LPS+INF-γ. A Griess reagent method will be used to detect NO and MTT. An alamar blue assay will be used to check the cell viability (CV) and ELISA used to detect TNF-α production in cell culture based assay.

AIM 2 To investigate inhibition of glycated protein-induced inflammation by different antioxidants.

AIM 3 To investigate inhibition of glycated protein-induced inflammation by receptor-mediated compounds.

AIM 4 To investigate inhibition of glycated protein-induced inflammation by hydrogen peroxide scavenging enzyme and NADPH oxidase inhibitors.
CHAPTER TWO

MATERIALS AND METHODS
2.1 Methods

2.1.1 Cell culture
All work was carried out under sterile conditions to avoid contamination with microorganisms. This includes the use of a sterile fume hood, disinfection of surfaces with 70% ethanol and the use of sterile solutions and working materials.

2.1.2 Cell maintenance
RAW 264.7 (Kirubakaran Shanmugam, Ph. D. student, Pharmacology Laboratory, UWS) and J774 (Balb/c monocyte macrophage) murine macrophages (Megan Steele, Ph. D. student, Pharmacology Laboratory, UWS) were grown in 75 cm$^2$ and 175 cm$^2$ flasks on Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) and 2 mM L-Glutamine in addition to penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). All cell lines were maintained in 5% CO$_2$ at 37°C in a humidified incubator.

2.1.3 Freezing and thawing of cells
Two cell lines were stored for several months at −80°C and for a longer period in N$_2$ at −170°C. Prior to freezing, cells were grown to confluency in 75 cm$^2$ or 175 cm$^2$ flasks. The cells were removed from the flask by scraper. After centrifuging and removing the supernatant, the cells were resuspended in 3 mL (10% DMSO, 30% FCS, and DMEM). The suspended cells were divided into 3 of (1 mL cryotubes). The suspended cells were kept in −80°C or in liquid nitrogen for the appropriate time of storage. To thaw the cells, the vial was thawed in a water bath at 37°C. The defrosted vial was then emptied into a 10 mL Falcon tube that had 8 or 9 mL of 37°C medium in it and washed by DMEM/0.1% FCS by spinning down at 900 rpm for 5 minutes.

2.1.4 Cell culture techniques
Cells were grown in 75 cm$^2$ or 175 cm$^2$ sterile tissue flasks for 24 hours until confluency in DMEM/5% FCS. They were maintained as described in section 2.1.1.
For experiments with CEA-AGEs or BSA-AGEs or (INF-γ and LPS), cells were seeded onto 96-well tissue culture plates at different densities (see Figures 3.1, 3.4 and 3.6) and, after optimisation, a density of $75 \times 10^3$ cells per well was used. Before every experiment, the cell culture medium was replaced with medium containing 0.1% FCS for one hour to minimise the interference of growth factors in the serum within the experiment. NO production was induced by the addition of CEA-AGEs or BSA-AGEs (1 mg/mL or in different doses; see Figure 3.3) for 24 hours. As a positive control for NO production, LPS (25 µg/mL) was added for 24 hours. Negative control wells contained BSA or CEA (1 mg/mL or in different doses) or medium. All experiments were performed in triplicate.

2.1.5 Concentration of the cells
Once the cells were grown to confluency within the culture flask, they were removed using a cell scraper. The cell suspension was then concentrated by centrifugation for 3 minutes at 900 rpm and resuspended in 5 mL of DMEM containing 0.1% FCS.

2.1.6 Cell count
Equal volumes of resuspended cell solution and Trypan blue (0.1%) were mixed to a total volume of 20 µL. Half of the mix was placed onto a Neubauer chamber counting slide. Sixteen squares of equal size were viewed and live cells were counted under a light microscope. The number of live cells per microlitre of cell solution was calculated by using the following equation:

$$\text{Cell count} \times 2 \times 10 = \text{number of cells per microlitre.}$$

2.1.7 Dispensing of cells into plates
Once the concentration of the cell suspension was determined, the volume of media was adjusted to the required concentration. This was achieved by dividing the total number of cells required by the number of cells per microlitre. 100 µl of cell suspension was administered to each well of a 96-well plate to give the desired cell total of $75 \times 10^3$ cells per well. For experiments using 6-well plate, $54 \times 10^4$ cells per
well were distributed into each well in a final total volume of 2 mL per well. Once the concentration of the cell suspension was determined, the volume of media was adjusted by a factor determined by dividing the total number of cells required by the number of cells per mL. Subsequently, cell concentration was adjusted and 100 µL of cell suspension were dispensed into the 60 inner wells of 96-well plates to give the desired 75 × 10³ cells per well.

2.1.8 Dilution of cell assay components

All components to be added to the wells were diluted as described in Table 2.1 to a concentration that was twice that of the desired final well concentration. This meant that 50 µL additions in a total well volume of 100 µL resulted in the correct concentration of the sample used.

2.1.9 Glycated protein characterisation and preparation

2.1.9.1 Production of advanced glycation end products

To produce AGEs, either CEA or BSA (10 mg/mL), 50 mM fructose, 500 mM glucose and 3 mM methylglyoxal in 100 mM PBS (pH = 8) were added together and incubated at 65°C for 4–6 weeks under sterile conditions. The pH was adjusted to 7.4 every week by adding sodium hydroxide (1 M). This adjustment was crucial as the reaction produces an extra positive charge of hydrogen (Figure 1.1) and a basic solution helps to precede the reaction (Ulrich and Zhang, 1997).

2.1.9.2 Preparation of dialysis tube

The glycated modified protein was subjected to dialysis to remove excess sugars. To prepare the dialysis tube (pore size of 12,000 Daltons), the desired length of tubing was boiled for 10 minutes in 0.5 L of tubing preparation solution I (see section 2.2.4), washed in distilled H₂O and boiled for 10 minutes in 0.5 L of tubing preparation solution II (see section 2.2.4). The AGEs in the tubes were then subjected to 0.9% sodium chloride in water for a week at 4°C to remove the extra sugars.
2.1.9.3 Dilutions
The concentration of the CEA-AGE or BSA-AGE was calculated on the basis of the end volume and the start concentration. The concentration was adjusted to 7 mg/mL or 10 mg/mL and sterile filtered with a disk filter (0.45 µm) before being added to the cells. 50 µL was added to each well and then adjusted to 100 µL either by experimental samples including anti-RAGE antibodies, RAGE antagonist, plant extract, catalase and acetovanillone or DMEM/0.1% FCS.

2.1.9.4 Storage of glycate protein
Glycated proteins, CEA-AGE and BSA-AGE were kept at −80°C for 6 months. Ready solutions were kept at 4°C during the experiments. Freezing and thawing were avoided to reduce the degradation over time (Goh and Cooper, 2008).

2.1.10 Sample preparation
The plant extracts including apigenin, resveratrol, Polygonum cuspidatum and NADPH oxidase inhibitors including diphenyl iodonium chloride and acetovanillone were dissolved in 1% DMSO/DMEM-0.1% FCS (Table 2.1). The undissolved plant powder part was sedimented and the supernatant was used for the experiments after sterile filtration with disk filter 0.45 µm. Plant extract was stored at 4°C and dissolved in 1% DMSO/DMEM-0.1% FCS prior to use. All antibodies were kindly provided by Abbott Neuroscience. The antibodies were aliquoted in sterile PBS and stored at a concentration of 100 µg/mL and 1 mg/mL at −80°C. All diluted antibodies were kept at −80°C until the experiment. For all experiments, the antibodies were thawed and kept at 4°C for 2–3 weeks. The concentrations of the antibodies were adjusted to 100 µg/mL and diluted in sterile PBS under sterile conditions. RAGE antagonist (TTP488) powder was kept at 4°C. TTP488 was diluted in DMEM/0.1% FCS and was vortexed to be dissolved. The dissolved TTP488 was kept as a stock solution with a concentration of 2 mM at −20°C until use. That solution was diluted to a final concentration of from 1 to 100 µM in the well prior to use. The aliquoted dissolved TTP488 was used only once during each experiment. Catalase was dissolved in fresh sterile PBS. Acetovanillone and
diphenyl iodonium chloride were dissolved in 1% DMSO/DMEM-0.1% FCS and HA was dissolved in DMEM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>1% DMSO/DMEM-0.1% FCS</td>
</tr>
<tr>
<td><em>Polygonum cuspidatum</em></td>
<td>1% DMSO/DMEM-0.1% FCS</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1% DMSO/DMEM-0.1% FCS</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Sterile PBS</td>
</tr>
<tr>
<td>TTP488</td>
<td>DMEM-0.1% FCS</td>
</tr>
<tr>
<td>Acetovanillone</td>
<td>1% DMSO/DMEM-0.1% FCS</td>
</tr>
<tr>
<td>Diphenyl Iodonium Chloride</td>
<td>1% DMSO/DMEM-0.1% FCS</td>
</tr>
<tr>
<td>Catalase</td>
<td>Sterile PBS</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>DMEM</td>
</tr>
</tbody>
</table>

2.1.11 Treatment of cells with anti-RAGE antibody and antagonist

The cells were seeded as described in section 2.1.7. After 24 hours incubation in a humidified incubator at 37°C and 5% CO₂, the medium was removed and the cells were treated with anti-RAGE antibody at concentrations between 0 and 50 μg/mL. Concentrations up to 1 mg/mL were tested and it was shown that this concentration did not affect the results (data not shown).

The concentrations of the anti-RAGE antibody added to the wells were twice the desired concentrations in the wells. The desired final concentrations of 25, 12.5, 6.25, 3.125, 1.56 and 0.78 μg/mL were calculated as 50, 25, 12.5, 6.25, 3.125 and 1.56 μg/L. An incubation time of 1 hour was not effective in the initial experiment (data not shown); however, increasing the incubation time to 3 hours showed a reduction in NO and TNF-α concentration (Figures 3.16 and 3.19). Consequently,
after incubation with antibody for three hours, the CEA-AGE (2 mg/mL) was added to the cells to make the total volume in the well of 100 μL. RAGE antagonist was dissolved in DMEM and the cells were treated with antagonist for 30 minutes prior to treatment with CEA-AGE (1 mg/mL) in the same pattern as that used for the RAGE antibody. In addition, HA low molecular weight (LMW) and HA high molecular weight (HMW) were incubated for 30 minutes at increasing concentrations between 0 and 1 mg/mL and then CEA-AGE (1 mg/mL) was added as an activator, which was followed by checking NO production with the Griess reagent method (section 2.1.14) and checking TNF-α production with ELISA (section 2.1.16) after an incubation time of 24 hours.

