DISRUPTION OF RAGE SIGNALING PREVENTS SYMPATHETIC NEURON

MALFUNCTION IN HIGH GLUCOSE CONDITIONS

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ABSTRACT

Diabetes, which is characterized by elevated plasma glucose, can have a devastating effect on peripheral nerves frequently leading to the clinical symptoms of neuropathy. Diabetic autonomic neuropathy (DAN) results from damage to autonomic nerves, and the most troubling forms of DAN often lead to cardiovascular abnormalities and premature death. Despite the prevalence of DAN and the impact to quality and life expectancy, the precise mechanisms underlying these pathologies are poorly understood. Recently, a new model for the onset of DAN was proposed where hyperglycemia-induced oxidative stress inactivates nicotinic acetylcholine receptors (nAChRs), the main receptor driving autonomic synaptic transmission at sympathetic ganglia. This inactivation leads to the depression of synaptic transmission, and consequently triggers the onset of autonomic neuropathy in diabetic mice. However, the source and pathways contributing to the elevation of reactive oxygen species (ROS) and oxidative stress remained unclear.

In recent years it has been shown that the accelerated formation of advanced glycation end products (AGEs) and activation of their receptor (RAGE) in diabetes play a major role in the induction of oxidative stress in sensory nerve damage. Thus, we hypothesized that the activation and up-regulation of RAGE during high glucose conditions is a major source of ROS production in sympathetic neurons leading to the inactivation of nAChRs and autonomic malfunction. In this thesis we show for the first time that RAGE is expressed in cultured sympathetic neurons and is also up-regulated during high glucose conditions. Our results further demonstrate that direct RAGE activation by its natural ligands leads to an increase in cytoplasmic ROS which in turn induces the inactivation of nAChRs in sympathetic neurons. We also report that high glucose-induced ROS generation and subsequent inactivation of nAChRs is prevented in sympathetic neurons from RAGE knock-out mice. The results of this dissertation suggest RAGE to be a

pivotal source of ROS production leading to the functional deficits observed in sympathetic

neurons during high glucose conditions.

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AAG	autoimmune autonomic ganglionopathies
ACh	acetylcholine
AGE	advanced glycation end product
ANS	autonomic nervous system
AO	anti-oxidants
ATP	adenosine triphosphate
AU	arbitrary units
BSA	bovine serum albumin
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
DAN	diabetic autonomic neuropathy
DRG	dorsal root ganglia
EGTA	ethylene glycol-bis (β-aminoethyl ester) – N,N,N', N'- tetracetic acid
ELISA	enzyme linked immunosorbent assay
EPSP	excitatory postsynaptic potential
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-etheansulfonic acid
HG	high glucose
HMGB1	high mobility group box 1/amphoterin
HNE	4-hydroxyl-2-nonenal
IPSP	inhibitory postsynaptic potential
KCl	potassium chloride

KO	knock out
KOH	potassium hydroxide
L-15	leibovitz L-15 medium
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	nuclear factor - κB
NGF	nerve growth factor
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PI-3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SCG	superior cervical ganglia
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMG	superior mesenteric ganglia
sRAGE	soluble RAGE
STZ	streptozotocin
TTX	tetrodotoxin
UDP-GlcNAc	uridine diphosphate-N-acetylhexosamine

CHAPTER 1

Diabetes mellitus is characterized for causing elevations in plasma glucose resulting from an inadequate production of insulin (type 1) or insulin resistance (type 2). Currently in North America, 19 million people live with diabetes mellitus and of those, approximately 90-95% suffer from the type 2 form. Diabetes is considered an epidemic in developed countries and it is predicted by 2025 that there will be over 300 million people living with this disease (World Health Organization). A common characteristic of diabetes is high blood sugar, or hyperglycemia, and this leads to a range of neurological complications that worsen with duration. Hyperglycemia-induced nerve damage, clinically referred to as diabetic neuropathy, is the most common diabetic complication affecting nearly 70% of patients (American Diabetes Association; http://www.diabetes.org/diabetes-basics/diabetes-statistics/?loc=DropDownDB-stats). Sensory and autonomic dysfunction are two common symptoms observed in patients with diabetic neuropathy. Diabetic sensory neuropathy is the result of hyperglycemia-induced damage to sensory nerves, usually in the arms and legs, leading to abnormal sensory function. This can lead to serious complications such as sensory loss, incapacitating pain, foot ulceration, gangrene, and infection (Boulton et al., 2004). This often leads to lower leg amputations as over half the leg amputations last year in the USA were diabetes related (Fernyhough et al., 2010). People with diabetes also often suffer from complications of the autonomic nervous system (ANS), clinically called diabetic autonomic neuropathy (DAN), resulting in conditions such as cardiac arrhythmias, orthostatic hypotension, gastrointestinal abnormalities, ocular deficiencies, and urinary tract problems (Duby et al., 2004; Vinik and Zeigler, 2007). Despite the prevalence of DAN and the impact it has on the lives of those affected, it still remains one of the least understood and studied complications associated with diabetes (Vinik et al., 2001, 2003).

Extensive research has described the structural and biochemical alterations that occur in peripheral nerves as a result of diabetes (Schmidt, 2002; Tomlinson & Gardiner, 2008). However, the exact sequence of events – from hyperglycemia to the occurrence and progression of symptoms typical of clinical neuropathy – has not yet been elucidated. It is the goal of this thesis to shed light on the molecular pathways that underlie the pathogenesis of diabetic autonomic complications.

1.1 Autonomic Nervous System

1.1.1 General Overview

The major function of the ANS is to maintain organ system homeostasis. The ANS plays a crucial role in maintaining system homeostasis by signaling through a network of neurons that works predominately without voluntary or conscious control. Accordingly, the name 'autonomic' comes from the Greek word *autonomia* meaning 'self-governing' (Richerson, 2009). The ANS is responsible for the innervation and control of all tissues and organs in the body, except skeletal muscle, which is governed by the somatic motor system (Bear et al., 2007). Among the tissues targeted by the ANS are smooth muscle, cardiac muscle, secretory epithelia and gland cells. Examples of ANS function include modulating pupil diameter, dilation and constriction of blood vessels, cardiac output, heart rate, and maintaining normal body temperature (Richerson, 2009). In addition, autonomic output can be regulated centrally by a distributed network of nuclei contained mainly in the hypothalamus and brain stem (Bear et al., 2007).

1.1.2 Function of the ANS

The ANS is comprised of three divisions: sympathetic, parasympathetic, and enteric (Richerson, 2009). The sympathetic and parasympathetic divisions are the major efferent pathways of the ANS and often produce opposite physiological outcomes. For example, the sympathetic nervous system controls the "fight-or-flight" response, which is an adaptive reaction to harmful or dangerous stimuli. The surge in sympathetic output causes an increase in heart rate, cardiac output, respiration, pupil dilation, gluconeogenesis, perspiration, and an increase in blood flow to the heart and muscles (Tortora and Derrickson, 2009). Thus, the fight-or-flight response primes the body to respond to emotional or physical stress in a fashion that enhances the organism's survival chances. Conversely, the parasympathetic nervous system is often referred to as the "rest-and-digest" division due to its function to replenish energy stores and aid in digestion (Tortora and Derrickson, 2009). Both sympathetic and parasympathetic divisions innervate most organ systems simultaneously and while they can function independent of one another, they generally work cooperatively to maintain homeostasis (Richerson, 2009).

1.1.3 Organization and anatomy of the ANS

All three divisions of the ANS have marked differences in their organization and anatomy. The sympathetic and parasympathetic divisions arise from preganglionic neurons, whose cell bodies are located in the spinal cord and brain stem of the central nervous system (CNS; Bear et al., 2007). These preganglionic neurons send myelinated axons to postganglionic neurons organized in autonomic ganglia outside the CNS, which in turn send unmyelinated axons to the target organs (Richerson, 2009). In contrast, the organization of the enteric division is more diffuse comprising a neuronal network that regulates gastrointestinal homeostasis.

In the sympathetic division, the preganglionic fibers originate from the first thoracic segment (T1) to the third lumbar segment (L3) of the human spinal cord. These preganglionic fibers leave the ventral roots of the spinal cord to form synapses with postganglionic neurons housed in sympathetic ganglia (Horn and Swanson, 2013). The human body usually houses 25 pairs of sympathetic ganglia that are located on either side of the vertebral column (Atri et al., 2012). The most rostral ganglia in the chain, and the one used in this thesis, is the superior cervical ganglion (SCG) which results from the fusion of cervical ganglia C1-C4 (Atri et al., 2012). It sends its postganglionic fibers to the head and face and innervates the pupil, salivary glands, lacrimal glands, sweat glands, and blood vessels. The SCG also provides sympathetic innervation to the cerebral vasculature (Horn and Swanson, 2013). The variety of functions modulated by the SCG as well as its easy accessibility has facilitated its characterization in multiple animal models and thus makes it an excellent model to study sympathetic function.

In contrast to the sympathetic ganglia, most parasympathetic ganglia lie within or near the organ they innervate, and as a result, their axonal fibers are shorter compared to the sympathetic fibers. Parasympathetic preganglionic fibers arise from neuronal cell bodies housed in the brain stem and sacral segments of the spinal cord, and are often very long since parasympathetic ganglia are not close to the spinal cord like the sympathetic ganglia. An example of a major parasympathetic tract is the 10th cranial nerve, which is also known as the vagus nerve. The vagus nerve provides parasympathetic innervation to major organs such as the heart, lungs, kidneys, and gastrointestinal system (Richerson, 2009).

The enteric division has a more diffuse organization when compared to the sympathetic and parasympathetic divisions. The enteric division is a complex interconnected system of sensory neurons, interneurons, and autonomic neurons that surrounds and controls the

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gastrointestinal system (Bear et al., 2007). It is the most complicated division of the ANS and is estimated to have as many as 100 million neurons in humans (Horn and Swanson, 2013). The enteric division is controlled by two major networks of interconnected neurons: the myenteric plexus and the submucous plexus. These two networks work together to control both the transport and digestion of food (Bear et al., 2007). In addition, sympathetic and parasympathetic innervation of the enteric nervous system can also modulate its activity; however, basic enteric functions are still maintained when all innervation to the CNS is removed (Bayliss et al., 1899; Furness, 2012).

1.1.4 Synaptic Physiology of the ANS

Preganglionic neurons of both the sympathetic and parasympathetic nervous system release the same primary neurotransmitter, acetylcholine (ACh), to the postganglionic neurons. ACh binds to ionotropic nicotinic acetylcholine receptors (nAChRs), located on postganglionic autonomic neurons, triggering the flow of mostly sodium and potassium ions (a small increase in calcium permeability also occurs). The immediate result is a net inward current that produces a fast excitatory postsynaptic potential (EPSP) that is capable of triggering action potential firing (Bear et al., 2007). This is the primary mode of neurotransmission in autonomic ganglia. However, ACh is also able to activate other receptors expressed on the postganglionic autonomic neurons that can modulate the EPSP generated by nAChRs. Activation of M_1 muscarinic receptors by ACh, which are G protein-coupled receptors that activate second messenger cascades to carry out a response, results in a slow EPSP that can last for several seconds. Conversely, activation of the dopaminergic receptors by dopamine or the α-adrenergic receptors by norepinephrine produces a slow inhibitory postsynaptic potential (IPSP). This membrane

hyperpolarization is thought to occur by ACh activation of the M_2 muscarinic receptor. Lastly, peptides such as angiotensin and substance P that are co-released with ACh can cause a depolarization lasting for several minutes called the late, slow EPSP (Atri et al., 2012). In summary, fast EPSPs mediated by nAChRs drive autonomic synaptic transmission while the other three types of synaptic signals modulate the efficiency of this communication.

