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Molecular Integrators of Neuropathy and Pain in Diabetes Mellitus

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Abstract Glycation of nucleotides, proteins and phospholipids contributes to the development of late diabetic complications, including the most debilitating one——diabetic neuropathy. Reactive intermediates of AGE formation such as glyoxal, methylglyoxal (MG) and other dicarbonyls are detoxified by the glyoxalase-system. However little is known about the regulation and nature of the mechanisms underlying neuropathology. Therefore we decided to focus on the role of MG-glyoxalase 1 (GLO-1) system in modulation of painful diabetic neuropathy.

Key words diabetic neuropathy, pain, ion channels, methylglyoxal, glyoxalase system
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1 Diabetic neuropathy-significance and mechanisms

Diabetic neuropathy (DN) is a painful and debilitating disorder, arisen from diabetes mellitus associated complications in the autonomic and peripheral nervous systems. The incidence rate of DN is higher in type-1 diabetes with the symptoms often being more severe than those in type-2. Neuropathic foot ulcer in diabetic patients is the primal cause of hospitalisation and doubles the risk of amputation of the lower limb[1]. However the eventual deformation or injury of the neuropathic foot increases the risk of amputation twelve-fold. More than half of the non-traumatic amputations are a result of diabetic neuropathy[2].

Depending on the diagnostic criteria used, the class of disorders included in the DN diagnosis are essentially defined as subclinical-, diffuse-neuropathy and focal syndromes [3]. Reduced nerve conduction velocity and abnormal quantitative sensory perception for vibration, tactile and thermal thresholds are usually defined as subclinical neuropathy.

Diffuse neuropathies are characterized by impairment in the autonomic and peripheral nervous systems and lead to functional deficits in the cardiovascular, gastrointestinal, sweating and urinary systems. Diffuse neuropathies are also paralleled by impaired metabolism and often cause symptoms such as orthostatic hypertension, heart rate unresponsiveness to respiration and tachycardia during rest[4].

Distal symmetric polyneuropathy (DSPN) affects about 25% of all diabetic patients and is the most frequent peripheral neuropathic complication seen in the clinics [5]. In DSPN reduced nerve conduction of large fibers leads to sensory impairment of touch sensation and paresthesia. DSPN associated reduction in small nerve fiber conduction results in higher pain perception, sensation of burning, morbidity and a higher mortality rate [4]. In the somatic and autonomic parts of the peripheral nervous system, nerve fibers are damaged by the effects of hyperglycemia resulting from diabetes. The hyperglycemia induces inflammation, damages metabolic function, increases

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apoptosis and impairs the microvasculature of the tissue[3]. In the later phases of diabetes mellitus damage of nerve fibers and neurons causes complete loss of pain perception.

Primal focal neuropathy occurs as vascular obstruction or mononeuritis, which is then naturally resolved. Repeated entrapment episodes are proceeding with progressive minor injuries [6]. Vulnerability is often presented in the wrist, elbow, knee, and shoulder involving median, ulnar, peroneal and plantar nerves. Carpal tunnel syndrome complication is three times more frequent in diabetes[7].

At present there is no effective treatment for DN except the glycaemia control and dedicated foot care. Although intensive therapy can delay or partly prevent the development of polyneuropathy and autonomic diabetic neuropathy, it cannot completely stop it [8]. Growing evidence suggests that a number of metabolic and biochemical pathways are involved in the multifactorial pathogenesis of DN. Targeting metabolic and biochemical mechanisms behind the pathogenesis of DN could provide clinicians with new and more effective treatments for DN.

2 Cellular pathways involved in diabetic neuropathy

An important metabolic enzyme affected by hyperglycaemia is aldose reductase (AR). AR serves many important functions such as the reduction of glucose to sorbitol in the polyol pathway. Hyperglycaemia activates AR and causes an accumulation of intracellular sorbitol which is known to result in cellular damage and lead to diabetic complications [9]. AR uses NADPH as a cofactor for reducing glucose to sorbitol. NADPH is a known redox co-factor that plays an important role in alleviation of oxidative stress. At physiological glucose levels of ~5.5 mmol/L, AR reduces less than 3% of the glucose being utilized in the cell [10]. But under hyperglycemic conditions (glucose 20 mmol/L) more than 30% of the glucose is reduced by AR[11] and thus generates intracellular NADPH depletion. Another putative mechanism for development of complications is the AR activity dependent glutathione (GSH) depletion in neuronal tissue [12]. Inhibition of AR activity recovers decreased conduction velocity, restores GSH levels and block lipid peroxidation[9, 13–14]. Nevertheless mouse models with low or complete lack of polyol pathway activity are still not protected from functional and structural DN[15].