Cell viability as well as NO and TNF-α production were tested after 20 hours incubation with RAGE antibody and 24 hours with RAGE antagonist. Cell viability was checked by MTT (section 2.1.13) or alamar blue assay (section 2.1.13) after removing the supernatant. NO production was measured by the Griess reagent method (Larsen et al., 2008) by adding 50 μL Griess reagent and 50 μL of supernatant, followed by transferring to the 96-well tissue culture plates.

The other half of supernatant was kept at −80°C for further TNF-α concentration detection. Antibodies numbers one to four were tested.

2.1.12 Treatment of cells with antioxidants

Samples were dissolved as previously described in section 2.10. The concentration of the dissolved plant extract was calculated as two times that of the desired concentration in the wells. The cells were counted as 75,000 cells per well after optimisation (see Figures 3.1, 3.4 and 3.6) and were seeded into 96-well tissue culture plates. After 24 hours, the medium was removed and the cells were incubated for 30 minutes with different doses of plant extracts at 37°C and 5% CO₂ in a humidified incubator.

After incubation with plant extract for 30 minutes, CEA-AGE with a concentration of 1 mg/mL was added to the cells so that the total volume of the wells was 100 μL. The cells with medium were used as negative controls in triplicate experiments. The
CEA-AGE (1 mg/mL) was established as the 100% level of TNF-α and NO production. A decrease in TNF-α and NO level were determined as percentages by dividing the TNF-α and NO concentration minus the negative control concentration by the positive control minus the negative control concentration, multiplied by 100. The number of cells and the concentration of CEA-AGE were optimised previously (Figures 3.1, 3.4 and 3.6).

After 24 hours incubation at 37°C and 5% CO₂, 50 µL of supernatant was transferred to the 96-well tissue culture plates. It was then mixed with 50 µL of Griess reagent and read at 540 nm using a Bio-Rad 680 plate reader. The other 50 µL of supernatant was transferred to another 96-well tissue culture plate for further TNF-α concentration determination. The MTT cell viability assay was done after removing the supernatant. Cell viability, NO and TNF-α tests were performed as described below.

2.1.13 Cell viability assays

- **MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) Assay**

This test measures the metabolic activity of cells via the intracellular reduction of MTT, a yellow, soluble tetrazolium salt to a violet, insoluble product (formazan) with an absorption maximum of 590 nm (Freimoser et al., 1999). The reaction takes place in the inner mitochondrial membrane of intact cells via the respiratory enzymes succinate dehydrogenase and cytochrome dehydrogenase (Bernas et al., 2002). The amount of formazan produced is proportional to the number of living cells. Initially, 75 × 10^3 cells/well were seeded onto 96-well cell culture plates and grown for 24 hours. The medium was then changed and the test substances were added (100 µL end volume). Following incubation for 24 hours, the medium was removed and kept for NO and TNF-α determination and the cells were washed with 1 × PBS, after which 100 µL of a MTT stock solution (1 mg/mL) were added. After four hours incubation, the supernatant was removed and the formed formazan crystals dissolved by the addition of EtOH (96%). Dissolution of the crystals was maximised by placing the plates on a rotary shaker for 10 minutes. Absorption of the formed dye
was measured with a 96-well plate Bio-Rad 680 plate reader at 590 nm. The reduction in cell activity of treated cells was calculated as a percentage of untreated control cells, which were set as 100%. Each sample was tested in each well, once in triplicate.

- **Alamar blue assay**

  The redox dye resazurin (also known as alamar blue) detects cell viability by converting from a non-fluorescent blue dye to the highly fluorescent red dye resorufin in response to chemical reduction of the growth medium caused by cell growth (Al-Nasiry et al., 2007). The fluorescent or colourimetric signal generated from the assay is proportional to the number of living cells in the sample (O'Brien et al., 2000). Cells were incubated with a 100 µL alamar blue solution (section 2.2.4) for 1.5 hours in a 96-well plate. The fluorescence unit was read at wavelengths of $\lambda_{\text{ex}} = 545$ and $\lambda_{\text{em}} = 595$ nm.

2.1.14 Determination of nitric oxide production

Nitric oxide is determined by the Griess reagent and by quantification of nitrite, one of its stable reaction products. The Griess reagent was made of equal volumes of 1% sulphanilamide in water and 0.1% naphthyl ethylene-diamine in 5% HCl and, in the presence of nitrite, the reagent forms a violet colour. Media supernatant (50 µL) was removed from each well and by means of a multi-channel pipette was transferred to another 96-well plate in which it was mixed with 50 µL of Griess reagent. The absorbance was read at 540 nm using a Bio-Rad 680 plate reader and enabling the calculation of nitrite concentration by using a standard curve (Figure 2.1) produced from known concentrations of sodium nitrite (NaNO$_2$). The remaining 50 µL of media were removed and placed in fresh 96-well plates and frozen at $-20^\circ$C for detection of TNF-α concentration at a later time.

2.1.15 Standard curve for sodium nitrite

Concentrations of 0–50 µM sodium nitrite in DMEM were measured by Bio-Rad 680 plate reader at 540 nm using the Griess reagent method. Measurement of nitric oxide
formation was done by measuring nitrite ($\text{NO}_2^-$), one of two primary, stable and non-volatile breakdown products of NO (Caimi et al., 2008) (Figure 2.1), as described in section 2.1.14.

![Figure 2.1 Standard curve for sodium nitrite.](image)

Different nitrite concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56 (µM) and 0 versus optical density (OD) used to illustrate the nitrite standard curve. The values represent means ± SD of triplicate tests ($n = 1$).

2.1.16 Murine tumor necrosis factor-alpha (TNF-α) measurement by ELISA kit (Catalogue number: 900-K54, Peprotech)

2.1.16.1 Buffers

Wash Buffer
0.05% Tween-20 in PBS, pH = 7.4

Block Buffer
1% BSA in PBS, pH = 7.4
Diluent
0.05% Tween-20, 0.1% BSA in PBS, pH = 7.4

Buffer for substrates tetramethylbenzidine (TMB)
25.7 mL 0.2 M dibasic sodium phosphate (Na$_2$HPO$_4$)
24.3 mL 0.1 M citric acid
50 mL ddH$_2$O

TMB solution
1 mg/mL TMB in DMSO was mixed and vortexed to produce a clear solution.

Reaction mixture
9.5 mL of 0.05 M phosphate-citrate buffer plus 0.5 mL of TMB solution plus 1 µL of
30% H$_2$O$_2$.

2.1.16.2 Murine TNF-α ELISA kit contents

Capture Antibody
Kit contained 100 µg of capture antibody that was reconstituted in 1 mL sterile water
to a concentration of 100µg/mL.

Detection Antibody
Kit vial contained 25 µg biotinylated antigen-affinity purified goat anti-murine TNF-
α + 2.5 mg D-mannitol. Vial was centrifuged prior to opening and contents
reconstituted to a concentration of 100 µg/mL.

Murine TNF-α Standard
The vial contained 1 µg of recombinant murine TNF-α + 2.2 mg BSA + 11.0 mg D-
mannitol. Vial was centrifuged prior to opening and reconstituted in 1 mL of sterile
water to a concentration of 1 µg/mL.

2.1.16.3 Storage of buffers, antibodies and solutions
During experiments, the reconstituted components were kept for 2 weeks within a
range of 2°C–8°C. Components were reconstituted, aliquoted and stored at −20°C
for up to 6 months. Block buffer and diluent were sterile filtered (disk filter 0.45 µm)
and stored at 4°C for up to one week. TMB powder was kept at 4°C.
2.1.16.4 TNF-α ELISA modification

The concentration of murine TNF-α in cell culture medium was determined by ELISA. The supplier’s guide accompanying the ELISA kit (PeproTech #900-K54) was altered slightly. All kit protocols were followed except that the concentrations of capture antibody, detection antibody and peroxidase conjugate were reduced to half of the recommended concentration. This allowed for more assays to be performed by one kit.

2.1.16.5 ELISA method

Initially, 100 µL of capture antibody was added to each well of a flat bottom, 96-well ELISA plate. The plate was then sealed with parafilm and incubated overnight at room temperature (RT). It was washed three times with wash buffer by shaking with a programmed plate washer. Then 300 µL of block buffer were added to each well. The plate was then covered and incubated for one hour at RT and RT definition differs among countries, but is typically defined as between 20°C and 25°C. Here, the ELISA plate was kept in an oven with the temperature controlled among and during the different steps of the experiments at 27°C, slightly higher than the typical room temperature range. Following incubation, the plate was washed three times with shaking by a programmed plate washer. For standard curve development, serial dilutions of TNF-α standard from 0 to 2,000 pg/mL in diluent were prepared. Four microlitres of stock in 1000 µL of diluent (4 ng/mL) was the initial concentration, followed by two fold dilution in the diluent. For the samples, the dilution factor was adjusted prior to the experiments with 75,000 cell numbers in order to be able to see the signal difference between lower and higher concentrations (and to avoid saturation, which leads to false negative results). The samples were then diluted in diluents accordingly. This was followed by addition of 100 µL of diluted samples or different TNF-α concentration of standard to the 96-well ELISA plate. The plate was covered and incubated for 2.5 hours at RT. The plate was then washed four times by shaking with a programmed plate washer. The detection antibody was diluted in diluents at a concentration of 50 µg/mL. Then 100 µL of the detection antibody were added to each well, covered and incubated for 2.5 hours at RT. The plate was washed four times with shaking by a programmed plate washer. Subsequently, avidin
peroxidase conjugate was diluted 1:4000 in diluent and 100 µL of it was added to each well. This was then covered and incubated for 30 minutes at RT, followed by four washings using the plate washer.