While all preganglionic autonomic neurons release ACh to drive synaptic transmission, postganglionic sympathetic and parasympathetic neurons use different neurotransmitters and receptors to drive the activity of their target cell. Sympathetic postganglionic neurons generally release norepinephrine which acts on adrenergic receptors. The two main types of adrenergic receptors are α and β which can bind both norepinephrine and epinephrine. On the other hand, postganglionic parasympathetic neurons are considered cholinergic because they use ACh as their primary neurotransmitter. Parasympathetic release of ACh on their target cells induces a response by activating muscarinic receptors (Tortora and Derrickson, 2009).

1.2 Nicotinic acetylcholine receptors in autonomic ganglia

The nAChRs are ligand-gated ion channels (ionotropic receptors) that are members of the pentameric Cys-loop receptor family (Richerson, 2009), characterized by a highly conserved pair of cysteine residues that forms the ligand binding pocket (Leonard and Bertrand, 2001; Albuquerque et al., 2009). Together with the nAChRs, this family also includes serotoninergic type 3, glycine, and $GABA_A$ receptors. The binding of two ACh molecules to the nAChR results in the opening of a non-selective cation pore with strong inward rectification (Dani and Bertrand, 2007; Atri et al., 2012; Krishnaswamy and Cooper, 2012). The nAChRs are known to drive fast synaptic transmission in autonomic ganglia (David et al., 2010).

Five of the eleven neuronal nAChR subunit genes identified so far are found to be expressed in mouse sympathetic ganglia : α3, α5, α7, β2, and β4 (Mandelzys et al., 1994; De Koninck and Cooper, 1995). In the SCG, nAChRs can occur as homo-pentamers (made up of only the α7 subunit) or hetero-pentamers (can contain the α3, α5, β2, and β4 subunits). The hetero-pentameric receptors can form with a variety of different subunit combinations, each with different functional properties (Mandelzys et al., 1995; McGehee and Role, 1995; Corringer et al., 2000, Putz et al., 2008). The most prevalent nAChR in all sympathetic ganglia is the one comprised of the α3/β4 subunit combination (Covernton et al., 1994; Lewis et al., 1997; Skok et al., 2002).

Since nAChRs are essential for normal sympathetic function, disruption in the functioning of this ion channel often leads to autonomic impairments. For example, patients with autoimmune autonomic ganglionopathies (AAG) develop antibodies against the α3 containing nAChRs and consequently suffer from severe autonomic complications (Vernino et al., 2008). These complications result in common symptoms associated with autonomic failure such as orthostatic hypotension, abnormal thermoregulation, gastroparesis, and decreased lacrimation. Interestingly, these dysautonomias occur without compromising the structural integrity of the ganglia as they remain anatomically intact and are without any signs of degeneration (Vernino et al., 2004, 2008, 2009).

The critical role of nAChRs in autonomic function was also explored in the context of diabetes. Recent studies show that accumulation of reactive oxygen species (ROS), which occur in diabetes, target intracellular cysteine residues in α3-containing nAChRs expressed in sympathetic neurons from the SCG (Campanucci et al., 2008; 2010). This results in the depression of sympathetic synaptic transmission and the occurrence of symptoms typical of

sympathetic failure (e.g. abnormalities in heart rate and thermoregulation; Campanucci et al.,

2010). The sympathetic problems occurring both in AAG and in diabetes are examples of

dysautonomias that can be attributed to dysfunctional $\alpha 3-$ containing nAChRs rather than

general structural deteriorations. These examples show that nAChRs are vital for the proper

functioning of the ANS.

1.3 Diabetic Autonomic Neuropathy

1.3.1 Clinical symptoms and treatments

DAN is a major complication of diabetes leading to nerve dysfunction of the ANS. The

main risk factors for DAN are hyperglycemia, long duration of diabetes, increasing age, female

sex, and having a higher body mass index (Vinik and Erbas, 2001). DAN can affect any area of

the ANS and thus is considered a system-wide disorder affecting many organ systems throughout

the body. Typical symptoms associated with DAN include resting tachycardia, exercise

intolerance, orthostatic hypotension, constipation, gastroparesis, erectile dysfunction, and

impaired neurovascular function (Vinik et al., 2003). Cardiovascular problems are among the

most troubling autonomic complications as it causes abnormalities in heart rate as well as in

central and peripheral vascular dynamics. This often is a leading source of morbidity and

mortality in diabetic patients predisposing them to malignant arrythmogenesis and to sudden

cardiac death (Vinik and Erbas, 2001).

Autonomic neuropathy is one of the most common complications arising from diabetes

with some clinical studies reporting a prevalence rate as high as 100% (Ziegler et al., 1992).

Many major clinical symptoms do not manifest until after many years with diabetes but when

they do arise, 25% to 50% of the patients die within 5 to 10 years of diagnosis (Ewing et al.,

1991; Rathmann et al., 1993). Subclinical symptoms of DAN can often be detected within 1 year of the onset of type 2 diabetes and within 2 years of type 1 diabetes (Pfeifer et al., 1984). DAN significantly increases the chance for mortality as patients are three times more likely to pass away within five years of diagnosis compared to diabetic patients without autonomic neuropathy (O'Brian et al., 1991).

The best option to date for treatment of DAN is preventive measures such as intense glycemic control, diet, and exercise (Vink et al., 2003). The Diabetes Control and Complication Trial Research Group (DCCT Research Group, 1998) showed that tight glycemic control could prevent or slow autonomic dysfunction over time in patients with type 1 diabetes. An interesting new drug to treat autonomic neuropathy that has recently been approved to be prescribed to patients is the antioxidant α-lipoic acid. Early therapy with this antioxidant has shown to slow or even reverse the progression of autonomic dysfunction (Ziegler et al, 1999). Other antioxidants such as vitamin E have also been shown to improve autonomic function in those with type 2 diabetes (Manzella et al., 2001). β-blockers and aldose reductase inhibitors seem to also show positive results in mitigating the detrimental effects diabetes has on autonomic function (Vinik et al., 2003). There are many different drugs on the market to treat DAN and the specific organ affected however, as mentioned above, preventive measures and lifestyle changes has shown to trump most pharmacological interventions. Also, since the development of DAN is such a multifactorial process, a combination of treatments should be further explored.

1.3.2 Diabetic autonomic neuropathy and sympathetic ganglia

Evidence from both animal and human studies point that a common neuropathological consequence of DAN in most autonomic ganglia is dendritic and axonal pathology without the occurrence of much neuronal loss (Schmidt, 2002). The distal portion of axons, especially those

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of longer length, are commonly found to have undergone degenerative or dystrophic changes (Schmidt, 2002; Vinik et al., 2003). Interestingly however, these anatomical changes seem to be selective for only certain autonomic ganglia. For example, the superior mesenteric (SMG) and celiac ganglia (CG) often develop these anatomical alterations while the SCG seems to be spared. The findings observed in human diabetic tissue also seem to correspond well with that of experimental animal studies. In the rat model of type 1 diabetes in which streptozotocin (STZ) is used to selectively destroy insulin secreting pancreatic cells, a loss of neurons in sympathetic ganglia is not observed even after 10 months (Schmidt, 2001). Also consistent with observations in human tissue, rodent models of diabetes show neuroaxonal dystrophy in the SMG and CG however, this is not observed in the SCG (Schmidt and Plurad, 1986). Why anatomical alterations are not observed in the SCG during diabetic conditions is not known.

1.3.3 Pathogenesis of Diabetic Autonomic Neuropathy

Despite the prevalence of DAN and its detrimental effect on the patient, it is one of the least understood and recognized disorders of diabetes. Evidence for detrimental metabolic pathways contributing to nerve damage (Greene et al., 1988; Hoeldtke et al., 2002; Vinik et al., 2001), neurovascular insufficiency (Cameron et al., 1997; Low et al., 1997), abnormal axonal transport (Tomlinson, 1983), excessive immune response (Pittenger et al., 1999), and a reduction or increase in neurotrophic growth factors (Apfel et al., 1994) have all been linked and attempted to explain the etiology of diabetic neuropathy (Vinik et al., 2003). While several hypotheses exist explaining the pathogenesis of peripheral nerve damage in diabetes, a single unifying mechanism has not been put forth and accepted. For example, one hypothesis is that all autonomic complication in diabetes is a result of the diminished blood flow due to microvascular

abnormalities (Schmidt, 2002). This diminished blood flow is thought to result in neuronal

ischemia and trigger degenerative processes for autonomic neurons. However, this global

explanation for the pathogenic mechanisms of DAN is confounded by the fact that certain

autonomic ganglia in diabetes, like the SCG, do not show any signs of structural degeneration

(Schmidt et al., 1993; Schmidt, 1996). It is likely that diabetes causes neuronal dysfunction

through different mechanisms depending on neuronal phenotype.

1.4 Four main molecular mechanisms of how hyperglycemia induces cellular damage

Glucose neurotoxicity is a serious consequence of diabetes and often leads to diabetic

neuropathies. In order to function properly, neurons demand a high amount of glucose. But

unlike muscle and fat cells, where glucose uptake is carried by the insulin-dependent GLUT4

transporter, in neurons glucose uptake is mostly mediated by the insulin-*independent* GLUT1

transporter. Thus, glucose transport into neurons follows the glucose concentration gradient and

insulin regulated glucose transport into brain cells has not yet been shown to occur. This makes

neuronal cells especially vulnerable to damage via hyperglycemia leading to diabetic neuropathy.

These neuropathies are especially prevalent in the peripheral nervous system where the neurons

are not protected from elevated glucose levels by the blood-brain barrier (Tomlinson and

Gardiner, 2008).

In diabetes, intracellular glucose levels are increased fourfold triggering four main

pathophysiological mechanisms leading to nerve damage (Brownlee, 2001; Tomlinson and

Gardiner, 2008). These are: increased polyol pathway flux; increased advanced glycation end

products (AGEs) formation; activation of protein kinase C (PKC) isoforms; and increased

hexosamine pathway flux. These four pathways are linked together by a single hyperglycemia-

induced process: oxidative stress by the overproduction of reactive oxygen species (Brownlee, 2001).

1. Increased activation of polyol (sorbitol) pathway

In normal euglycemic conditions, such as in non-diabetic people, most of the glucose in peripheral nerves is phosphorylated by hexokinase and enters into the glycolytic pathway as glucose-6-phosphate. However, in diabetes, high blood glucose saturates hexokinase resulting in glucose to be diverted into the polyol pathway (van Heyningen et al., 1959; Gabbay et al., 1966). The first enzyme in this pathway, aldose reductase, reduces glucose to sorbitol by consuming NADPH. In hyperglycemia, a significant amount of sorbitol accumulates intracellularly and is not cleared due to its low plasma membrane permeability. Several mechanisms have been proposed to explain how hyperglycemia-induced increases in polyol pathway flux contribute to glucose neurotoxicity. These include osmotic stress caused by intracellular sorbitol accumulation, decreased (Na^+ - K^+) ATPase activity, an increase in cytosolic nicotinamide adenine dinucleotide (NADH/NAD^+), and finally decreases in cytosolic NADPH (Brownlee, 2001; Tomlinson & Gardiner, 2008). Activation of the polyol pathway may also indirectly damage neurons by decreasing nerve blood flow (Greene et al., 1988).

2. Increased activation of protein kinase C

Intracellular hyperglycemia increases diacylglycerol concentration which ultimately activates protein kinase C (PKC; Koya et al., 1998). PKC activation is also thought to be indirectly mediated by RAGE-ligand interaction (Portilla et al., 2000) and the increased flux of glucose through the polyol pathway (Keogh et al., 1997). Studies have shown that abnormal

PKC activation is involved in several of the mechanisms thought to underlie diabetic neuropathies. This includes the reduced formation of nitric oxide and the increased production of endothelin-1 which contributes to vasoconstriction (Ishii et al., 1996). Consequently, this leads to reduced nerve blood flow, a characteristic commonly seen in diabetic patients (Cameron et al., 1997). PKC activation also increases vascular permeability, promotes angiogenesis, and induces a pro-inflammatory response (Brownlee, 2001; Tomlinson & Gardiner, 2008).