Activation of hexosamine pathway is also implicated in development of insulin resistance[16]. The up regulation of glutamine-fructose-6-phosphate-amido-transferase towards energy storage leads to hyperlipidemia and obesity. On other hand O-linked N-acetylglucosamine (O-GlcNAc) transferase highly expressed in β-cells is catalysing O-linked glycosylation of proteins [17], and thus implicates the potential involvement of the hexosamine pathway in β-cell function. O-glycosylation of SP1 transcription factor is hampering the transport of thiamine and thus deactivates transketolase, an enzyme with a pivotal role in the detoxification process through the pentose phosphate pathway[18]. O-GlcNAc levels are increased by streptozotocin in islets, demonstrating further potential damage of β-cells[19].

Hyperglycaemia also mediates diabetic complication by activating protein kinase C (PKC). As a result of the increased PKC activation vascular permeability, neovascularisation and blood flow is reduced [20]. Ohshiro *et al*. (2006) demonstrated the critical role of the PKC-β isoform activation for diabetes-induced tubular hypertrophy and glomerular enlargement [21]. In agreement with the findings by Ohshiro *et al*. it has been shown that inhibition of PKC- β with ruboxistaurin successfully corrects nerve conduction velocity and neuronal blood flow in diabetic rats[22].

A high production of intracellular superoxide ($O_2^{\cdot-}$) is seen with the DN pathology. The high superoxide levels increase the amount of free radicals that oxidizes the membrane lipids and lead to increased oxidative stress in the cells. Production of reactive oxygen species (ROS) and auto-oxidation under hyperglycaemic conditions leads to depletion of antioxidant defence mechanisms [23–24]. High levels of superoxide and elevated uptake of NAD^+ due to NADH inhibits the activity of GAPDH, which results in accumulation of reactive metabolites and thereby initiating the formation of advanced glycation end products (AGEs) [25–26]. A number of scavenger molecules such as butylated hydroxytoluene, producol, taurine and α-lipoic acid have been shown to prevent the loss of motor- and sensory nerve conduction velocity. The scavenger molecules also reduce the latency of pain perception in diabetic animal models[22].

Although the optimal therapeutic dosage of antioxidants is not known yet, α-lipoic acid has been licensed and is being used for treatment of DSPN in clinical studies[8].

High glucose levels accelerate non-enzymatic glycation and glycooxidation processes. The formation of AGEs alters the assembly of the cytoskeleton, induces protein aggregation and modifies nuclear proteins and nucleic acids. The concentration of AGEs is directly correlative with loss of myelinated fibres and elevated demyelinization. Binding of AGEs to their cognate receptor RAGE initiates pro-inflammatory signalling through activation of the JNK/SAPK and p38MAPK pathways. This activation leads to increased NF-κB signalling and thus AGEs serve an important role in regulating the immune responses[25, 27]. It is clear that perpetual inflammation initiate cellular dysfunction and vascular endothelium damage [26, 28], while the accumulation of AGEs is related with the extent of diabetic complications[29–31]. The AGE formation inhibitor aminoguanidine has been shown to reduce the AGE aggregation but did not restore nerve conduction velocity in animal models. However studies targeting the AGE/RAGE/NF-κB-axis in genetically modified animals may provide the basis for new therapeutic options[27, 32].

In the future, novel therapeutic targets at the level of pain receptors, and voltage-gated sodium channels are likely to reduce significantly the incidence of DN.

Despite a large number of mechanisms contributing to loss of nerve conduction in DN have already been investigated, very little is still known about the molecular pathways underlying neurodegeneration.