2.1.16.6 Colour development using tetramethylbenzidine (TMB)

For colour development, 100 µL of reaction mixture was added to the wells after the washing program in the programmed plate washer. $\text{H}_2\text{O}_2$ was added after preparing a complete reaction mixture solution and the solution was mixed vigorously. The plate was then incubated at RT. Colour development (blue) was monitored at 655 nm and a reading was taken every 5 minutes to determine the linear phase of the reaction. The supplier’s (PeproTech) protocol does not recommended the addition of a reaction stopping solution, but after 30 minutes the reaction was stopped by adding 0.5 M sulphuric acid (100 µL per well). The absorbance was then measured at 450 nm with a reference wavelength at 650 nm. Following the reaction the blue colour changes to yellow.

2.1.16.7 Standard curve for TNF-α

TNF-α concentration ranging from 2 to 0 ng/mL in diluent were prepared. Different TNF-α concentrations were added to each well in triplicates. Colour development measured as optical density (OD) versus concentration was reported at 655 nm (Figure 2.2) and 450 nm with the reference wavelength of 655 nm (Figure 2.3). The standard curve is non-linear and a sigmoidal dose response (variable slope) model was used to fit the standard curve in Graphpad Prism. The sample’s OD was measured and calculated by Graphpad Prism to a correspondent concentration by using the analysing option in Graphpad Prism 4 and 5. The equations and calculations for reactions with and without a reaction stop (see section 2.1.16.6) are shown in appendices 1 and 2. The equations were used for further concentration measurements.
Figure 2.2  Standard curve for TNF-α without the reaction stop solution.

Six different TNF-α concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL versus OD at 655 nm illustrated as a TNF-α standard curve. The values are expressed as mean ± SD of triplicate tests. The sigmoidal dose-response (variable slope) curve fit of Graphpad Prism was used (n = 1).
Figure 2.3  Standard curve for TNF-α with a reaction stop solution.
Six different TNF-α concentrations of 2000, 1000, 500, 125, 62.5, 31.25 and 0 pg/mL versus OD at 450 nm with reference wavelength 650 nm illustrated as the TNF-α standard curve (sigmoidal dose response fit provided by Graphpad Prism). The values are expressed as mean ± SD of triplicate tests (n = 1).
2.1.17 Statistical analysis
Statistical analysis was performed by using Student’s $t$-test as included in Graphpad Prism 4 (see Figures 3.11 and 3.18).

2.1.18 Hydrogen peroxide measurement by Amplex Red assay
Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) is a non-fluorescent molecule that, when oxidised by H$_2$O$_2$ in the presence of horse radish peroxidise (HRP), changes to resorfurine (Figure 2.4), a highly fluorescent product (Votyakova and Reynolds, 2004). This reaction may be used to detect low amounts of H$_2$O$_2$ in biological samples. Here, cells were seeded in a 6-well tissue culture plate with 540,000 cells per well and 2 mL DMEM/0.1%FCS. The reason for the high cell numbers was that at lower numbers the concentration of H$_2$O$_2$ was too low to detect (data not shown) but by increasing the number of cells, H$_2$O$_2$ could be detected. After 24 hours of incubation, the medium was removed and CEA-AGE (1 mg/ml) or LPS (25 µg/mL) in PBS (pH = 7.4) was added to the wells. With a total volume of 2 mL, DMEM could not be used as it interfered with fluorescence detection. The Amplex Red solution was added to a new 96-well tissue culture plate prior to the experiments. Supernatant (10 µL) of the cells treated with either CEA-AGE (1 mg/mL) or LPS (25 µg/mL) at different time points were added to 100 µL of Amplex Red solution in a 96 well tissue plate at 5 min intervals in triplicate. A waiting time of 30 minutes for detection was established and all samples were detected with an interval of 5 minutes by plate reader. The fluorescence was read at $\lambda_{ex} = 563$ and $\lambda_{em} = 587$ nm wavelengths. A solution containing no H$_2$O$_2$ was used to determine the background fluorescence level, a measure that was subtracted from all experimental values. Furthermore, the absorbance at 540 nm was used as another method of H$_2$O$_2$ detection because of the unavailability of equipment in a certain period. Similar results from both methods were obtained.
2.1.19 **Standard curve for hydrogen peroxide**

Different concentrations of H$_2$O$_2$ from 0 to 100 µM in a volume of 10 µL in PBS were added to Amplex Red solution (100 µL) in a 96-well plate and the fluorescence unit was read at $\lambda_{ex} = 563$ and $\lambda_{em} = 587$ nm and was used as the standard curve for further H$_2$O$_2$ measurement (Figure 2.6). Absorbance at 540 nm was also used for detection (Figure 2.5) of different concentrations of H$_2$O$_2$ because of the unavailability of equipment in a certain period. The standard curves were used for further H$_2$O$_2$ concentration measurement.
Figure 2.5 Standard curve for hydrogen peroxide using the absorbance method.

Different $H_2O_2$ concentrations of 125, 62.5, 31.25, 15.62 and 0 µM versus OD at 540 nm are fitted with a linear curve and presented as a $H_2O_2$ standard curve. The values are expressed as mean ± SD in triplicate tests ($n = 1$).
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Figure 2.6 Standard curve for hydrogen peroxide using the fluorescence method.

Correlation between the fluorescence levels at $\lambda_{ex} = 563$ and $\lambda_{em} = 587$ nm with different $H_2O_2$ concentrations of 30, 15, 3.75 and 1.67 and 0 µM are illustrated. The values are expressed as mean ± SD in triplicate tests ($n = 1$).

2.1.20 Treatment of cells with hydrogen peroxide

Cells were seeded at 75,000 cells per well and incubated for 24 hours. After 24 hours the media was removed and $H_2O_2$ in PBS was added to the cells and incubated for one hour at 37°C. After the hour of incubation, the solution was removed and cells were treated with CEA-AGE (1 mg/mL). The cell viability was measured by MTT assay (see section 2.1.13) after 24 hours (see Figure 3.33) and expressed as a percentage using calculation of $\left[\frac{(OD \text{ sample} - OD \text{ negative control})}{(OD \text{ positive control} - OD \text{ negative control})}\times 100$. 

$Y = 214.9X + 106.8$

$R^2 = 0.991$
2.2 Materials

2.2.1 Equipment

Bench centrifuge 5424
Big Scraper
Cell culture flask (75 cm\(^2\) and 175 cm\(^2\))
Cell culture plate, 6-wells
Cell culture plate, 96-wells
Centrifuge 5810
Cryotube
Disposable disk filter 0.2 and 0.45 µm
ELISA tissue culture plate, flat bottom
Fluorescence plate reader
Gloves
Hemocytometer
Incubator
Power supplies
pH meter 3505
Plate washer ELX405
Scale AFA-210LC
Syringe and needles
Small Scraper
Spectrophotometer plate reader
Stepper
Stepper pipette
Vortex
Water bath

2.2.2 Software

Adobe Acrobat 8.1 was used to produce Figure 1.2. Graphpad Prism 4 or GraphPad Prism 5 were used to draw all other graphs as well as to perform calculations and statistical analyses. Microsoft Office Word was used to prepare the text and Microsoft PowerPoint 2003 or Microsoft PowerPoint 2007 were used to draw Figure
1.3. ACD ChemSketch Freeware 10 was used to draw the chemical structures Figures 1.1 and 1.4. Endnote X1 was used for the reference list and text citations.

### 2.2.3 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alamar blue</td>
<td>BioSource</td>
</tr>
<tr>
<td>Amplex red (10-Acetyl-3, 7-dihydroxyphenoxazine)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Apigenin</td>
<td>Nutrafur</td>
</tr>
<tr>
<td>Acetovanillone</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Catalase</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Chicken Egg Albumin (CEA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>DMEM</td>
<td>GIBCO</td>
</tr>
<tr>
<td>Diphenyl idodnium chloride (DPI)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3,3′-[(3,3′-Dimethyl[1,1′-biphenyl]-4,4′-diyl]bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt (Trypan Blue)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid (EDTA)</td>
<td>AMRESCO</td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>D-(-) Fructose</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>D- (+) Glucose</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Interferon gamma (INF-γ)</td>
<td>PeproTech</td>
</tr>
<tr>
<td>Hyalouronic acid (HA)</td>
<td>Lifecore</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>Merck</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Monobasic dehydrate sodium phosphate</td>
<td>AMRESCO</td>
</tr>
<tr>
<td>N (-1-naphtyl) ethylenediamine dihydrochloride</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td><em>Polygonum cuspidatum</em></td>
<td>Lipa Pharmaceutical</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Lipa Pharmaceutical</td>
</tr>
</tbody>
</table>
Sodium Chloride AMRESCO
Sodium dihydrogen orthophosphate, monohydrate AMRESCO
Sodium phosphate dibasic anhydrous AMRESCO
Sulfanilamide Sigma Aldrich
Tetramethylbenzidine (TMB) Sigma Aldrich
TTP488 Sanofi Aventis
Tubes Dialysis Sigma Aldrich
Tween-20 AMRESCO

2.2.4 Buffers, media and solutions

Alamar blue Solution
2.5 mg/L resazurin sodium salt in DMEM

Amplex Red Solutions
Horse radish peroxidase (HRP) 10 U/mL and 100 µM Amplex Red (AR) in PBS

Griess Reagent
Equal volumes of 1% sulfanilamide in water and 0.1% napthyl ethylene-diamine in 5% HCl

MTT Solution
1 mg/mL MTT in PBS, pH = 7.4

100 mM Sodium phosphate buffer (PBS), pH = 8 and pH = 7.4
0.2 M Sodium phosphate dibasic anhydrous (Na$_2$HPO$_4$)
0.2 M monobasic dehydrate sodium phosphate (NaH$_2$PO$_4$, 2H$_2$O)

Tubing preparation solution I
2% w/v sodium bicarbonate + 1 mM EDTA, pH = 8

Tubing preparation solution II
1 mM EDTA, pH = 8
CHAPTER THREE

RESULTS
3. Results

3.1 Induction of inflammation by glycated proteins, interferon gamma and lipopolysaccharide

The synergistic effects of LPS and INF-γ on nitric oxide production in macrophages were investigated and it was confirmed that the combination of LPS and INF-γ stimulates the production of inflammatory markers (Figure 3.5). In addition, the effects of glycated modified proteins such as BSA-AGE and CEA-AGE on nitric oxide production were compared.