3. Increased flux through the hexosamine pathway

In hyperglycemic conditions, fructose-6-phosphate from glycolysis is diverted into the hexosamine pathway. Eventually, fructose-6-phosphate is converted into uridine diphosphate-N-acetylhexosamine (UDP-GlcNAc) which is known to bind to serine and threonine residues on intracellular proteins. This interaction between UDP-GlcNAc and serine and threonine is known to cause protein dysfunction and also promotes gene transcription factors, such as specificity protein 1 (Sp1), which are involved in causing hyperglycemia-induced neuronal damage (Brownlee, 2001; Tomlinson & Gardiner, 2008).

4. Increased AGE formation

AGEs are mostly formed by the non-enzymatic glycation of proteins, lipids, and nucleotides (Lee and Cerami, 1989; Brownlee, 2000). Increased AGE accumulation is a normal process of aging but AGE production is accelerated under hyperglycemic conditions (Brownlee, 2000; Ulrich and Cerami, 2001; Thornalley, 2002). Current research attributes a major role of AGEs in the pathogenesis of neurological complications caused by diabetes. AGE levels are proportional to the duration of diabetes and often excessive amounts lead to oxidative stress,

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inappropriate transcription signals, interruption of cellular pathways, and inflammatory-like neuronal damage. AGE buildup also leads to protein cross-linking and aggregation both intra- and extracellularly (King, 2001; Thornalley, 2002; Wang et al., 2003; Layton and Sastry, 2004). In addition to the direct structural effects of AGE, there is interaction of AGE with cell surface receptors. Many of these receptors bind and destroy AGEs while others, like the receptor for AGEs (RAGE), mediate key intracellular signaling processes (Neeper et al., 1992; Schmidt et al., 1996; Singh et al., 2001; Thornalley, 2002). RAGE is the main receptor for AGE-mediated signaling and has been implicated in several diabetic complications including atherosclerosis, retinopathy, nephropathy, and neuropathy. RAGE activation is also thought to significantly contribute to oxidative stress in hyperglycemic conditions (Huang et al., 2001; Wautier et al., 2001; Zill et al., 2001; Singh et al., 2001; Ishihara et al., 2003; Xu and Kyriakis, 2003; Shaw et al., 2003; Zill et al., 2003; Li et al., 2004; Toth et al., 2007). A more detailed discussion of RAGE will be discussed in section 1.6.

AGEs can be formed via three main ways: intracellular auto-oxidation of glucose to glyoxal; slow, spontaneous rearrangement of the Amadori product to 3-deoxyglucosone; and conversion of the products of glycolysis, glyceraldehyde-3-phosphate and dihydroxyacetone, to the highly reactive methyglyoxal (Brownlee, 2001). These reactive dicarbonyls – glyoxal, deoxyglucosone, and 3-deoxyglucosone – mainly react with the amino group of extracellular and intracellular proteins to form AGEs (Yamagishi et al., 2008). There is substantial evidence implicating the accumulation of AGEs as a critical biochemical event underlying the development of diabetic neuropathies. These observations are supported by both animal and clinical research that shows that the AGE formation inhibitor aminoguanidine partially prevents nerve damage associated with diabetes (Brownlee, 2001).

Abnormal AGE accumulation has been found to exist in both the central and peripheral nervous system under hyperglycemic conditions (Toth, 2007). A study by Misur et al. (2004) found that AGEs were highly expressed on the axons and myelin sheaths of diabetic peripheral nerves and the amount of AGE expression was proportional with the severity of morphological alterations. AGE-related modifications of proteins in peripheral nerves can lead to dysfunction of several cellular proteins such as tubulin, neurofilament, and $(Na^+ + K^+)$ ATPase (Toth, 2007). It has also been reported that AGE modifications of proteins impaired myelin synthesis in peripheral nerve, which may correlate with the thinning of myelin noticed in mouse models that develop diabetic neuropathy (Misur et al., 2004).

1.5 Role of oxidative stress in diabetic neuropathy

All four mechanisms that contribute to glucose neurotoxicity – increased flux through the polyol and hexosamine parthways, PKC activation, and AGE formation – are linked together by their tendency to increase oxidative stress in response to high glucose. These findings suggest oxidative stress as a common mechanism underlying the pathogenesis of diabetic neuropathy. Hyperglycemia overloads glucose metabolic pathways resulting in increased free radical generation and oxidative stress. If the free radical production exceeds the antioxidant capacity of a cellular system, oxidative stress occurs. Excessive free radical accumulation results in the damage of proteins, lipids, and nucleic acids by oxidative stress (Brownlee, 2001). Accumulation of such injury impairs biological activity of the cell and often leads to apoptotic cell death (Vincent et al., 2004).

Superoxide (O_2^-), hydrogen peroxide (H_2O_2), and superhydroxide (OH) are three of the most common oxygen free radicals, or reactive oxygen species (ROS), which accumulate in

response to hyperglycemic insult and causes oxidative stress. In high glucose conditions, the mitochondrial electron transport chain overproduces superoxide. Usually in normal metabolic conditions, superoxide is converted into the powerful oxidizer, hydrogen peroxide, and then enzymatically cleared via transformation into water by catalase. However, in hyperglycemia, increased glucose flux through the polyol pathway impairs this clearance mechanism and results in the accumulation of hydrogen peroxide. This in turn causes the increased production and accumulation of superhydroxide by way of the Fenton reaction (Brownlee, 2001; Tomlinson and Gardiner, 2008).

Hyperglycemia-induced oxidative stress is the most accepted hypothesis to explain the development of diabetic neuropathy. Studies have found that this oxidative stress triggers apoptotic mechanisms in sensory neurons and supporting glial cells and could be a unifying mechanisms leading to diabetic neuropathy (Vincent et al., 2004). More recently, it was also reported that oxidative stress plays a major role disrupting sympathetic function and triggering symptoms of DAN in diabetic mice (Campanucci et al., 2010)

Most people with diabetes develop complications of the ANS that adversely affect their quality of life and life expectancy. DAN causes cardiac arrhythmias, orthostatic hypotension and gastrointestinal abnormalities (Duby et al., 2004; Vinik and Zeigler, 2007). Yet, the cause of many of these diabetes induced complications are poorly understood. It was recently reported (Campanucci et al., 2010) that synaptic transmission in sympathetic ganglia was depressed in diabetic mice. To understand the mechanism behind this finding, the authors concentrated on nAChRs, the main receptor driving autonomic synaptic transmission, in cultured sympathetic neurons during diabetic conditions. Neurons that were exposed to high glucose conditions *in vitro* showed a use-dependent inactivation of nAChRs mediated by oxidative stress. This

inactivation was found to cause depression of autonomic synaptic transmission in sympathetic ganglia, which in turn led to the decrease in sympathetic drive in various animal models of diabetes. Moreover, the onset of autonomic neuropathy was prevented by manipulating the level of oxidative stress with the help of antioxidants or by genetically mutating nAChRs subunits, making these receptors insensitive to oxidation. Thus, hyperglycemia-induced oxidative stress inactivates nAChRs, which depresses autonomic function and triggers the onset of diabetic autonomic neuropathy. Through this research, it is now clear that hyperglycemia produces an accumulation of ROS in sympathetic neurons, however; the major mechanism underlying ROS generation has not yet been documented (Campanucci et al., 2008, 2010).
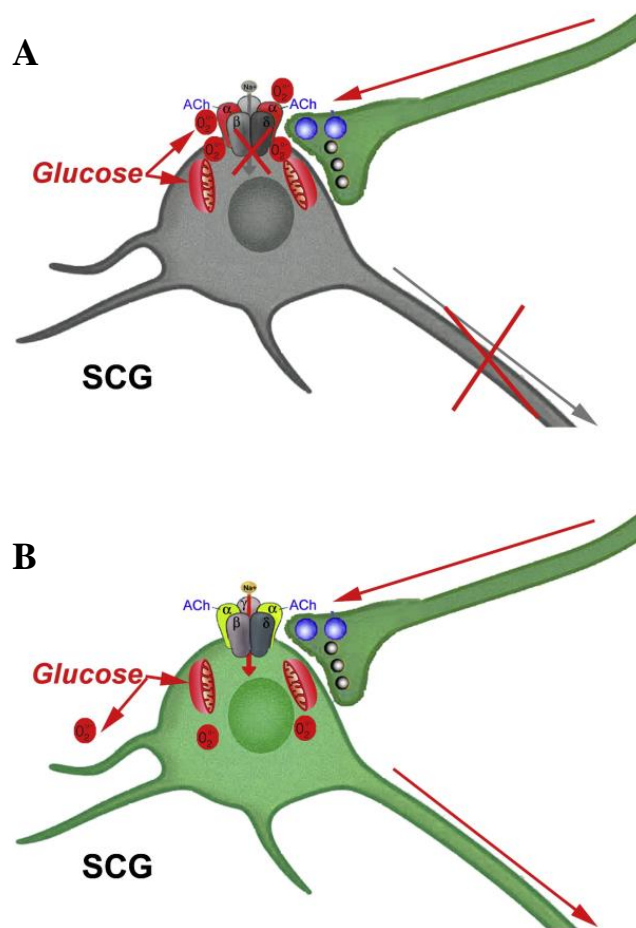
Figure 1-1. Effect of ROS on sympathetic synaptic transmission. (A) During sustained hyperglycemia, glucose-generated free radicals (O_2^-) oxidize cysteine residues in the α subunit (red) of the nAChR through which they diminish sympathetic synaptic transmission and trigger consequent alterations in peripheral tissue function. Glucose-generated ROS may arise from within SCG neurons via substrate oxidation or from extracellular sources. **(B)** Mutating the α subunit of the nAChR (yellow) by eliminating cysteine residues prevents free-radical-induced impairment of nAChR signaling allowing continued synaptic transmission despite hyperglycemia-induced free-radical generation (modified with permission from Diano and Horvath, 2010).

1.6 RAGE

RAGE is a multi-ligand receptor of the immunoglobulin family expressed in both the central and peripheral nervous systems (Toth et al., 2007). Under physiological conditions, RAGE plays a role in development, neuronal growth and survival, tissue repair, and the elimination of AGE-modified compounds (Vlassara, 2001; Rong et al., 2005). In contrast, it has also been implicated in various disease states such as diabetes, tumor outgrowth, chronic inflammation, and several neurodegenerative disorders such as Alzheimer's disease, stroke, and multiple sclerosis (Toth et al., 2007; Vincent et al., 2007). RAGE is composed of three extracellular domains, a single transmembrane domain, and a short cytoplasmic tail necessary for signaling (Fig. 1-2, Leclerc et al., 2009). RAGE interacts with several ligands such as the S100/calgranulin family, high-mobility group box 1 (HMGB1), amyloid-beta peptide, and AGEs. RAGE ligands are often released during cellular or physiological stress and interaction with RAGE often leads to the viscous inflammatory-related cycle of RAGE activation, up-regulation, and further activation (Toth et al., 2007; Vincent et al., 2007; Sims et al., 2010).