3 Advanced glycation end products-damage and clearance

Glycation of proteins, nucleic acids and lipids by glucose and/or other non-reducing sugars is a non-enzymatic reaction yielding a heterogeneous class of compounds collectively termed advanced glycation end products (AGEs). The class of AGEs consist of monolysyl adducts, hydroimidazolones, bis (lysyl) imidazolium cross-links and 3-deoxyglucosone derivatives. In parallel to monosaccharides, markedly contributing to formation of AGEs are α-oxo-carbonylic compounds as even more reactive as sugars[33]. One of the several mechanisms underlying carbonyl stress is glycolysis in which intermediates such as triose phosphates could be non-enzymatically degraded to form oxoaldehydes. Alfa-oxoaldehydes cause damage to the cells and tissue and is known to be mutagenic. The detrimental effects of α-oxoaldehydes have been shown to be particularly pronounced in diabetes and uraemia[34–35]. The strongly cytotoxic metabolite MG is produced primarily as a by-product of glycolysis through non-enzymatic phosphate elimination from the glycolytic pathway. The intermediary molecules of glycolysis dihydroxyacetone phosphate and glyceraldehyde 3-phosphate together with MG are the main source of intracellular and plasma AGEs[21].

Phagocytosis of myelin contributes to segmental demyelination of peripheral nerves due to aggregation of AGEs in the cell. AGEs mediated modification of essential axonal cytoskeletal proteins such as actin, tubulin and neurofilaments, result in axonal atrophy/degeneration and impaired axonal transport[36]. Moreover glycation of the extracellular matrix protein laminin impairs regenerative activity [37–38] while excessive intra- and extracellular accumulation of AGEs has been detected in perineural basal laminae, axons, Schwann cells and endoneural and epineural microvessels of diabetic subjects[31]. Extensive research body suggests activation of key cell signalling pathways with subsequent modulation of gene expression, and intracellular AGE formation, leading to quenching of nitric oxide and impaired function of growth factors. Activation of AGE-RAGE axis in vascular and neuronal tissue is supposed to promote late diabetic complications[25, 39-40].

The glyoxalase system (Figure 1) is the predominant cellular defence mechanism against MG preventing the formation of AGEs. The system is localized in the cytosol of the cell and consists of glyoxalase-1 and glyoxalase-2, together with the cofactor glutathione (GSH). It is the major detoxifying cellular mechanism, catalysing the conversion of reactive carbonyl and α-oxoaldehyde glycating agents into the non-toxic α-hydroxyacids. The most important reaction catalysed by glyoxalase-1 is the conversion of the precursor of a number of AGEs- MG to S-D-lactoylglutathione.

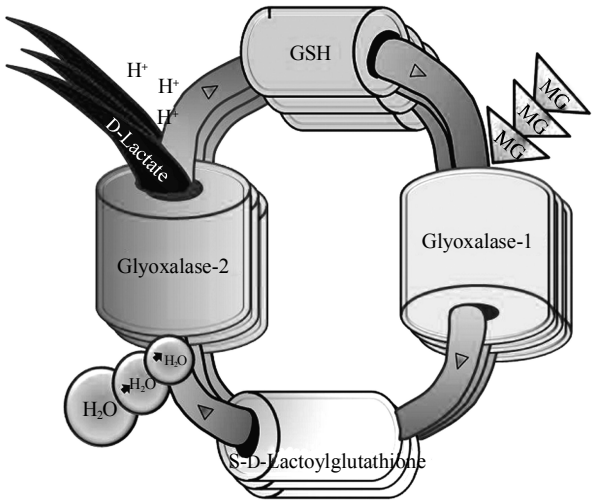

Fig. 1 The glyoxalase system

Glyoxalase-1 together with the co-factor glutathione (GSH) detoxifies methylglyoxal to S-D-Lactoylglutathione, which needs one water molecule (H_2O) for further metabolization to D-Lactate and H^+ by the glyoxalase-2.

Mouse studies show organ and tissue dependency of GLO-1 activation. According to Bierhaus *et al*. (2012) the highest activation of GLO-1 is occuring in the liver although it could be also detected in kidneys, spleen, lung and brain. The lowest GLO-1 activity was found in sciatic nerves, suggesting that they are particularly vulnerable to the toxicity of reactive α-oxoaldehydes metabolized by GLO-1. Alfa-oxoaldehydes levels and in particular MG increases in the state of hyperglycaemia and thus predisposes patients to even higher neuronal damage[34].

The degree of diabetic polyneuropathy correlates instantly with the concentration of MG derived AGEs [31], also recorded by measurement of GLO-1 down regulation in sciatic nerves of diabetic WT-mice [40]. The decrease of the GLO-1 is probably due to activation of the polyol pathway and formation of sorbitol which elevates the levels of ROS by reduction of glucose with NAPDH [41]. This on other hand influences the amounts of reduced glutathione and thus affects the production of s-D-lactoylglutathione from the glyoxalase system [42]. The polyol pathway could be an additional contributor to the down regulation of GLO-1 in diabetes through the receptor for AGEs-RAGE shown to play a considerable role in pathogenesis of diabetic complications.