3.1.1 Optimisation of cell numbers and cell viability assay

Cell number variations were tested to determine if such variation can affect the cell viability. Cell viability was checked in different numbers of cells in response to effective doses of CEA-AGE. There were no significant differences in the cell viability assay using different cell numbers and cell death increased with the highest number of dead cells in the high concentration of CEA-AGE (Figure 3.1). However, based on the results from various concentrations of TNF-α (Figure 3.6), a 75,000 cell number was estimated to be optimal.

3.1.2 Comparison of BSA-AGE and CEA-AGE for nitric oxide production

Two glycated proteins, BSA-AGE and CEA-AGE, were assessed and the potency of NO production and cell viability were compared. Non-glycated proteins (BSA and CEA 1 mg/mL) were used as negative controls (Figure 3.2, graphs A and B). The data show that glycation increased the production of the NO inflammation marker. Cell viability decreased at concentrations of more than 0.5 mg/mL of CEA-AGE to 75%. This indicates that glycation induces mild cell death (Figure 3.1). TNF-α concentrations were also tested (data not shown) and similar results were obtained.
Figure 3.1  Optimisation of cell numbers for MTT assay.

Cell viability (as %) of control in response to CEA-AGE treatment of the concentrations of 3.5, 1.75, 0.8, 0.4 and 0 mg/mL by MTT assay with different cell numbers of RAW 264.7 murine macrophages are shown. Percentages were calculated by \[\frac{(OD \text{ sample} - \text{background})}{(OD \text{ non simulated cells} - \text{background})} \times 100\]. All data are presented as the mean ± SD in triplicate tests (n = 1).
Figure 3.2  Comparison of cell viability and nitric oxide production with two glycated proteins: BSA-AGE and CEA-AGE.

Two different proteins were incubated with sugars (CEA-AGE with concentrations of 0, 0.312, 0.625, 1.25 and 2.5 mg/mL) and (BSA-AGE with concentrations of 0, 0.218, 0.43, 0.875, 1.75, 3.5 and 7 mg/mL). The potency for inflammatory response, measured as nitrite (in µM), along with the cell viability (in %) after 24 hours incubation with 75,000 cells. A. CEA-AGE and B. BSA-AGE. The values are expressed as mean ± SD of triplicate tests (n = 2).
3.1.3 Comparison of two glycated proteins
Two glycated proteins at the same concentrations (1 mg/mL) are compared for NO production in Table 3.1 and Figure 3.2. The results show that CEA-AGE produced slightly more NO than BSA-AGE. The experiment was repeated and produced a similar result (Table 3.1). Therefore after comparison, CEA-AGE was selected for further experiments. The TNF-α production using the two glycated proteins showed a similar result.

3.1.4 Optimisation of nitric oxide production with increasing concentrations of CEA-AGE in two cell lines
The best concentration of CEA-AGE for assessing NO production in two different cell lines of RAW 264.7 and J774 murine macrophages was investigated. As shown in Figure 3.3, a concentration of 1 mg/mL was selected as the optimum concentration for NO production. That was because 1 mg/mL produced enough NO to differentiate it in different experiments; moreover, the cell viability was about 90%. Therefore I used this concentration was used for all further experiments.

3.1.5 Optimisation of cell number and nitric oxide production
The optimum cell number for NO production in response to the effective doses of CEA-AGE was measured in RAW 264.7 murine macrophages (Figure 3.4). The results indicated that 75,000 cells were optimal for further experiments based on NO and TNF-α production (Figures 3.4 and 3.6).

3.1.6 Comparison of nitric oxide production with LPS and INF-γ in RAW 264.7 murine macrophage cell line
The combination of (LPS+INF-γ) for NO production was compared with that from glycated proteins (Figures 3.2, 3.3 and 3.4). LPS (1 mg/mL) and INF-γ (0.4
The LPS+INF-γ treatment produced a concentration of NO that was comparable to that from glycated proteins (Figures 3.4 and 3.5); however, a reduction of the potency was observed during the period of time. The potency of LPS+INF-γ with 20 µM NO production was the same as that of the glycated proteins (Figures 3.3 and 3.4). The potency of the glycated proteins decreased during the one year period over which testing occurred (Figures 3.2, 3.3 and 3.4).

Table 3.1 Concentration of nitric oxide, measured as nitrite production (µM) in cells treated with CEA-AGE (1 mg/mL), BSA-AGE (1 mg/mL) and in control cells in duplicate.

<table>
<thead>
<tr>
<th>Glycated proteins</th>
<th>Nitrite (µM) production</th>
<th>Nitrite (µM) production</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-AGE (1 mg/mL)/ control</td>
<td>35/2</td>
<td>32/4</td>
</tr>
<tr>
<td>CEA-AGE (1 mg/mL)/ control</td>
<td>48/11</td>
<td>43/8</td>
</tr>
</tbody>
</table>
Figure 3.3  Nitric oxide productions in two cell lines treated with CEA-AGE at different doses.

NO production, measured as nitrite (µM), in response to decreasing CEA-AGE concentrations of 3.5, 1.75, 0.8, 0.4 and 0 (mg/mL) in comparison to non-activated cells in two different cell lines with 75,000 cells per well after 24 hours incubation: RAW 264.7 and J774 murine macrophage cell lines. The values are expressed as mean ± SD of triplicate tests (n = 1).
Figure 3.4 Optimisation of nitric oxide production using different cell numbers.

NO production, measured as nitrite (µM), in response to different CEA-AGE concentrations of 1.75, 0.8, 0.4 and 0 (mg/mL) in different cell numbers in RAW 264.7 murine macrophage cell line. The values are expressed as mean ± SD of triplicate tests (n = 1).
Figure 3.5  Nitric oxide productions in response to different doses of LPS+INF-γ.

NO production, measured as nitrite (μM) in response to three combinations of LPS+INF-γ treatment compared with non-activated (in medium) in RAW 264.7 murine macrophage cells. The values are expressed as mean ± SD of triplicate tests (n = 1).
3.1.7  **Cell number optimisation and TNF-α measurement**

The TNF-α concentration was measured in different cell numbers in response to CEA-AGE (1 mg/mL) treatment. On the basis of the results (Figure 3.6), a 75,000 cell number was estimated to be the optimal cell number for determination of TNF-α concentration. Figure 3.6 shows TNF-α concentration saturation at the 100,000 and 85,000 cell numbers.

3.2.  **Inhibition of glycated protein-induced inflammation by different antioxidants**

In this section, results of testing of polyphenols of apigenin, *Polygonum cuspidatum* (PC) and resveratrol for their efficacy in reduction of TNF-α and NO in two macrophage cell lines are reported.

3.2.1  **Optimisation of dilution factors for TNF-α measurement**

Dilution is a way to detect cytokine level by ELISA in various biological samples (Miura et al., 2008). A dilution factor experiment was undertaken to determine the dilution factor most suitable for TNF-α concentration measurement (Figure 3.7). There was no difference in the resulting concentrations between the undiluted samples and the different concentrations of apigenin treated samples. All dilution factors could detect differences in concentration between different apigenin concentration treated samples.

3.2.2  **Optimisation of dilution factors by calculation**

To find the best dilution factor, different dilution factors with corresponding concentrations and the ratio of the maximum to minimum concentrations were calculated (Table 3.2). The results show that a dilution factor of 100 could
differentiate TNF-α concentrations most accurately and over a wider TNF-α concentration range; hence, the 100 dilution factor was used for further concentration measurements.

Figure 3.6  Optimisation of cell numbers for TNF-α production.

TNF-α (pg/mL) production in response to treatment with (1 mg/mL) of CEA-AGE using different cell numbers of the RAW 264.7 murine macrophage cell line in comparison to unstimulated cells (medium) is shown. The values are expressed as mean ± SD of triplicate tests (n = 1).
Figure 3.7 Optimisation of dilution factor for TNF-α detection.

TNF-α concentrations with different dilution factors in apigenin treated cells with (1 mg/mL) of CEA-AGE activation in comparison to concentrations in non-diluted samples are shown. The values are expressed as mean ± SD of triplicate tests (n = 1). This experiment was repeated twice and the results were similar.
Table 3.2  Optimisation of dilutions for TNF-α detection, measured by the ratio of highest to lowest sample concentrations in apigenin treated samples (data from Figure 3.7).

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>100</th>
<th>66</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum ± SD TNF-α (pg/mL)</td>
<td>1455 ± 341</td>
<td>1645 ± 301</td>
<td>2288 ± 262</td>
</tr>
<tr>
<td>Minimum ± SD TNF-α (pg/mL)</td>
<td>506 ± 118</td>
<td>611 ± 53</td>
<td>996 ± 114</td>
</tr>
<tr>
<td>Ratio of maximum/Minimum concentrations</td>
<td>2.8</td>
<td>2.69</td>
<td>2.2</td>
</tr>
</tbody>
</table>

3.2.3 Reproducibility of the half maximum inhibitory concentration (IC$_{50}$) values between experiments in one cell line for apigenin treatment

Reproducibility is an important factor in good cell culture practice (GCCP) (Van der Valk et al. 2010). This experiment was undertaken to check the reproducibility of the data in two independent experiments using the RAW 264.7 murine macrophage cell line. TNF-α and NO production were determined within one month in two independent experiments. Productions were compared based on percentages (Figure 3.8) and concentrations (Figures 3.9 and 3.10). The IC$_{50}$ values were calculated by plotting the curve in Graphpad Prism 4 or 5 and drawing a line for 50% inhibition and then checking the concentration along the X axis (results summarised in Table 3.3). Reproducibility of IC$_{50}$ values (see Table 3.3), as well as TNF-α (see Figure 3.9) and NO production (see Figure 3.10) was tested in RAW 264.7 murine macrophage cells. The results show a GCCP with reproducible results in two
independent experiments within one month. The results were consistent between the two experiments.