A considerable amount of evidence suggests that the interaction of RAGE with its ligands initiates signal transduction cascades involved in a variety of diabetic complications, including atherosclerosis, retinopathy, nephropathy, and neuropathy (Ritthaler et al., 1995; Hudson et al., 2003; Sakaguchi et al., 2003, Wendt et al., 2003; Flyvbjerg et al., 2004). One particular consequence of RAGE signaling that is the intense focus of current research is the production of ROS. RAGE ligands are significantly increased in diabetes and it has been proposed that activation of RAGE signaling pathways produce ROS, likely via nicotinamide adenine dinucleotide phosphate (reduced; NADPH) oxidase, which in turn results in further RAGE ligand formation and RAGE up-regulation (Yan et al., 2004). This enhancement of RAGE

signaling is at the center of current research aiming to understand the mechanisms of nervous system damage in diabetes.
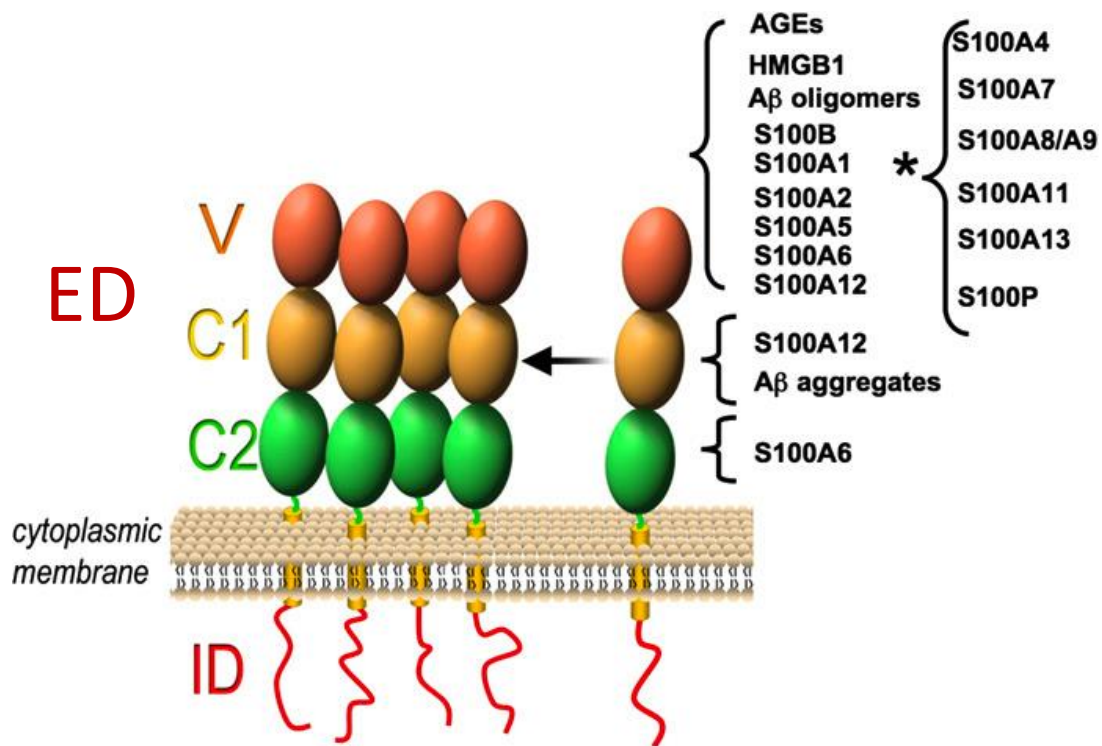
Figure 1-2. Schematic representation of RAGE and its ligands. The extracellular domain (ED) comprises three immunoglobulin-like domains: the V, C1 and C2 domain. A single transmembrane helix connects the extracellular domain with the short intracellular domain (ID). RAGE ligands interact with the extracellular domains as indicated (obtained with permission from Leclerc et al., 2009).

1.7 Objectives

The main objective of this thesis is to identify a major source of ROS production in cultured sympathetic neurons exposed to high glucose. We anticipate that if such a source is detected and blocked, the pro-oxidative signaling that has been shown to cause nAChR inactivation in sympathetic neurons will be diminished. This in turn should prevent the autonomic synaptic depression and consequent autonomic dysfunction observed in diabetic mice. Specifically, we hypothesize that RAGE signaling is the major contributor to high glucose induced ROS production in SCG neurons. In this thesis I test this hypothesis using electrophysiological, imaging, and biochemical techniques *in vitro*. The main goal of this study is to shed light on novel therapeutic approaches for the treatment and prevention of autonomic dysfunction caused by diabetes.

CHAPTER 2

MATERIALS AND METHODS

Neuronal primary cultures. All experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. SCG neurons were cultured from neonatal (P1-P3) C57 black 6 (C57BL6) wild-type or RAGE knock-out (RAGE KO) mice as previously described (Campanucci et al., 2008). Briefly, mice were euthanized by cervical transection in a sterile environment and SCGs were removed. Ganglia were placed in a Petri dish with serum-containing media (L15 supplemented with vitamins, cofactors, penicillin-streptomycin, 5 mM glucose, and 10% horse serum). Once cleaned, the ganglia were enzymatically dissociated at 37°C in Hanks balanced salt solution (HBSS) containing trypsin (180–200 U/ml; Worthington, Freehold, NJ, USA) and buffered with HEPES (adjusted to pH 7.4 with NaOH). The resulting cell suspension was washed twice in serum-containing medium, to inactivate the trypsin, and plated on laminin-coated coverslips attached to modified 35 mm tissue culture dishes. Neurons were grown in media consisting of L-15 supplemented with vitamins, cofactors, penicillin–streptomycin, 5 mM glucose, 5% rat serum and NGF (10 ng/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 95 % air-5 % CO_2 and fed every 3-4 days with growth media. To eliminate non-neuronal cells, cultures were treated with cytosine arabinoside (10 µM; Sigma, St. Louis, MO, USA) from days 2 to 4.

Culture conditions and treatments. After a waiting period of 1 week to allow recovery from stress and axotomy due to culturing procedures, neurons were either maintained in control conditions (5 mM glucose) or were switched to experimental treatments for an additional week.

To mimic hyperglycemic insult, cultures were treated with high glucose (HG)-containing growth media (25 mM glucose). In some experiments neurons were exposed to RAGE ligands, such as S100 protein (5-15 µg/ml; EMD Biosciences, San Diego, CA), HMGB-1 protein (1-5 µg/ml; Sigma), or AGE-BSA (200-300 µg/ml, BioVision). To block RAGE function during high glucose insult, a polyclonal antibody against RAGE (anti-RAGE) designed to block its function was used (30 µg/ml). For antioxidant treatment, 100 µM of the antioxidant α-lipoic acid and 1000 U/ml of catalase were prepared fresh on the day of the experiment and applied through the patch pipette during whole-cell recording.

Whole-cell recording. ACh-evoked currents were measured using whole-cell patch-clamp techniques. Membrane currents were recorded with an Axopatch 200B amplifier and a Digidata 1440A digitizer (Molecular Devices, Union City, CA). Series resistance compensation was not employed during data recording. Recordings were conducted at room temperature, sampled at 5 kHz, and stored on a personal computer. Recording electrodes had resistances of 5-12 MΩ and the cell membrane potential was clamped at -60 mV. pClamp 10.2 software (Molecular Devices) was used for data acquisition and analyses. Recording electrodes were filled with the following solution (in mM): 65 KF, 55 KAc, 5 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 2 MgATP, and 10 HEPES, and pH was adjusted to 7.2 with KOH. Cultured neurons were perfused with control extracellular solution containing (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.44 KH_2PO_4 , 2.8 $CaCl_2$, 0.18 $MgCl_2$, 10 HEPES, 5 glucose, 2 glutamine, and 5 µg/ml phenol red; pH was adjusted to 7.4 NaOH. ACh (100 µM) was dissolved in the perfusion solution and was applied under gravity using a perfusion fast-step system (SF-77B, Warner Instruments). Neurons were exposed repeatedly to 1 second applications of ACh-containing perfusion solution every 15 seconds. TTX

(1 µM; Alomone Labs Ltd., Jerusalem, Israel) was added to the perfusion solution to block

action potential generation during ACh applications. To quantify the run-down of the ACh-

evoked currents we plotted the ratio of the peak current (I) in response to the 90th application

(I_{90}) in a series to the peak current in response to the 1st application (I_1) in the series.

Intracellular ROS measurements. To quantify the changes in cytosolic ROS we used the ROS-

sensitive dye CM-H_2DCFDA (Molecular Probes, Burlington, Ontario, Canada) as previously

described (Campanucci et al., 2008; 2010). Cultures were incubated for 45 min at 37°C with

medium containing CM-H2DCFDA (10 µM) and subsequently washed three times with control

perfusion solution (see above). The cultures were then placed on the stage of an inverted

microscope (AxioObserver; Carl Zeiss, Germany) and viewed through a 40x (1.3 numerical

aperture) oil-immersion objectives (Zeiss) at room temperature. To obtain fluorescent images,

we excited the cultures with 470 nm wavelength using a Colibri 2.0 LED illumination system

(Zeiss) and collected 510-550 nm wavelength emissions with an AxioCam camera (Zeiss)

controlled by AxioVision software (Zeiss). LED intensity and exposure times were kept the same

for all neurons imaged. To quantify the fluorescent intensity, the difference in the intensity of the

region of interest (neuronal cell body excluding the nucleus) from the background was taken.

Immunocytochemistry. To measure RAGE or protein adducts of 4-hydroxyl-2-nonenal (HNE),

we fixed neurons with 4% paraformaldehyde for 1 hour at room temperature (Campanucci et al.,

2003). After washing 3 times with PBS (3 minutes each wash), the cells were then blocked with

10% horse serum for 30 minutes. Cultured neurons were then incubated overnight at 4°C with a

rabbit anti-RAGE (1:1000, Chemicon, Temecula, CA) or 4-HNE antibody (1:500, Alpha

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Diagnostics Intl., San Antonio, TX) diluted in PBS containing 1% BSA and 0.5% Triton X-100. The next day, the samples were washed in PBS (three times for 3 minutes each) and then incubated in the dark for 1 hour at room temperature with a goat anti-rabbit secondary antibody conjugated to FITC (1:500; Invitrogen). Samples were covered with an anti-photobleaching reagent (Vectashield; Vector Laboratories, Burlingame, CA) and viewed with an epifluorescence microscope as described above. Fluorescence intensity was analyzed by subtracting the region of interest from background.

Western Blotting. SCG neurons exposed to control or high glucose conditions were lysed using a 1% NP-40 lysis buffer containing various protease inhibitors. Protein concentration was determined by Bradford assay and samples were resolved by 10% SDS-PAGE and then electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The resulting membrane was incubated with the following primary antibodies overnight at 4°C: rabbit anti-RAGE (1:1000; Chemicon), rabbit anti-NFκB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-GAPDH (1:10,000; Abcam, Cambridge, MA). The membrane was then washed three times with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) before being probed with either a horseradish peroxidase-conjugated goat anti-rabbit or mouse secondary antibody (1:20000; Bio-Rad Laboratories). Protein signals were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology) and quantified by performing densitometry using ImageJ software (NIH, Bethesda, MD).

Statistics. All statistical analyses were done using Graphpad InStat 3.0. All values are reported as mean±s.e.m. and p values less than or equal to 0.05 were considered statistically significant.

For comparisons of the increase in RAGE immunofluorescence and RAGE/GAPDH ratio between control and high glucose groups, we used the Mann-Whitney U test. To determine the statistical differences between the I_{90}/I_1 ratios, where appropriate, either the Mann-Whitney U test or Kruskal-Wallis Test followed by post hoc comparisons using the Dunn's Multiple Comparisons test was employed. Statistical comparison between control and S100 treatment for mean CMH_2-DCFDA and HNE fluorescent intensities were determined using the Mann-Whitney U test. For comparisons between control and HG conditions for mean CMH_2-DCFDA and HNE fluorescent intensities, the nonparametric Kruskal Wallis Test was used followed by Dunn's post hoc analysis.

CHAPTER 3

RESULTS

3.1 SCG neurons show increased expression of RAGE in high glucose conditions.

Previous reports on sensory neurons show that RAGE protein is expressed at low levels in control conditions and is up-regulated when exposed to high glucose conditions (Vincent et al., 2007). Since RAGE activation is associated with the generation of ROS, here we propose that RAGE expression in sympathetic neurons is up-regulated during high glucose conditions and is a major source of ROS production contributing to nAChR inactivation. To test this, RAGE expression was observed and quantified by immunofluorescence. Recent findings from our laboratory (Chang and Campanucci, unpublished observations) show that cultured sympathetic neurons from the SCG were found to express a basal level of RAGE in control conditions that appeared broadly distributed over the soma and processes. Exposure to high glucose (from 24 hrs up to 8 days) caused a significant increase ($p<0.05$) in RAGE immunofluorescence (RAGE-IF), which plateaued by 48 hours and remained elevated for the length (8 days) of the experiment (Fig. 3.1A). Exposure to high glucose for 6 (not shown) to 12 hours had no effect on the level of RAGE expression ($p>0.05$). These data were further supported by the detection of a significant increase in the level of RAGE protein after 1 week of high glucose treatment using Western Blotting (Fig. 3.1B). A single RAGE band was observed at approximately 50 kDa and high glucose (0.55 ± 0.16) treatment resulted in more than a 4 fold increase in RAGE expression relative to GAPDH when compared to control (0.13 ± 0.01).

3.2 The RAGE ligand S100 increases oxidative stress markers in SCG neurons.

Reports from several different cell types, including sensory neurons (Vincent et al., 2007), endothelial cells (Brownlee, 2000) smooth muscle cells, and mononuclear phagocytes (Stern et al., 2002), have shown that RAGE activation directly leads to oxidative stress. To confirm this in cultured SCG neurons, we activated RAGE by exposing the cells to the natural RAGE ligand S100 protein (15 µg/ml) for 1 week. Working concentration for S100 was determined using electrophysiological experiments (see section 3.3D). Increase in cytoplasmic ROS levels and protein adducts of 4-hydroxy-2-nonenal (HNE), a major by-product of endogenous lipid peroxidation, are common markers for cellular oxidative stress (Halliwell and Whiteman, 2004; Eruslanov and Kusmartsev, 2010). Thus, we quantified the cell's oxidative state by monitoring cytoplasmic ROS levels by CM-H_2DCFDA fluorescence or HNE immunofluorescence. As expected, exposure to S100 protein caused a significant increase in both DCFDA (Control: 1545±50 AU; S100: 3587±73 AU) and HNE (Control: 4182±76 AU; S100: 4610 ± 66 AU) levels quantified as an increase in fluorescent intensity (Fig. 3.2). These results suggest that activation of RAGE leads to pro-oxidative signaling in SCG neurons.
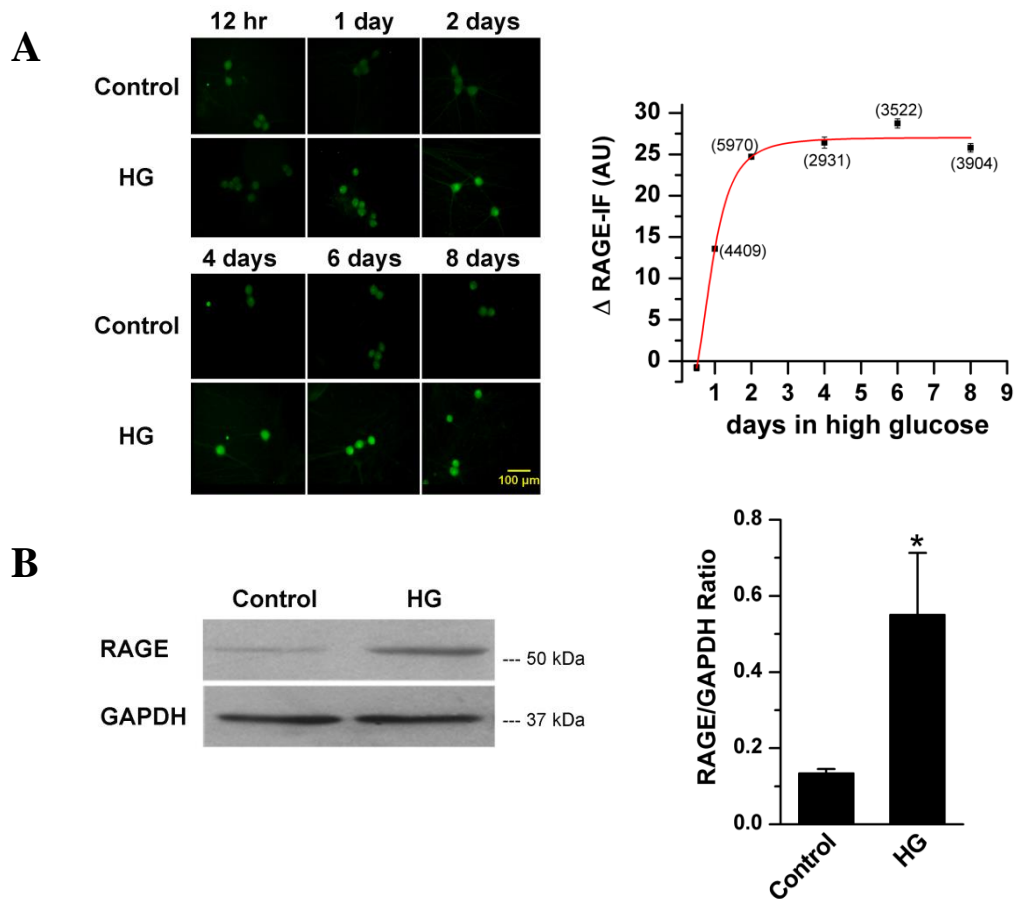
Figure 3.1: SCG neurons show increased expression of RAGE in high glucose conditions.
(A) Fluorescent images of RAGE immunostained SCG neurons exposed to control and high glucose conditions for 12 hrs and up to 8 days. Exposure of these neurons to high glucose resulted in a time-dependent increase in RAGE immunofluorescence (RAGE-IF). The line graph summarizes in arbitrary units the change in RAGE–IF measured as the difference in mean pixel intensity compared to control. The increase in RAGE-IF was significantly different by 24 hrs and plateaued by 2 days in high glucose ($p < 0.001$). In contrast, neurons exposed to 12 hrs of high glucose or kept in control conditions did not show any significant change in RAGE-IF. The scale bar represents 50 μm. Findings are the work of Christine Chang. **(B)** Representative immunoblots showing RAGE protein expression in control and high glucose conditions in cultured SCG neurons. The bar graph shows the average integrated densities of the RAGE band normalized to that of GAPDH for neurons cultured in control (n=4) or in high glucose (n=4) conditions. Each point in (A) and bar in (B) represents the mean ± s.e.m.; *$p < 0.05$.

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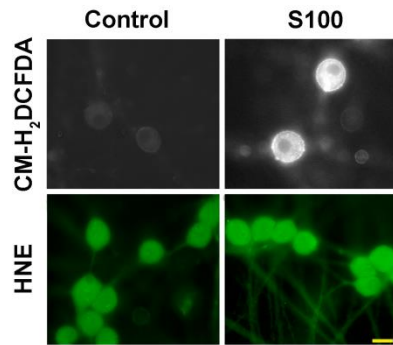
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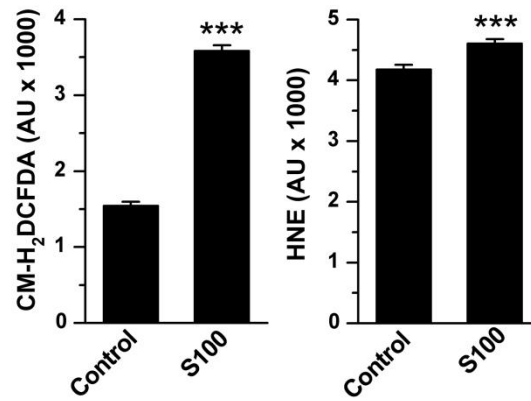
Figure 3.2: The RAGE ligand S100 increases oxidative stress markers in SCG neurons.
(**A**) Fluorescent images of neurons cultured in control conditions or incubated with S100 protein
(15 μg/ml) for 1 week and loaded with CM-H$_2$DCFDA or immunostained for protein adducts of
HNE. The scale bar is 50 μm. (**B**) The bar graphs show the average fluorescence intensity in
arbitrary units for neurons cultured in control (n=348 neurons from 3 dishes) or in the presence
of S100 protein (n=540 neurons from 3 dishes) for ROS detection with DCFDA (left) or HNE
immunofluorescence respectively (right; n= 100 neurons from 3 dishes for each bar). Each bar
represents the mean ± s.e.m.; ***p<0.001.

3.3 RAGE ligands induce oxidation-mediated inactivation of nAChRs in SCG neurons.

Previous studies have shown that cytoplasmic ROS accumulation induces the inactivation of nAChRs in SCG neurons, which results in autonomic dysfunction (Campanucci et al., 2008, 2010). Given that direct activation of RAGE by S100 protein induces ROS accumulation, the question arose whether direct RAGE activation also induces the use-dependent inactivation of nAChRs in cultured SCG neurons. To explore this possibility we exposed cultured SCG neurons to increasing concentrations of the natural RAGE ligands S100 and HMGB1 proteins and determined that 15 µg/ml and 5µg/ml, respectively, significantly induced the run-down of ACh-evoked currents (Fig. 3.3A). The effects of 1 week exposure to S100 (15 µg/ml; (I_{90}/I_1: 0.64 ±0.07) or HMGB1 (5 µg/ml; I_{90}/I_1: 0.51 ±0.05) are summarized in figure 3.3 (B&C). We also wanted to determine if exposure to BSA-AGEs, the most studied RAGE ligand contributing to a myriad of diabetic complications (Goh and Cooper, 2008), induces the inactivation of nAChRs. As expected, exposing SCG neurons for 1 week to 300 µg/ml of BSA-AGE, the approximate amount found in diabetic tissue (One et al., 1998), resulted in nAChR inactivation (I_{90}/I_1: 0.87 ±0.04; Fig. 3.3D). These results indicate that induction of RAGE function by its ligands causes nAChR inactivation in SCG neurons.

Next, since antioxidant treatment successfully prevented the oxidation-mediated inactivation of nAChRs in SCG neurons (Campanucci et al., 2010), we concentrated on the potential beneficial role of antioxidants to treat the RAGE-mediated effects. Thus, to determine whether the RAGE-mediated inactivation of nAChR is indeed caused by cytoplasmic ROS, we co-exposed sympathetic neurons to S100 protein and to the antioxidants α-lipoic acid (100 µM) and catalase (1000 units/ml) for 1 week. The antioxidant mix successfully prevented the inactivation of nAChR currents induced by the S100 treatment (S100+AO I_{90}/I_1: 0.96±0.07; Fig.