3.2.4 Inhibition of nitric oxide production induced by CEA-AGE by apigenin
The reduction of NO production by apigenin (5 µM) treatment in comparison to that in controls (CEA-AGE 1 mg/mL treated cells) was investigated (Figure 3.11). Graphpad Prism was used to perform the statistical analysis (Student’s t-test). Figure 3.11 shows that apigenin treatment significantly reduced inflammation ($p < 0.0001$).

3.2.5 Reproducibility of the experiments between two cell lines
The reproducibility of apigenin experiments between two cell lines of RAW 264.7 and J774 murine macrophages was also tested (Figure 3.12). The results show that the IC$_{50}$ values were at concentrations of less than 10 µM in both cell lines. A similar result was shown in a comparison of concentrations (Figure 3.13). Figures 3.12 and 3.13 show that the reduction of NO production is less than 10 µM in both cell lines.
Figure 3.8 Effect of apigenin on CEA-AGE treated RAW 264.7 murine macrophages.

Inhibition of inflammation induced by CEA-AGE (1 mg/mL) by different concentrations of apigenin, 5, 2.5, 1.25 and 0.62 and 0 μM after 24 hours incubation and reproducibility in two independent experiments with 75,000 cell numbers in RAW 264.7 murine macrophage cells are shown. The values are expressed as mean ± SD of triplicate tests. The data are shown as percentages of control (A and B).
Chapter Three. Results

A.

![Graph A](image)

B.

![Graph B](image)

**Figure 3.9** Apigenin effect on TNF-α production induced by CEA-AGE.

Inhibition of inflammation induced by CEA-AGE (1 mg/mL) with different concentrations of apigenin treatment, 5, 2.5, 1.25 and 0.62 and 0 µM after 24 hours incubation and reproducibility in two independent experiments with 75,000 cell numbers in a RAW 264.7 murine macrophage cell line are shown. The values are expressed as mean ± SD of triplicate tests. TNF-α concentration is in pg/mL. Graphs A and B reflect the data presented in graphs A and B of Figure 3.8.
Figure 3.10  Apigenin effect on nitric oxide production induced by CEA-AGE. Inhibition of inflammation induced by CEA-AGE (1 mg/mL) with different concentrations of apigenin, 5, 2.5, 1.25 and 0.62 and 0 µM after 24 hours incubation and reproducibility in two independent experiments with 75,000 cell numbers in a RAW 264.7 murine macrophage cell line are illustrated. The values are expressed as mean ± SD of triplicate tests. The NO concentration is shown as nitrite (µM). Graphs A and B reflect the data presented in graphs A and B of Figure 3.8.
Table 3.3 IC\textsubscript{50} values in two independent experiments of apigenin treated cells in the RAW 264.7 murine macrophage cell line.

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{50} of Apigenin (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-\alpha</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td>5.03</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.844</td>
</tr>
<tr>
<td></td>
<td>1.07</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values from two independent experiments are shown. The data were extracted from Figure 3.8.
Figure 3.11  Apigenin effect on CEA-AGE induced nitric oxide production. RAW 264.7 murine macrophages were pre-incubated with apigenin (5 µM) prior to treatment with CEA-AGE (1 mg/mL) and compared to CEA-AGE (1 mg/mL) activated cells. The difference was significant ($p < 0.0001$), after 24 hours incubation. The values are expressed as mean ± SD of triplicate tests ($n = 2$).
Figure 3.12  Effect of apigenin on CEA-AGE induced nitric oxide production and cell viability in two cell lines.

Inflammation reduction by apigenin 0–10 µM in two cell lines after 24 hours incubation with CEA-AGE (1mg/mL) in A: RAW 264.7 (see also Figure 3.8) and B: J774 murine macrophage cell lines. The values are expressed as mean ± SD of triplicate tests (n = 2). The data are shown as percentages of control levels.
Chapter Three. Results

A. RAW 264.7

B. J774

Figure 3.13  Effect of apigenin on CEA-AGE induced nitric oxide production in two cell lines.

Inflammation reduction by apigenin in two cell lines in concentrations between 0–10 μM after 24 hours incubation with CEA-AGE (1 mg/mL) in A: RAW 264.7 and B: J774 murine macrophage cell lines are illustrated. The values are expressed as mean ± SD of triplicate tests (n = 2). The data are shown as nitrite concentrations (μM). Data extracted from graphs A and B of Figure 3.12.
3.2.6 Effect of resveratrol on inflammation induced by CEA-AGE
The effect of resveratrol on inflammation induced by CEA-AGE (1 mg/mL) was confirmed on the basis of its ability to reduce nitric oxide production to 50% (Bi et al., 2005). Further, the anti-inflammatory effect of resveratrol on NO production induced by CEA-AGE (1 mg/mL) along with its effect on cell viability was determined. A slight inhibition of NO production (about 40%) was observed with the addition of 2 mM of resveratrol (see Figure 3.14).

3.2.7 Effect of Polygonum cuspidatum on inflammation induced by CEA-AGE
The anti-inflammatory activity of Polygonum cuspidatum is dependent on the extraction method (Ishihara et al., 2000). In this experiment, the anti-inflammatory effect of a Polygonum. cuspidatum extract containing resveratrol on NO production induced by CEA-AGE (1 mg/mL) along its effect on cell viability were investigated. A maximum 50% inhibition with 90% cell viability with Polygonum cuspidatum concentration (1 mg/mL) was observed (Figure 3.15). PC is known as a source of resveratrol (Wang et al., 2011) and this additional inhibitory effect of PC in comparison to purified resveratrol may be attributed to other unknown compounds present in the plant extract.
A.

B.

Figure 3.14  Effect of resveratrol treatment on CEA-AGE induced nitric oxide production and cell viability.

Inhibition of inflammation induced by CEA-AGE (1 mg/mL) with increasing concentrations of resveratrol of 0, 0.02, 0.2 and 2 mM after 24 hours incubation. The values are expressed as mean ± SD of triplicate tests (n = 1). The data are shown as percentages of control (graph A) and resveratrol concentrations in µM (graph B).
Figure 3.15 Effect of *Polygonum cuspidatum* on CEA-AGE induced nitric oxide production and cell viability.

Inhibition of inflammation induced by CEA-AGE (1 mg/mL) with increasing concentrations of *Polygonum cuspidatum* extract, 0, 0.05, 0.5, 5, 50 and 500 µg/mL after 24 hours incubation is shown. The values are expressed as mean ± SD of triplicate tests (n = 2). The data are shown as percentages of control (graph A) and nitrite concentrations (µM) (graph B). The X axes are in logarithmic scale.
3.3 Inhibition of AGE-induced inflammation by receptor mediated compounds

In this section, the effect of natural and synthetic RAGE antagonist and RAGE antibody on inflammation induced by glycated protein in cell based assay is investigated.

3.3.1 Effect of different antibodies on nitric oxide production induced by CEA-AGE

Expression of RAGE-induced NO production by glycated protein has been reported to be decreased by RAGE antibody (Chen et al., 2009). Four different antibodies (two RAGE antibodies and two controls, all obtained from Abbott Neuroscience), were investigated for inhibition efficacy on NO production induced by CEA-AGE (Figure 3.16) and it was a blind study to the experimenter. The results of the blind study show that antibodies number one and two could be RAGE antibody candidates for further development. First, the antibodies were tested following 30 minutes of incubation and there was no change in inflammation level (data not shown). However, by increasing incubation time to three hours, a reduction was observed indicating that the antibodies have a slow affinity effect. The effect of the antibodies decreased during the period tested with the early test periods showing the greatest reduction (graph A, Figure 3.19).

3.3.2 Inhibitory effect of two effective antibodies on nitric oxide production induced by CEA-AGE

Two antibodies received from Abbott Neuroscience were investigated for inhibition efficacy on NO production (measured as nitrite concentration, µM) (Figure 3.17). The results show that both antibodies reduced the NO production up to 10 µM in comparison to positive control of 20 µM.
3.3.3 Comparison of IC$_{50}$ values for antibodies number one and two

Table 3.4 shows the IC$_{50}$ values determined for antibodies number one and two for inhibition effect on NO production.

3.3.4 Inhibitory effect of nitric oxide production induced by CEA-AGE by RAGE antibody number one

The reduction of NO production by RAGE antibody in comparison to that in controls (CEA-AGE treated cells) is shown in Figure 3.18. and the Statistical analysis was done by Student’s $t$-test.
Figure 3.16 Effect of four antibodies on nitric oxide production and cell viability.

RAW 264.7 murine macrophages were pre-incubated for 3 hours with different concentrations of four antibodies (0–30 µg/mL) and their effects on nitric oxide (NO), TNF-α and cell viability (CV) after induction by CEA-AGE (1 mg/mL) and following 20 hours incubation are illustrated. The values are expressed as mean ± SD in triplicate tests (n = 2) and are shown as percentages (A, B, C and D are for the four antibodies tested).
Figure 3.17  Effect of antibodies number one and two on CEA-AGE induced nitric oxide production.

The effects of antibodies one and two on NO production, measured as nitrite (µM), induced by CEA-AGE (1 mg/mL) after 20 hours incubation are shown. The values are expressed as mean ± SD in triplicate tests (n = 2). Concentrations tested for antibody number one were 0, 0.6, 1.25, 3.75, 7.5, 15 and 21.5 µg/mL and for antibody number two were 0, 0.78, 1.56, 2.12, 6.25, 12.5 and 25 µg/mL. Nitrite (µM) levels in A and B derived from graphs A and B in Figure 3.16.
Table 3.4  \( IC_{50} \) values of antibodies one and two for inhibition of nitric oxide production.

<table>
<thead>
<tr>
<th></th>
<th>( IC_{50} ) of antibody number one</th>
<th>( IC_{50} ) of antibody number two</th>
</tr>
</thead>
<tbody>
<tr>
<td>( IC_{50} )</td>
<td>21.5 (µg/mL)</td>
<td>25 (µg/mL)</td>
</tr>
</tbody>
</table>

The \( IC_{50} \) values are determined for antibodies one and two for inhibition of NO production induced by CEA-AGE (1 mg/mL) (see Figure 3.16, graphs A and B).

*** \( p < 0.005 \)

![Graph](image)

Figure 3.18  Effect of antibody number one on CEA-AGE induced nitric oxide production.