3.3B). These data further supports the role of RAGE as a major source of cytoplasmic ROS

during high glucose conditions.
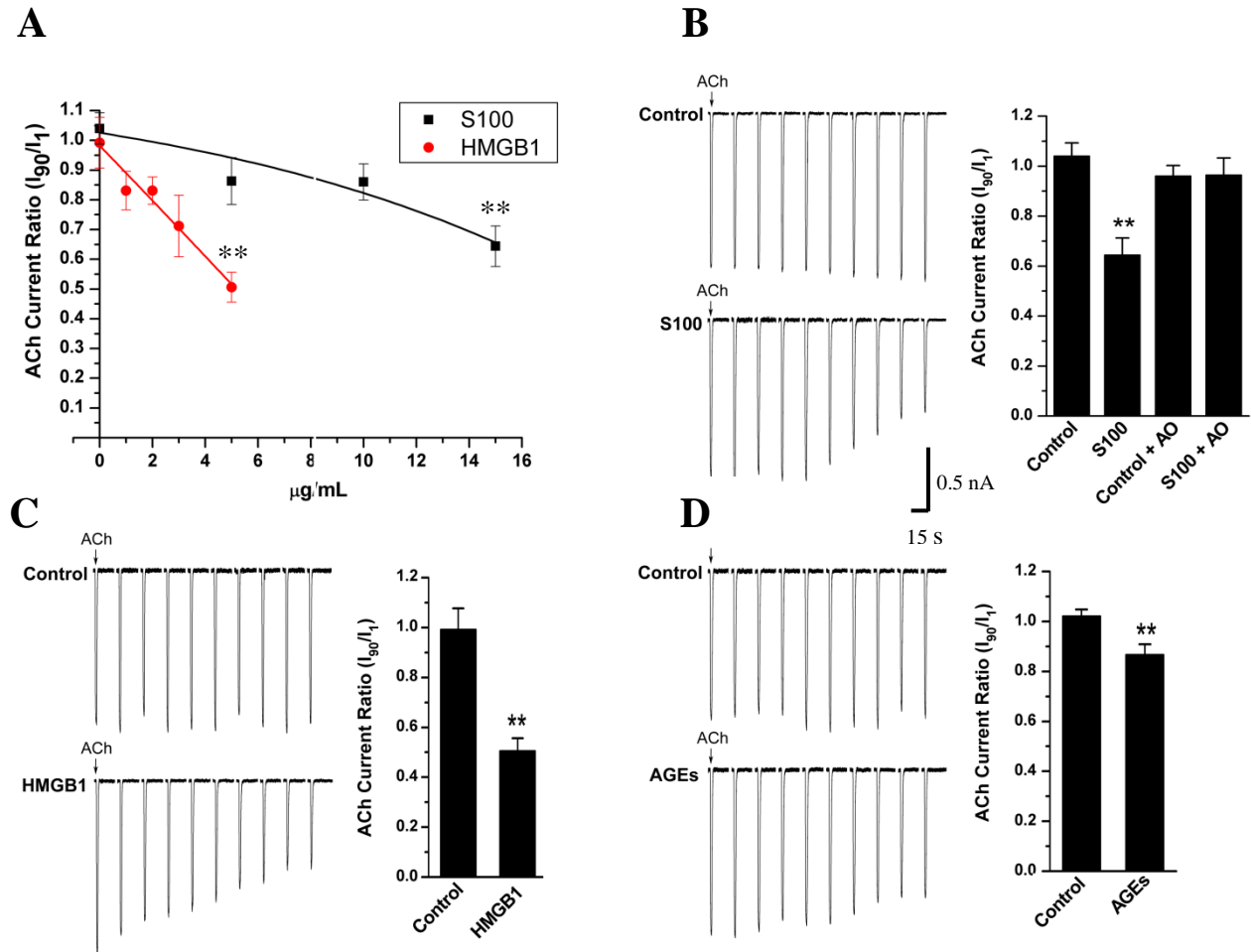
Figure 3.3: Activation of RAGE signaling induces nAChR inactivation and this effect is prevented by antioxidant treatment. (A) Each point represents the mean $I_{90}/I_1 \pm$ s.e.m for SCG neurons exposed to various concentrations of S100 or HMGB1. The points are fitted using an exponential decay function (S100: $r^2=0.93$; HMGB1: $r^2=0.96$). ** refers to $p < 0.01$. **(B)** Currents evoked by a series of ACh (100 μM) applications on SCG neurons from wild-type mice in control conditions, incubated for 1 week with 15 μg/ml S100, control conditions and intracellularly perfused with an antioxidant mix (α-lipoic acid and catalase), or 1 week incubation with 15 μg/ml S100 protein and intracellularly perfused with the antioxidant mix. To help visualize the effect of the treatments on ACh-evoked currents, only one every ten traces are shown. Histogram summarizes the ratio of the 90th ACh-evoked current to the first in the series (I_{90}/I_1) for SCG neurons incubated for 1 week in control (n=7), S100 (n=12), control plus antioxidants (n= 6), and S100 plus antioxidants (n=8). Each bar is the mean $I_{90}/I_1 \pm$ s.e.m; ** refers to $p < 0.01$. **(C)** Similar experiment as in (A) but comparing the effect of incubating cultured SCG neurons from wild type mice in media containing HMGB1 (5 μg/ml; n = 7) for 1 week versus control (n = 5). Each bar is the mean $I_{90}/I_1 \pm$ s.e.m; ** refers to $p < 0.01$.**(D)** Similar experiments as in (A) and (B) but in this case comparing the effect of incubating cultured SCG neurons from wild type mice in media containing BSA-AGEs (300 μg/ml; n = 13) for 1 week versus control (n = 10). Each bar in (B), (C) and (D) is the mean $I_{90}/I_1 \pm$ s.e.m; ** refers to $p < 0.01$. The n values reported in (B), (C) and (D) were obtained from 3-6 dishes for each condition.

3.4 High glucose induced inactivation of nAChRs is prevented in SCG neurons with disrupted RAGE signaling.

A recent report has shown that high glucose heightens the oxidative state of SCG neurons which results in nAChR inactivation (Campanucci et al., 2010). As shown above, high glucose induced the up-regulation of RAGE and RAGE activation increased oxidative markers in sympathetic neurons and caused nAChR inactivation. Thus, in order to determine whether RAGE plays a role in causing nAChR inactivation under high glucose, we used a RAGE neutralizing antibody to disrupt RAGE signaling. Exposure to 30 μg/ml of the RAGE neutralizing antibody blocks approximately 50% of RAGE radio-ligand binding (R & D Systems). This is consistent with unpublished observations from a collaborator (Dr. Fernyhough at the University of Manitoba) who shows that 30 μg/ml of antibody is sufficient to significantly disrupt the RAGE-mediated neurite outgrowth of embryonic sensory neurons *in vitro*. Our results show that when SCG neurons were co-incubated with the RAGE neutralizing antibody under high glucose conditions for 1 week, this treatment partially prevented the run-down effects of high glucose on ACh-evoked currents (Control I_{90}/I_1: 1.05 ± 0.04; HG I_{90}/I_1: 0.77 ±0.06; HG + Ab I_{90}/I_1: 0.92 ±0.05; Fig. 3.4).

3.5 High glucose fails to induce oxidative stress and nAChR inactivation in SCG neurons from RAGE KO mice.

The findings shown so far strongly suggest that RAGE signaling has a pivotal role in the generation of oxidative stress and inactivation of nAChRs in SCG neurons exposed to high glucose conditions. Therefore, to confirm this possibility we took advantage of mice that lack exon 1 in the RAGE gene, resulting in global RAGE knock-out (RAGE KO) (Myint et al., 2006).

Our results indicate that high glucose induced-ROS formation (Control: 1486±36 AU; HG: 2089±65 AU) and lipid peroxidation (Control: 4050±53 AU; HG: 4837±63 AU) was prevented in SCG neurons from RAGE KO mice (ROS: 1089±32 AU; HNE 4152±84 AU; Fig. 3.5) suggesting that RAGE is required for the deleterious effects of high glucose. Interestingly, we observed that basal cytoplasmic ROS measured as CM-H_2DCFDA fluorescence levels was significantly lower in SCG neurons from RAGE KO mice (1032±20 AU) than from wild-type mice (1486±36 AU) maintained in the same control (5 mM glucose) media (Fig. 3.5A). These results suggest that RAGE signaling may contribute to the basal redox state of autonomic neurons.

Using RAGE KO mice we also confirmed that RAGE is required to impair nicotinic function under high glucose conditions. When SCG neurons cultured from RAGE KO mice were exposed to high glucose for 1 week, they showed stable ACh-evoked currents (HG I_{90}/I_1: 1.00 ±0.08) suggesting that high glucose failed to induce nAChR inactivation (Fig. 3.6). Passive membrane properties (membrane potential, Vm; membrane capacitance, Cm; and access resistance, Ra) were not significantly different in wild type and RAGE KO neurons exposed to both control and high glucose conditions (Table 3.1). We also incubated SCG neurons from RAGE KO mice for 1 week with S100 protein (15 µg/ml), HMGB1 (5 µg/ml) or AGEs (300 µg/ml), and in all these cases, ACh-evoked currents were not significantly affected (S100 I_{90}/I_1: 1.03 ± 0.11; HMGB1 I_{90}/I_1: 0.81 ±0.08; AGEs I_{90}/I_1: 1.02 ± 0.03; Fig. 3.6). However, although not statistically significant, HMGB1 was still able to cause some decline of ACh-mediated currents. This effect could be explained by the known pro-oxidative signaling of HMGB1 through its other binding sites, such as toll-like receptors (Gill et al., 2010).
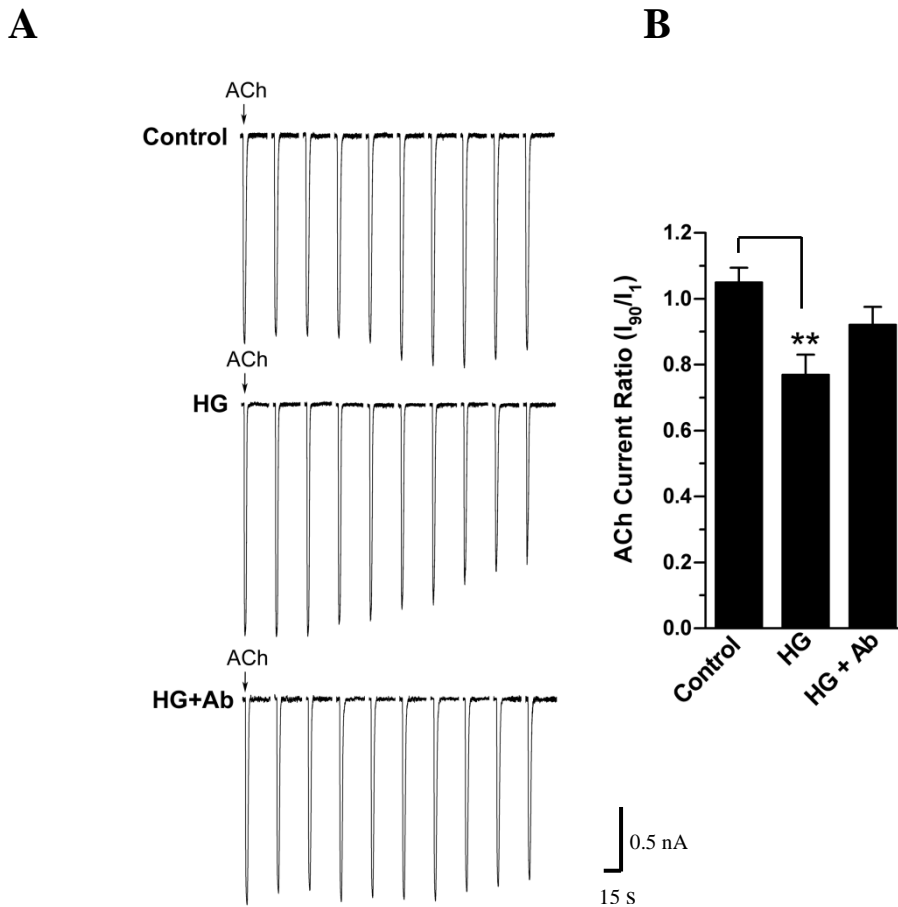
Figure 3.4: High glucose induced inactivation of nAChRs is prevented by a RAGE function blocking antibody. (**A**) Currents evoked by ACh (100 μM) applications on SCG neurons from wild type mice maintained in control glucose concentration, 1 week in high glucose (HG), or 1 week in high glucose plus anti-RAGE antibody (30 μg/ml). After the first trace, every tenth trace is shown for clarity. (**B**) Histogram summarizes the ratio of the 90th ACh-evoked current to the first in the series (I_{90}/I_1) for SCG neurons incubated for 1 week in control glucose concentration (n=10 neurons from 4 dishes), high glucose (n=14 neurons from 7 dishes), and high glucose plus anti-RAGE antibody (n=11 from 5 dishes). Each bar is the mean $I_{90}/I_1 \pm$ s.e.m; ** refers to p < 0.01.
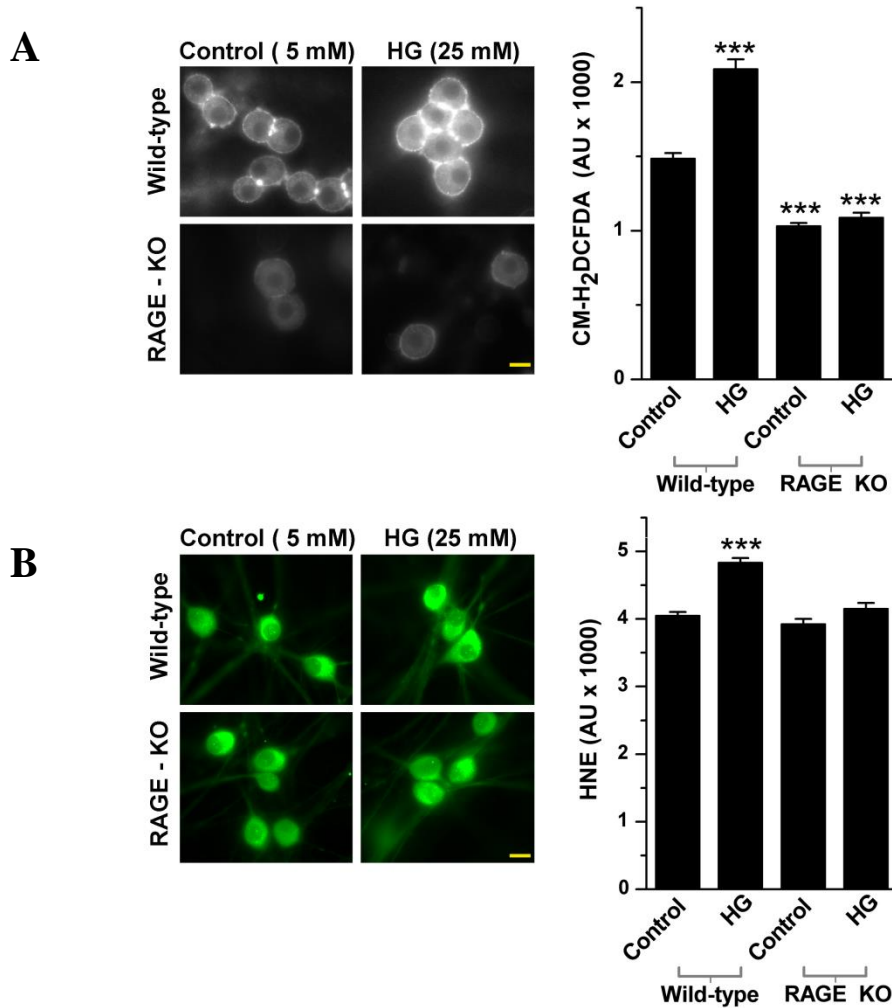
Figure 3.5: High glucose fails to induce ROS accumulation and HNE-protein adduct formation in SCG neurons from RAGE KO mice. (A) Fluorescent images of ROS detection in SCG neurons from wild-type and RAGE KO mice maintained in either control or high glucose conditions for 1 week. The bar graph shows the average DCFDA fluorescence intensity in arbitrary units for neurons from wild-type mice in control (n=129) or high glucose (n=140) conditions, and neurons from RAGE KO mice in control (n=118) or high glucose (n=119) conditions. Data was collected from 3 dishes for each condition. **(B)** Immunofluorescent images for HNE protein adducts in SCG neurons from wild-type and RAGE KO mice maintained in either control or high glucose conditions for 1 week. The bar graph shows the average fluorescence intensity for HNE in arbitrary units for neurons from wild-type and RAGE KO mice in control or high glucose conditions (n= 100 neurons from 3 dishes for each bar). Each bar in (A) and (B) represents the mean ± s.e.m.; ***p<0.001. The scale bar in (A) and (B) is 50 µm