RAGE antibody number one effect on CEA-AGE (1 mg/mL) induced inflammation in comparison with medium and with CEA-AGE-only (1 mg/mL) treatments after 24 hours incubation are shown. The values are expressed as mean ± SD in triplicate tests (n = 1).
3.3.5 Effect of RAGE antibodies on CEA-AGE induced inflammation in three independent experiments

Antibody number one was used for further experiments as a RAGE antibody based on the results. The reduction of NO and TNF-α production by antibody number one was investigated in three independent experiments using the same activator in different batches, CEA-AGE (1 mg/mL) from different aliquoted antibodies (Figure 3.19).

3.3.6 TNF-α reduction with RAGE antibodies in two independent experiments

The reduction of TNF-α concentration (pg/mL) in response to CEA-AGE (1 mg/mL) and RAGE antibody number one treatment was investigated (Figure 3.20).

3.3.7 Effect of RAGE antibody number one on CEA-AGE induced nitric oxide production in three independent experiments

A reduction of NO production, measured as nitrite (μM) in response to CEA-AGE (1 mg/mL) and antibody number one treatment was detected (Figure 3.21).

3.3.8 IC$_{50}$ values determined for antibody number one

IC$_{50}$ values extracted for antibody number one with potency for NO reduction was tested with three different batches of CEA-AGE (1 mg/mL) within a period of a year. The results are compared in Table 3.5. This table shows that different CEA-AGEs with different potencies gave different IC$_{50}$ values and IC$_{50}$ values were ligand dependent.
A.

![Graph A](image)

- NO
- CV
- TNF-α

B.

![Graph B](image)

- NO
- CV
Figure 3.19  Effect of RAGE antibody number one on CEA-AGE induced inflammation along with cell viability measure in three batches.

RAGE antibody number one effects on NO and TNF-α production, induced by CEA-AGE (1 mg/mL) with different potencies were measured after 20 hours incubation. The values are expressed as mean ± SD in triplicate tests (n = 2). The values are shown in percentages of control levels (graphs A, B and C). Antibodies with different potencies are shown in graph A with concentrations of 0, 1.300, 2.650, 5.370 and 10.750 µg/mL, graph B with concentrations of 0, 0.60, 1.25, 3.75, 7.50, 15.00 and 21.5 µg/mL and graph C with concentrations of 0, 0.625, 1.250, 2.500, 5.000 and 10.000 µg/mL were tested.
Figure 3.20 Effect of RAGE antibody number one on TNF-α induced production by CEA-AGE.

RAGE antibody number one effect on TNF-α production, induced by CEA-AGE (1 mg/mL) with different potencies was measured after 20 hours incubation. The values are expressed as mean ± SD in triplicate tests (n = 2). TNF-α value are shown as concentrations (pg/mL). (graphs A and B) derived from graphs A and B Figure 3.19.
A.

![Graph A](image1)

B.

![Graph B](image2)
Figure 3.21  Effect of antibody number one on nitric oxide production induced by CEA-AGE.

CEA-AGE (1 mg/mL) with different potencies was added to RAW 264.7 murine macrophages. The cells were pre-incubated with antibody number one for 3 hours prior to activation. NO production was detected after 20 hours incubation with CEA-AGE (1 mg/mL). The values are expressed as mean ± SD in triplicate tests (n = 1). The data are shown as nitrite concentrations (µM). (graphs A, B and C are derived from Figure 3.19.)
Table 3.5  IC₅₀ values for anti-RAGE antibody number one based on activation with various antibodies batches.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>IC₅₀ of antibody number one for Nitrite (µg/mL)</th>
<th>CEA-AGE (1 mg/mL) potency for Nitrite production (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment one</td>
<td>1.3</td>
<td>20</td>
</tr>
<tr>
<td>Experiment two</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Experiment three</td>
<td>21.5</td>
<td>39</td>
</tr>
</tbody>
</table>

3.3.9 TTP488 effect on inflammation markers
Receptor mediated compounds such as RAGE antagonist have been introduced as a potential treatment for AD in animal models (Webster et al., 2008). This experiment assessed the anti-inflammatory effect of RAGE antagonist TTP488 on markers of inflammation induced by CEA-AGE (1 mg/mL) (Figure 3.22). The data show reductions of all inflammatory markers and cell viability; however, no adverse effect was reported by RAGE antagonist in a clinical trial (Sabbagh et al., 2008).

3.3.10 TTP488 effect on TNF-α and nitric oxide production
The effect of TTP488 RAGE antagonist on markers of inflammation induced by CEA-AGE (1 mg/mL) was tested. The data show reductions in inflammation marker TNF-α and NO concentrations (Figure 3.23). The test confirmed that TTP488 has an anti-inflammatory effect on glycated protein induced inflammation.
3.3.11 Effect of hyaluronic acid with different molecular weights on inflammation markers

Hyaluronic acid high molecular weight has been reported to be a reducing agent of cytokines induced by glycated proteins at the mRNA level (Neumann et al., 1999). It was hypothesised that molecular weight of hyaluronic acid decreases during ageing in the human body and it can be a basis for increasing the effect of glycated proteins in aged persons (Goldin et al., 2006). Hyaluronic acid with two different molecular weights LMW (3.1 × 10^4 kDa) and HMW (1.7 × 10^6 kDa) were tested (Figure 3.24). With HMW treatment a reduction was observed but not with LMW treatment. This reduction could be attributed to the MW of hyaluronic acid but hyaluronic acid is also known as a bio-adhesive polymer (Barbault-Foucher, 2002).
Figure 3.22  Effect of TTP488 on inflammation induced by CEA-AGE and on cell viability.

RAW 264.7 murine macrophages were incubated for 30 minutes with TTP488 RAGE antagonist with increasing concentrations of 0, 0.9, 0.18, 0.7, 2.5, 5, 11, 23 and 46 µM prior to incubation with CEA-AGE (1 mg/mL) for 24 hours. Cell viability, TNF-α and NO production were measured. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentages of control. The X axis is in logarithmic scale.
Figure 3.23  Effect of TTP488 on CEA-AGE induced inflammation.
RAW 264.7 murine macrophages were incubated for 30 minutes with RAGE antagonist TTP488 at increasing concentrations prior to incubation with CEA-AGE (1 mg/mL) for 24 hours. NO and TNF-α concentrations were measured. The values are expressed as mean ± SD in triplicate tests (n = 2). TNF-α (pg/mL) and nitrite (µM) concentrations are shown. Graphs A and B data derived from Figure 3.22. The X axis is in logarithmic scale.
A. HMW

![Graph showing the effect of HA HMW on NO and CV](image)

B. LMW

![Graph showing the effect of HA LMW on NO and CV](image)

Figure 3.24  Effect of hyaluronic acid on CEA-AGE induced inflammation.

Effect of increasing concentrations of hyaluronic acid with different molecular weights (A, hyaluronic acid HMW and B, hyaluronic acid LMW) on NO production induced by CEA-AGE (1 mg/mL) and effect on CV after 24 hours incubation were measured. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentages of control levels.
3.3.12 Effect of hyaluronic acid with different molecular weights on nitric oxide production

The effect of LMW and HMW on NO production induced by CEA-AGE induced (Figure 3.25) was investigated. The data show a reduction in NO production by hyaluronic acid HMW but not with hyaluronic acid LMW. Hyaluronic acid LMW reduced NO production by up to 40% from the control levels while the reduction was only 10% for hyaluronic acid HMW.

3.3.13 IC\textsubscript{50} values determined for TTP488, hyaluronic acid HMW and anti-RAGE antibody number one

The IC\textsubscript{50} values for hyaluronic acid HMW and TTP488 were calculated by plotting data in Graphpad Prism and IC\textsubscript{50} value was calculated as described in section 3.2.3 (Table 3.6).

<table>
<thead>
<tr>
<th></th>
<th>Hyaluronic acid (HMW) (mg/mL)</th>
<th>TTP488 (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} for NO production</td>
<td>2.5</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 3.25  Effect of hyaluronic acid on nitric oxide production induced by CEA-AGE.

Effect of increasing concentrations of hyaluronic acid with different molecular weights on NO production-induced by CEA-AGE (1 mg/mL) after 24 hours incubation was measured. A, LMW and B, HMW. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown in nitrite concentrations (µM). Graphs A and B derived from Figure 3.24.
3.4 Inhibition of AGE-induced inflammation by hydrogen peroxide scavenging enzyme

The following tests were performed to obtain a better understanding of the role of oxidative stress in cell signalling by glycated proteins. Catalase, acetovanillone and diphenyl iodonium chloride were tested. In addition, hydrogen peroxide effects in response to CEA-AGE (1 mg/mL) and LPS (25 µg/mL) were tested.

3.4.1 Catalase effect on cell viability and nitric oxide production induced by CEA-AGE in J774 murine macrophage cell line

This experiment investigated the effect of H$_2$O$_2$ elimination and CEA-AGE treatment. Catalase was investigated for its effect on reduction of NO production induced by CEA-AGE (1 mg/mL) and on cell viability (Figure 3.26) in J774 murine macrophage cell line. The data show that a reduction of NO production by catalase treatment is independent of cell viability.

3.4.2 Catalase effect on cell viability and nitric oxide production induced by LPS and INF-γ

The catalase effect on reduction of NO production induced by LPS 12.5 µg/mL and INF-γ 0.4 U/mL along with cell viability in RAW 264.7 murine macrophage cell line was assessed (Figure 3.27).
A.

Figure 3.26  Effect of Catalase on CEA-AGE induced inflammation.
Effect of increasing concentrations of 0, 250, 500, 1000, 2000 and 4000 U/mL catalase on J774 murine macrophages incubated with CEA-AGE (1 mg/mL) for 24 hours. NO production was measured as nitrite (µM) and the cell viability (%) was measured by MTT assay. The values are expressed as mean ± SD in triplicate tests (n = 1). The data are shown as percentages of controls (graph A) and as nitrite concentrations (graph B).
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A.

![Graph A]

B.

![Graph B]

Figure 3.27  Effect of Catalase on LPS and INF-γ induced inflammation.