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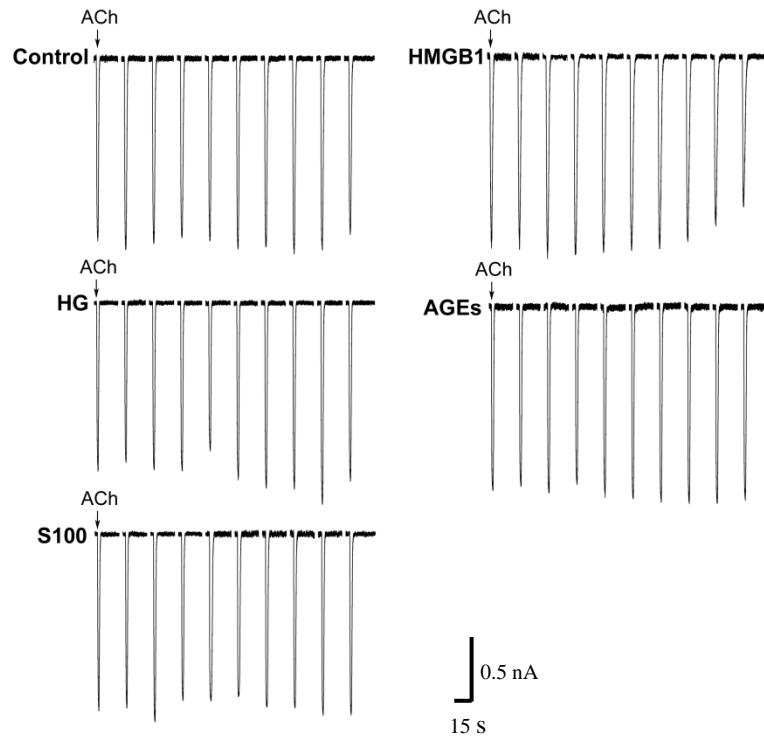
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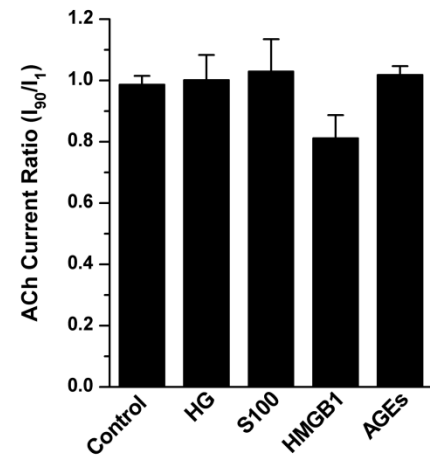
Figure 3.6: Sympathetic neurons lacking RAGE do not show nAChR inactivation when incubated with high glucose or RAGE ligands. (A) Currents evoked by a series of ACh (100 μM) applications on SCG neurons from RAGE KO mice maintained in either control conditions or incubated for 1 week with high glucose, 15 μg/ml S100 protein, 5 μg/mL HMGB1, or 300 μg/mL BSA-AGEs. After the first trace, every tenth trace is shown for clarity. (B) Histogram summarizes the ratio of the 90th ACh-evoked current to the first in the series (I_{90}/I_1) for RAGE KO SCG neurons maintained in either control conditions (n=6) or incubated for 1 week with high glucose (n=11), 15 μg/ml S100 protein (n=7), 5 μg/mL HMGB1 (n=7), or 300 μg/mL BSA-AGEs (n=8). Data was collected from 3-5 dishes for each condition. Each bar is the mean $I_{90}/I_1 \pm$ s.e.m.

	Wild-type		RAGE KO	
	5 mM	**25 mM**	**5 mM**	**25 mM**
Vm (mV)	-38.25±2.9	-40.31±2.27	-49.5±2.53	-44.5±3.82
Cm (pF)	25.56±2.71	27.49±2.30	34.84±4.16	23.9±2.25
Ra (MΩ)	32.4±2.78	29.72±2.28	28.95±1.87	32.98±3.14

Table 3.1: Passive membrane properties. Comparison of mean passive membrane properties for cultured SCG neurons from wild type and RAGE KO mice exposed to both, control (5 mM) and high glucose (25 mM) conditions. Note, mean values were not significantly different. N values are the same as those reported above for the respective ACh-evoked current recording.

CHAPTER 4
DISCUSSION

In this study we use an *in vitro* approach to highlight RAGE as a major source of high glucose-triggered oxidative stress leading to sympathetic neuron dysfunction. Recently, a new model for the onset of dysautonomia in diabetes was proposed where hyperglycemia-induced oxidative stress inactivates nicotinic acetylcholine receptors (nAChRs), the main receptor driving autonomic synaptic transmission in sympathetic ganglia. This inactivation leads to the depression of synaptic transmission, and consequently triggers the onset of autonomic neuropathy in diabetic mice (Campanucci et al., 2010). However, the source and pathways contributing to the elevation of ROS and generation of oxidative stress remained unclear. We show here that RAGE protein levels are up-regulated in high glucose conditions and that ligand activation of RAGE increases cellular oxidative stress leading to nAChR inactivation in cultured sympathetic neurons. We then go on to report that in the absence of RAGE expression, using RAGE KO mice, the accumulation of ROS and subsequent inactivation of nAChRs that occurs in high glucose conditions were prevented. These results shed light on RAGE as a major source of ROS production in hyperglycemic conditions and suggest a pivotal role of RAGE in the development of diabetic autonomic neuropathy.

RAGE protein expression is up-regulated in high glucose conditions

Several studies have previously reported the detrimental effects of RAGE up-regulation and activation in contributing to both vascular and renal dysfunction observed in diabetes (Yamamoto et al., 2000; Stern et al., 2002). However, very few studies have looked at the involvement of RAGE expression and signaling in diabetic neuropathy. In DRG neurons, which are affected in diabetic sensory neuropathy, exposure to RAGE ligands caused an increase in

RAGE expression and activation resulting in severe oxidative stress and neuronal apoptosis (Vincent et al., 2007). It was observed in mouse models of long-term diabetes an increased RAGE expression in neurons and especially in Schwann cells (Toth et al., 2007). Patients with diabetic sensory neuropathy have also been reported to have increased RAGE and NF-κB activation in peripheral nerves (Bierhaus et al., 2004). This increased expression corresponds with the observation that RAGE blockade in animal models prevented the majority of sensory deficits caused by diabetes (Bierhaus et al., 2004). While the above work has examined the contribution of RAGE towards the sensory abnormalities observed in diabetes, the purpose of this thesis was to investigate whether RAGE plays a similar pathological role in autonomic neurons exposed to diabetic conditions.

Here we show for the first time by immunocytochemistry (Chang and Campanucci, unpublished observations) and western blotting that RAGE is expressed in cultured sympathetic neurons and is up-regulated by high glucose in a progressive, time-dependent fashion (Fig. 3.1). However, we do not know the molecular mechanisms mediating this increase in RAGE expression. RAGE signaling often leads to sustained activation of NF-κB, a pro-inflammatory transcription factor (Yan et al., 1994; Bierhaus et al., 2004), suggesting that the deleterious effects of RAGE are linked to inflammation. Interestingly, the RAGE promoter contains binding sites for NF-κB, allowing NF-κB activity levels to have a positive feed-back effect on RAGE expression (Li and Schmidt, 1997). This may explain why RAGE up-regulation and signaling often produces a vicious inflammatory cycle where activation leads to further RAGE up-regulation. This RAGE-mediated chronic inflammatory cycle has been observed in sensory neurons (Feldman et al., 2007), vascular endothelial and carcinoma cells (Li and Schmidt, 1997; Stern et al., 2002). However, these reports contrast with our preliminary findings which do not

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show changes in phosphorylation of NF-κB (data not included) in our cultured sympathetic neurons exposed to high glucose. It is possible that RAGE is being up-regulated by another transcription factor such as specificity protein 1 (SP-1), which also binds to the RAGE promoter independently from NFκB (Li and Schmidt, 1997). Also, even though increased NF-κB activity is observed with long-term diabetes in both human and animal models (Bierhaus et al., 2001), rat models with diabetes for a moderate amount of time has shown diminished NF-κB activity (Purves and Tomlinson, 2002). Therefore, it is possible that exposing SCG neurons to high glucose for 1 week was too short of a period for an observable NF-κB increase. Further experiments will be required to clarify this observation.