Effect of increasing concentrations of 0, 500, 1000, 2000 and 4000 U/mL catalase on RAW 264.7 murine macrophages incubated with LPS 1 µg/mL and INF-γ 0.4 U/mL for 24 hours. NO was measured as nitrite (µM) and cell viability (%) by MTT assay. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentage of controls (graph A) and as nitrite concentrations µM (B).
3.4.3 Catalase effect on nitric oxide production and cell viability induced by CEA-AGE in RAW 264.7 murine macrophage cell line

The catalase effect on reduction of NO production by CEA-AGE (1 mg/mL) along with cell viability in RAW264.7 murine macrophage cell line was investigated (Figure 3.28). The results show that catalase can reduce inflammation marker and that of the hydrogen peroxide is involved in this inflammation.

3.4.4 Diphenyl iodonium chloride effect on cell viability and nitric oxide production induced by CEA-AGE

This experiment examined the proposed signalling pathway. NADPH oxidase is a key enzyme for ROS production including $H_2O_2$ and inhibition of this enzyme by diphenyl iodonium chloride may give an insight into whether the enzyme is involved in inflammation induced by glycated proteins. The effect of the NADPH oxidase inhibitor, diphenyl iodonium chloride, on CV and NO production induced by CEA-AGE (1 mg/mL) in RAW264.7 murine macrophage cell line was investigated (Figure 3.29). The results show that it can inhibit the inflammatory marker as it was expected.

3.4.5 Acetovanillone (Apocynin) effect on cell viability and nitric oxide production induced by CEA-AGE

The effect of another NADPH oxidase inhibitor, acetovanillone, also called apocynin, on the reduction of NO production induced by CEA-AGE (1 mg/mL) and on cell viability in RAW264.7 murine macrophage cell line was investigated (Figure 3.30). The data show that this inhibitor can reduce inflammation, but only up to 30% of total concentration.
3.4.6 IC\textsubscript{50} values determined for DPI, Catalase and Acetovanillone

Table 3.7 represents the IC\textsubscript{50} values calculated from data presented in Figures 3.26, 3.27, 3.28, 3.29 and 3.30, as described in section 3.2.3. The result show that oxidative stress is involved in stimulation of inflammation marker.
Figure 3.28  Effect of catalase on CEA-AGE induced inflammation.
Effect of increasing concentrations of 0, 250, 500, 1000, 2000 and 4000 U/mL catalase on RAW 264.7 murine macrophages, incubated with CEA-AGE (1 mg/mL) for 24 hours was investigated. NO production was measured as nitrite (µM) and the cell viability (%) was measured by MTT assay. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentages of control levels (graph A) and as nitrite concentrations µM (graph B).
A.

![Graph A: % of control vs. [DPI] (µM)]

B.

![Graph B: [Nitrite] (µM) vs. [DPI] (µM)]

**Figure 3.29 Effect of diphenyl iodonium chloride on CEA-AGE induced inflammation.**

Effect of increasing concentrations of 0, 1.5, 3, 6.125, 12.250, 24.5, 49 and 98 µM of diphenyl iodonium chloride on RAW 264.7 murine macrophages, incubated with CEA-AGE (1 mg/mL) for 24 hours was investigated. NO production was measured as nitrite (µM) and cell viability (%) was measured by MTT assay. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentages of controls (graph A) and as nitrite concentrations (µM) (graph B).
Figure 3.30  Effect of acetovanillone on CEA-AGE induced inflammation.

Effect of increasing concentrations of 0, 1.187, 2.375, 4.750, 9.500 and 19.00 mM of acetovanillone on RAW 264.7 murine macrophages, incubated with CEA-AGE (1 mg/mL) for 24 hours was investigated. NO production was measured as nitrite (µM) and the cell viability (%) measured by MTT assay. The values are expressed as mean ± SD in triplicate tests (n = 2). The data are shown as percentages of controls (graph A) and in nitrite concentrations (µM)(graph B).
Table 3.7  IC<sub>50</sub> values of experimentally tested compounds (data based on Figures 3.26, 3.27, 3.28, 3.29 and 3.30).

<table>
<thead>
<tr>
<th>Compounds and concentration</th>
<th>Activator</th>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI (µM)</td>
<td>CEA-AGE (1 mg/mL)</td>
<td>RAW 264.7 murine macrophage</td>
<td>3.25</td>
</tr>
<tr>
<td>Catalase (U/mL)</td>
<td>CEA-AGE (1 mg/mL)</td>
<td>RAW 264.7 murine macrophage</td>
<td>1608</td>
</tr>
<tr>
<td>Catalase (U/mL)</td>
<td>CEA-AGE (1 mg/mL)</td>
<td>J774 murine macrophage</td>
<td>2942</td>
</tr>
<tr>
<td>Catalase (U/mL)</td>
<td>LPS (25 µg/mL)/ INF-γ (10 U/mL)</td>
<td>RAW 264.7 murine macrophage</td>
<td>375</td>
</tr>
<tr>
<td>Acetovanillone (mM)</td>
<td>CEA-AGE (1 mg/mL)</td>
<td>RAW 264.7 murine macrophage</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.4.7 Hydrogen peroxide measurement in response to CEA-AGE treatment

The role of H$_2$O$_2$ as a signal mediator has been extensively investigated (Saberi et al., 2008). To check the role of H$_2$O$_2$ as a messenger its production in response to activators was tested. Concentration of H$_2$O$_2$ was measured in response to CEA-AGE (1 mg/mL) treatment at different times based on absorbance because of the unavailability of fluorescence plate reader (figure 3.31). The data show that there was an increase in concentration in the first minutes after CEA-AGE treatment (Figure 3.31).

3.4.8 Hydrogen peroxide measurement in response to LPS

The concentration of H$_2$O$_2$ was measured in response to LPS (25 µg/mL) treatment at different times using a fluorescence-based method (Figure 3.32). The data show that there was an increase in concentration in the first minutes after LPS treatment in the same manner as glycated protein treatment (Figure 3.32). The graphs (Figures 3.31 and 3.32 show a rapid release of H$_2$O$_2$ in the first minutes of testing in response to the different activators.

3.4.9 Toxicity of prior addition of hydrogen peroxide on CEA-AGE (1 mg/mL) treatment

This experiment was carried out to check the role of H$_2$O$_2$. The synergistic effect of prior H$_2$O$_2$ treatment with CEA-AGE (1 mg/mL) on cell viability is shown in Figure 3.33. The results show that toxicity increased with prior H$_2$O$_2$ treatment. This could be explained by the signalling molecule role of H$_2$O$_2$ for the cells.
Figure 3.31 Measurement of hydrogen peroxide production in response to CEA-AGE treatment.

RAW 264.7 murine macrophages incubated with CEA-AGE (1 mg/mL) for 40 minutes. $H_2O_2$ release was measured at five-minute intervals by absorbance at 540 nm. The values are expressed as mean ± SD in triplicate tests ($n = 1$). The data are shown as $H_2O_2$ concentration (µM) per 540,000 cells.
Figure 3.32  Measurement of hydrogen peroxide in response to LPS.

RAW 264.7 murine macrophages incubated with LPS (25 μg/mL) for 30 minutes with measurement intervals of five minutes. H$_2$O$_2$ release was measured by fluorescence in $\lambda_{ex} = 563$ and $\lambda_{em} = 587$nm. The values are expressed as mean ± SD in triplicate tests ($n = 2$). The data are shown as H$_2$O$_2$ concentration (μM) per 540,000 cells.
A.

![Graph](image)

B.

![Graph](image)

Figure 3.33 Toxicity of CEA-AGE after prior treatment with hydrogen peroxide for 30 minutes.

Effect of increasing concentrations 0, 3.9, 7.8, 15.62, 31.25 and 62.50 µM of hydrogen peroxide  A. without incubation with CEA-AGE and B. with incubation with CEA-AGE (1 mg/mL) for 24 hours are shown. It was measured by MTT assay as a reduction of the cell viability (%) in comparison to controls (non-activated cells with CEA-AGE or medium). The values are expressed as mean ± SD in triplicate tests (n=1).
CHAPTER FOUR

DISCUSSION
4. Discussion
Protein modification is broadly known as a factor in ageing and protein glycation is of particular interest as protein glycation is found in AD brain (Li et al., 1998). While there are several studies on glycated proteins, there are few articles on the signalling network triggered by glycated proteins. This study was carried out to provide an insight into the components of the network that is triggered by glycated proteins.

The involvement of oxidative stress and inflammation in the network triggered by glycated proteins was reported previously (Wolff et al., 1991; Heidland et al., 2001; Maillard-Lefebvre et al., 2009). This study hypothesised a particular signalling pathway (see Figure 1.3).

4.1 Induction of inflammation by glycated proteins, interferon gamma and lipopolysaccharide
To examine the signalling network induced by glycated proteins, different proteins were glycated under the same conditions. The results in chapter three, section 3.1.2 show that CEA-AGE is more potent than BSA-AGE for NO production, which is a marker of inflammation, and also show that the native protein had virtually no effect (Figure 3.2 and Table 3.1). In addition, NO productions in the two different murine macrophage cell lines were similar at approximately 20 µM, especially with treatments up to 1 mg/mL (Figure 3.3). This dose level was selected for further experiments. In addition, the optimum cell number for inflammation induced by glycated proteins and for TNF-α detection while avoiding saturation was estimated to be 75,000 cells (Figure 3.6). A modified ELISA-based approach was developed and the results showed that temperature was one of the crucial factors for TNF-detection: the higher the temperature, the stronger the signal was. Another important factor for TNF-α detection was cell number. A change in cell number of around 10,000 cells changed the TNF-α signal dramatically. TNF-α concentration with 85,000 cells was 880 pg/mL whereas with 70,000 cells was 600 pg/mL (Figure 3.6). Moreover, a difference of 15,000 cells resulted in a 280 pg/mL difference. Those results indicate that cell number was crucial for consistent measurements, in particular for TNF-α
detection. In contrast, with NO production cell number changes from 85,000 to 70,000 resulted in a 4 µM difference (Figure 3.4). This suggests that while a difference of 10,000 cells produces a difference of about 35% in the total TNF-α concentration, such a difference was approximately 12% for NO production (Figure 3.4). The results also suggest that TNF-α measurement is more accurate with the addition of a reaction stopping solution (sulfuric acid). Such an addition was not part of the commercial protocol. In addition, use of a wider optical density range (OD) was able to differentiate between different concentrations of TNF-α (Figures 2.2 and 2.3).

4.2 Inhibition of glycated protein induced inflammation by different antioxidants

The effect of apigenin was investigated (section 3.2) and the results show that undiluted samples treated with different concentrations of apigenin did not exhibit any reduction and the effect of apigenin was investigated as described (Figure 3.7). Dilution factors of 25, 66 and 100 showed that there was a reduction related to the different apigenin concentrations and the CEA-AGE treatment whereas there was no effect with no dilution of apigenin (Figure 3.7). A reduction was observed with the 25, 66 and 100 dilution factors, with slight differences between the three dilution factors (Table 3.2). The IC\textsubscript{50} values for apigenin were about 5 µM for TNF-α and 1 µM for NO reduction (Table 3.2). The therapeutic index, which is the ratio of lethal dose to effective dose, of apigenin was high and there was no cytotoxicity up to 10 µM. Reduction of inflammation markers was also observed (Figures 3.8, 3.9 and 3.10). The IC\textsubscript{50} values were reproducible in two independent experiments in RAW 264.7 murine macrophage cell line and were also reproducible between two cell lines of (RAW 264.7 and J774) murine macrophages (Table 3.3 and Figure 3.13). Additionally, inhibition by apigenin 5 µM was significant at a high level ($p < 0.0001$; Figure 3.11). By increasing the concentration of resveratrol, cell death was observed (Figure 3.14). Scuro et al. (2004) showed an apigenin IC\textsubscript{50} value of 10 µM in mouse peritoneal macrophage cell line. Here, the IC\textsubscript{50} of apigenin was close to other
published data, but the other study used LPS and/or INF-γ as activators (Scuro et al., 2004).

*Polygonum cuspidatum* is known to be a source of resveratrol (Wang et al., 2010). Here, the results show a greater level of NO reduction by in comparison to resveratrol (Figures 3.14 and 3.15). This indicates that the reduction might be partly related to the resveratrol content of *Polygonum*, but that there may be other compounds that are responsible for the additional reduction shown by *Polygonum treatment* in comparison with purified resveratrol treatment. Here, resveratrol by itself did not reduce NO production significantly. Unpublished data of the group show that 50% of *Polygonum* contains resveratrol and the data indicate that *polygonum* with 900 µg/mL of resveratrol (equal to 450 µg/mL) can reduce NO production by the same percentage as 50%–60% of resveratrol with 450 µg/mL (2mM).

Additionally, the apigenin with high inhibition activity and a flavone structure (Scervino et al., 2006) was compared with resveratrol with low inhibition activity and a stilbene structure (Joseph et al. 2008). This comparison supposes that the flavone structure is an important part of polyphenols for their production of anti-inflammatory activity. However, more investigations are needed into the structurally related activities of different plant extracts with various structures to confirm that a flavone structure is essential for high inhibition activity.

### 4.3 Inhibition of AGE-induced inflammation by receptor mediated compounds

Hyaluronic acid was tested as a natural reported RAGE antagonist (Neumann et al., 1999). A decrease in the molecular weight of HA has been related to ageing and the harmful effect of glycation (Newmann et al., 1999). Two different molecular weights (3.1 × 10⁴ kDa and 1.7 × 10⁶ kDa) of HA were tested. HA is also known as a natural polymer (Price et al., 2007) and a natural RAGE antagonist at the mRNA level (Neumann et al., 1999). This was confirmed at the protein level by determination of a change in NO production (Figures 3.24 and 3.25). The effects observed in cell
culture could be due to the bio-adhesive property of the polymer and further experiments are needed at the protein level to determine if the reduction is related to the RAGE receptor antagonising activity of HA. Interestingly, a RAGE antibody provided by Abbott Neuroscience shows binding to RAGE in \textit{in vitro} experiments by partial inhibition of inflammation markers.

Here, anti-RAGE antibodies showed reduction of NO and TNF-\(\alpha\) production. A maximum reduction of 10\% was observed for NO production within the concentration range of 0–10 \(\mu\)g/mL (graph A, Figure 3.19) and a maximum reduction of TNF-\(\alpha\) with 90\% inhibition at concentrations up to 10 \(\mu\)g/mL (graph A, Figure 3.19). However, the effective dose was found to be different, based on different CEA-AGE batch results (Table 3.5). These results was also confirmed at the same time by Chandler et al. (2010) with anti-RAGE antibody in the N11 cell line. This inhibition activity level is consistent with the mild results in a clinical trial and an animal model indicating a beneficial effect of RAGE antagonist and antibody on AD (Rafii and Aisen, 2009). The incubation time needed for the anti-RAGE antibody was greater than that for the other compounds tested. The reason for that difference may be related to its bulk structure and its affinity.

A RAGE antagonist, known by the brand name TTP-488 (or PF-04494700) is marketed by Sanofi Aventis (Chen et al., 2008). It has been shown that the RAGE antagonist family could reduce cytokine levels in cell culture medium (Vilayur and Harris, 2009). In this study, RAGE antagonist (TTP488) demonstrated reduction of NO and TNF-\(\alpha\) production (Figure 3.22). RAGE antagonist has also been reported to be an agent for lowering A\(\beta\) in AD patient brain (Rafii and Aisen, 2009). Here, the TTP488 IC\(_{50}\) value was found to be 1 \(\mu\)M for NO reduction and 8.5 \(\mu\)M for TNF-\(\alpha\) reduction induced by glycated proteins (Figure 3.22). According to National Institute of Health (NIH), TTP488 may be used in clinical trials at a concentration of 60 mg/mL. There are however, few published studies of cell-based assays. This present study confirmed the anti-inflammatory effect of TTP488, but it also demonstrated a reduction in cell viability. Moreover, the therapeutic index of TTP488 was low. There were however no side effects reported for RAGE antagonist in animal and
clinical trials (Webster et al., 2008). Here, however, cell death was observed in the cell culture model (Figures 3.22 and 3.23).

4.4 Inhibition of AGE-induced inflammation by inhibitor of NADPH oxidase, hydrogen peroxide scavenging enzyme and effect of prior hydrogen peroxide treatment on cell viability

In the signalling pathway triggered by glycated proteins, ROS has a significant role (Figure 1.3). Results showed that H$_2$O$_2$ was produced after treatment by glycated proteins. Interestingly, the results showed that H$_2$O$_2$ production levels after LPS and CEA-AGE treatment were similar (Figures 3.31 and 3.32). After 5 minutes, there was an increase in H$_2$O$_2$ production, but that increased level decreased after 10 minutes. This could be in accordance with the known role of H$_2$O$_2$ as a messenger (Rhee et al., 2005). More experiments are needed to determine the level of H$_2$O$_2$ production at more evenly time intervals points. In addition, overall ROS and superoxide production should be measured. Moreover, DPI and acetovanillone as NADPH oxidase inhibitors, and catalase, as a hydrogen peroxide scavenging enzyme are also known as antioxidant enzymes (Hellemans et al., 2003). Both showed reductions in the CEA-AGE induced inflammation with presented IC$_{50}$ values (Table 3.7). Acetovanillone reduced the inflammation induced by glycated proteins with an IC$_{50}$ value of 3.25 µM. This suggests that oxidative stress is involved in the AGE-RAGE pathway, in support of previous work (Evans et al., 2001).

To confirm that H$_2$O$_2$ works as a messenger in the AGE-RAGE signalling pathway, cells were treated with H$_2$O$_2$ prior to stimulation by glycated proteins. Toxicity of CEA-AGE was observed with prior treatment with H$_2$O$_2$ for one hour (Figure 3.33). The results show that H$_2$O$_2$ can act as a signalling pathway messenger and increase the toxicity of glycated proteins, even after short incubation times (Lal et al., 2002).

In summary, these results support the hypothesis of an AGE-RAGE signalling pathway presented in previous studies. The pathway could form part of a possible signalling network and inhibition of inflammation may result through this signalling network (Figure 4.1). ROS scavenging enzymes, receptor mediated compounds and
antioxidants are possible compounds for slowing down the progression of inflammation that has been induced by glycated protein in different diseases; diseases that are associated with glycation in their disease development mechanism. Here, the RAGE antagonist TTP-488 showed the most reduction, however, research is needed into overcoming the possible adverse reaction might be associated with cell death that were observed in *in vitro* studies. The role of ROS should be further investigated in this signalling pathway. Moreover, more experiments at the protein level with ELISA and western blotting are needed to determine the inhibition effects of different introduced effective compounds, including anti-RAGE antibodies, RAGE antagonists such as TTP488, hyaluronic acid, apigenin, resveratrol and *Polygonum cuspidatum*. The role of hydrogen peroxide should also be studied in more detail, with other methods of measurement. In addition, detection of the superoxide anion should be investigated and its detection related to the measurement of hydrogen peroxide levels.
Figure 4.1 Schematic representation of signalling pathways induced by glycated proteins and inhibition effects of different compounds or extracts on this signalling pathway.
REFERENCES


References


References


advanced glycation end products in nondiabetic subjects. *Metabolism*, 55(9), 1227–1231.


Appendix 1  Four parameters logistic equation for TNF-α measurement for standard curve of TNF-α without reaction stop solution.

<table>
<thead>
<tr>
<th>Sigmoidal dose-response (variable slope)</th>
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<td>Number of points analysed</td>
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Six different TNF-α concentrations (2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL) versus OD at 655nm illustrated as the TNF-α standard curve. The sigmoidal dose-response (variable slope) curve fit (n = 1) and the calculated equation are used for concentration measurement by GraphPad Prism software.
Appendices

Appendix 2  Four parameters logistic equation for TNF-α measurement for standard curve of TNF-α with reaction stop solution.

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\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log EC50 - x) \cdot \text{Hill Slope}}} \]

Six different TNF-α concentrations (2000, 1000, 500, 125, 62.5, 31.25 and 0 pg/mL) versus OD at 450 nm with reference wavelength 650 nm illustrated as the TNF-α standard curve (sigmoidal dose response fit) (n = 1). The calculated equation is used for further concentration measurement by GraphPad Prism software.