Ligand activation of RAGE causes oxidative stress and nAChR inactivation

In this thesis it was demonstrated that exposure of SCG neurons to the natural RAGE ligand S100 causes an increase in oxidative stress (Fig. 3.2). This is in agreement with several studies in a multitude of cell types showing that ROS production and accumulation is a common downstream effect of sustained RAGE activation (Yan et al., 1994; Lander et al., 1997; Wautier et al., 2001; Xu et al., 2003; Ding et al., 2007 Vincent et al., 2007). A previous study has shown that elevations in cytosolic ROS induced nAChRs inactivation leading to synaptic depression in intact sympathetic ganglia (Campanucci et al., 2008). Thus, as we expected, SCG neurons exposed to three natural RAGE ligands (S100, HMGB1, and AGEs) resulted in the inactivation of nAChRs. To confirm this was mediated by oxidative stress, we successfully prevented the S100-induced nAChR inactivation by co-treating cells with antioxidants. Interestingly, AGEs (Brownlee, 1992; Ulrich and Cerami, 2001; Thornalley, 2002), S100 (Kosaki et al., 2004), and HMGB1 (Yao and Brownlee, 2009) have been shown to be increased in diabetic tissue and may

be one of the main contributors in ROS generation and the subsequent depression of autonomic synaptic transmission. Supporting this view are adipocytes, which have also been reported to be a major source of S100 secretion. Therefore, adipocyte dysfunction or obesity, which is intimately link to type II diabetes, may further add to autonomic dysfunction by excessive S100 secretion (Steiner et al., 2010).

Our findings revealing the deleterious effects of RAGE signaling on autonomic function could potentially have broader implications for our understanding of both the physiology and pathophysiology of the ANS. This is the case for satellite glial cells, which are specialized glial cells in autonomic ganglia found tightly wrapped to neurons and responsible for controlling the neuronal microenvironment (Hanani, 2005, 2010). Interestingly, satellite glial cells are known to highly express S100 proteins (Hanani, 2010), and since these cells may have a major role in influencing sympathetic synaptic transmission by controlling the composition of the neuronal microenvironment, it is possible that RAGE plays a role in mediating neuron-glia communication. We have shown here that incubating cultured SCG neurons with exogenous S100 proteins inactivates nAChRs, most likely acting through RAGE. For future research it would be interesting to explore the possibility whether S100 released from satellite glial cells contributes to the SCG synaptic depression reported in diabetes (Campanucci et al., 2010). However, we should also consider the potential contribution of other sources of S100 proteins that could further impact the pro-inflammatory microenvironment generated in diabetes, such as astrocytes (Gerlach et al., 2006; Tramontina et al., 2006; Ellis et al., 2007), macrophages (Ellis et al., 2007), and Schwann cells (Perrone et al., 2008).

Inhibiting RAGE function prevents high-glucose induced oxidative stress and nAChR inactivation

Here we showed that high-glucose failed to induce accumulation of cytoplasm ROS in cultured SCG neurons from RAGE KO mice (Fig. 3.5A). Furthermore, cytosolic ROS levels in neurons from RAGE KO mice showed a lower basal ROS fluorescence compared to wild-type. Therefore, these findings led us to propose that not only is RAGE the predominant source of oxidative stress under high glucose conditions in our system but also that it contributes to the normal cytoplasmic redox state of autonomic neurons. Another intriguing aspect of how RAGE can affect neuronal function under pathological conditions comes from the recent report showing RAGE expression in mitochondria in cancer cells (Kang et al., 2013). In cancer cells RAGE has pro-survival functions by enhancing mitochondrial activity and ATP production. However, if RAGE is indeed expressed in the mitochondria of SCG neurons and if it has any role in mitochondrial bioenergetics in either physiological or pathological conditions remains to be determined.

After revealing that RAGE expression was needed for high glucose to induce ROS accumulation, our next logical step was to determine if the high glucose mediated nAChR inactivation was prevented in neurons where RAGE function was hampered. We show that by co-exposing sympathetic neurons to high glucose and a function blocking RAGE antibody, the nAChR inactivation was partially prevented (Fig. 3.4). Further, when exposing SCG neurons from RAGE KO mice to high glucose, inactivation was completely prevented (Fig. 3.6). As expected from the above imaging experiments, nAChRs were insensitive to the effects of high glucose in neurons lacking RAGE. This is likely explained by the prevention of ROS generation in RAGE KO neurons, thus preserving the normal functioning of nAChRs.

A recent study by Campanucci et al. (2010) reported that hyperglycemia causes an irreversible inactivation of nAChRs in sympathetic neurons. An important point to note is that even though our general conclusions remain consistent with that of the recent study, the magnitude and time course of the high glucose-induced inactivation of ACh-evoked currents is markedly different. We observed a slow but consistent run-down of approximately 30% by the 90th ACh-evoked current while the previous report shows a faster and larger decline (70% run-down by the 30th application; Campanucci et al., 2010). This discrepancy can be accounted for by two main factors. First, the perfusion system during whole-cell recording was completely different between the two studies. In the previous report, agonist applications were performed with the help of a compressed-gas puffer pipette of just 2-5 µm in diameter. The main weaknesses of that system were that the final concentration of agonist applied to the cell depends on the distance that the puffer pipette is placed from the cell membrane, and that all currents are obtained from a small and discrete area of the cell body instead of from the vast dendritic arborization characteristic of SCG neurons. To circumvent these issues in the present thesis, agonist application was performed using a fast step continuous perfusion system. The fast step perfusion system generates a constant river of solution that switches back and forth commanded by the patch clamp amplifier and it uses a much larger diameter pipette (0.6 mm) resulting in currents evoked from the full cell body and dendrites of cultured neurons. In addition, we cannot discard the possibility that the discrepancies in magnitude and time course of the run-down of ACh-evoked currents are based on the health of our cultured neurons. The health of neurons in cultures that depend on NGF for survival is related to the amount of NGF used. In the current study we used 60% less NGF in our culture media than in Campanucci et al. (2010) and neurons were maintained healthy for up to 1 month; the current amount of NGF used was markedly

apoptotic for the neurons used in the original study (unpublished observations). Thus, it is possible that we are observing less nAChR inactivation compared to Campanucci et al. due to the fact our neurons are in a healthier state. Overall, we are confident that the discrepancy between the studies can be attributed to differences in experimental design instead of biological reasons.

Future Directions:

Two critical questions still remain unanswered – 1) how is RAGE being activated by high glucose in our *in vitro* experimental set-up; 2) What are the molecular mechanisms that produce oxidative stress in response to RAGE activation? To address the first question, we hypothesize that a significant portion of RAGE activation occurs because of the formation of AGEs due to high glucose in the culture media. An *in vitro* study of monocytes has shown that only two hours are needed to form AGEs and induce NF-κB signaling in response to hyperglycemia (Schiekofer et al., 2003). Thus, incubating sympathetic neurons with high glucose for one week should provide more than enough time to form enough AGEs *in vitro* for a significant increase in RAGE activation. Another source of RAGE activation may be the cultured sympathetic neurons themselves. Accumulating evidence suggests that S100 and HMGB1 can be secreted in response to certain stimuli and then act in an autocrine or paracrine fashion to induce RAGE activation (Wang et al., 1999; Schmidt et al., 2000; Scaffidi et al., 2002; Bonaldi et al., 2003; Donato, 2007; van Beijnum et al., 2013). Further experiments are needed to determine if high glucose stimulates the extracellular secretion of RAGE ligands from sympathetic neurons.

To address the second question we must focus on the RAGE downstream signaling cascade. The molecular pathways mediating RAGE signaling is an area of intense research that could one day have many therapeutic implications. However, there are still many questions

remaining about how RAGE is able to incorporate many diverse ligands into so many different cellular responses. Evidence suggests that RAGE activation results in the rapid generation of ROS by a mechanism that in part involves the activation of NADPH oxidase (Yan et al., 1994; Lander et al., 1997; Wautier et al., 2001; Vincent at al., 2007). Other studies have also shown RAGE signaling occurring primarily via the MAPK and PI-3K/Akt pathway which eventually leads to ROS formations via NADPH oxidase activation (Lander et al., 1997; Ishihara et al., 2003; Shanmugam et al., 2003; Xu et al., 2003; Zill et al., 2003; Vincent et al., 2007). Future experiments need to be designed to address if NADPH oxidase activity is increased in SCG neurons exposed to high glucose and if this is prevented in neurons from RAGE KO mice. Preliminary evidence from our lab also shows strong increases in PI-3K/Akt signaling pathway in SCG neurons exposed to high glucose for 1 week; however, no increase in the MAPK pathway was observed (data not shown).

A future and critical goal of this work is to test whether blocking RAGE function has a protective effect in maintaining normal sympathetic function in STZ-induced diabetic mice. Using similar measures of sympathetic activity as conducted by Campanucci et al. (2010), we plan to study if sympathetic function becomes depressed in STZ-induced diabetic RAGE KO mice. The results presented in this thesis strongly support a crucial role of RAGE in the onset and development of diabetic autonomic neuropathy. We anticipate that the diabetes induced sympathetic dysfunction would be milder or occur later in mice lacking RAGE.

Another future goal of this project is to propose RAGE as a potential therapeutic target for the treatment of DAN. There are currently only two main functionally relevant isoforms of RAGE: the full length RAGE in which signaling is achieved by the intracellular tail and the soluble isoform (known as sRAGE; Schmidt et al., 2000). The sRAGE isoform lacks the

transmembrane domain and can therefore act as a decoy receptor and block RAGE activation (Leclerc et al., 2009; Sims et al., 2010). sRAGE may act as a potential therapeutic agent against pathologies where there is excessive RAGE activation (Vincent et al., 2010). Interestingly, it has been found that plasma sRAGE is decreased in diabetic patients and it may contribute to diabetic complications by increasing the availability of RAGE ligands to interact with membrane-bound RAGE (Katakami et al., 2005). Thus, we are interested in testing the efficacy of sRAGE in treating the autonomic complications caused by diabetes. Preliminary steps have already been taken in order to achieve this goal in collaboration with Dr. Scott Napper by isolating and purifying sRAGE from mouse lung tissue using a previously published protocol (Englert et al., 2008). We anticipate that sRAGE will be helpful in preventing the deterioration of sympathetic function in STZ-induced diabetic mice. Previous studies have reported that sRAGE treatment was more efficient to ameliorate diabetic complications than the complete deletion of RAGE (Bierhaus et al., 2004). This is probably due to the fact that sRAGE prevents RAGE ligands from binding to RAGE and also to other binding sites capable of producing cellular dysfunction.

General Conclusions

The detrimental effects of RAGE activation and the subsequent downstream ROS production and accumulation have been shown in the context of diabetic sensory neuropathy. However, little is known about the involvement of RAGE in autonomic malfunction during hyperglycemia. Here we show in cultured sympathetic neurons that RAGE is expressed in control conditions but is up-regulated in response to high glucose. Further, we demonstrate that the high glucose induced ROS production and the following nAChR inactivation is prevented in sympathetic neurons where RAGE function is blocked. These results portray RAGE as a major

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source of ROS production in response to high glucose that eventually results in autonomic

neuron malfunction. We propose that the blockade of RAGE activation offers new therapeutic

approaches for treating DAN.

CHAPTER 5

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