The onset of diabetic neuropathy is mainly characterized by neuropathic pain, which subsequently develops into severe neurological deficits. A number of animal models have been established to study DN including spontaneous, induced or genetically based type-1 diabetes. Diabetes mellitus results in significantly elevated hyperalgesiac levels in mice and rats in Hot plate, Hargreaves and Tail flick assays [40, 43-44]. The correlation between development of hyperalgesia and the elevation of MG levels after administration of GLO-1 inhibition in WT mice provides a strong basis for argumentation about engagement of MG in the mechanism of induced thermal stimuli response and nociception in diabetes[40].

Furthermore overexpression of GLO-1 under hyperglycaemic conditions decreased nociception and attenuated levels of MG, implicating the significance of this defence mechanism for the cellular survival, viability and hindering the development of late diabetic complications[40].

4 Ion channels involved in diabetic neuropathy

The underlying mechanisms of persistent pain in DN remain elusive. The onset of pain begins in the early and middle phases of diabetes also termed: sensory peripheral neuropathy. In the periphery; sensation to afferent neurons is conducted by myelinated and unmyelinated nerve fibers. The missing blood nerve barrier in primary afferent neurons makes them uniquely sensitive to their environment and thus a very interesting subject for investigating the hyperglycaemic damage [45]. Primary sensory dorsal root ganglion (DRG) neurons with a diameter less than 25 μm are typically associated with unmyelinated C-fibers or thinly myelinated A-δ fibers responsible for conducting nociceptive stimuli. Neurons with large cell bodies having a diameter above 35 μm are typically associated with myelinated A-β fibers and transmit proprioception and vibrational sensation[46].

4.1 Transient receptor potential vanilloid receptor 1 (TRPV1)-structure and function

One of the most targeted mediators of pain under hyperglycaemic conditions is the noxious heat gated transient receptor potential vanilloid receptor 1 (TRPV1)[47]. TRPV1 is a Ca^{2+}-permeable, non-selective cation channel, encoded by 838 amino acids. The main gating stimuli of TRPV1 comprises: heat, low pH, capsaicin and endogenous ligands [47]. TRPV1 is

specifically located and expressed in small sized unmyelinated C-fiber DRGs responsible for nociception. Its major physiological role is general mediation of thermal nociception induced by tissue damage or inflammation. Diabetes increased capsaicin- and proton activated inward currents in rat DRGs also paralleled by up regulation of TRPV1 in the plasma membrane [48]. Hypoalgesic mice have shown down regulated TRPV1 expression implying further its functional significance for diabetes induced thermal nociception[49]. Blocking of TRPV1 activation is an intriguing issue in improvement of sensory perception in subjects with DN. Local capsaicin manifestation for 8 weeks showed significant recovery of total symptoms score in patients with DN[50].

4.2 Voltage gated sodium channels-structure and function

Recently, studies have shown that altered expression and function of voltage-gated sodium channels is involved in the development of neuropathic pain in animal models[43, 51–52]. The voltage-gated sodium channels consists of an approximate 260 ku α-subunit and a smaller auxiliary β-subunit[53]. The α-subunit is able to form both the trans-membrane channel and a functional voltage gate independently of the β-subunits. The β-subunits regulates the voltage kinetics, cellular localization and stability of the α-subunit.

The α-subunit consists of four domains (Ⅰ～Ⅳ), each containing six trans-membrane segments(S1～S6) and an additional pore loop between the S5 and S6 helices. The α-subunit has five distinct toxin binding sites, that form respectively site 1 for tetrodotoxin (TTX), saxitoxin (STX) and μ-conotoxin, site 2 for veratridine and site 3 for α-scorpion toxin [53–54]. To date, there are nine known isoforms of the α-subunit named Nav1.1 to Nav1.9. The isoforms are encoded by nine different genes (SCN1A- 5A and SCN8A- 11A).

When present, the β_1 or β_3 -subunit are found covalently attached to the C-terminal extracellular loop of the α-subunit near the α-scorpion toxin (α-ScTx) binding site as shown in Figure 2. The β_2- or β_4-subunits are found linked to the N-terminus of the α-subunit through a disulphide bond with their presence strongly dependent on species and tissue type [53]. All β-subunits are type 1 trans-membrane proteins harbouring a single membrane-spanning region, with the extracellular domain of the β-subunit containing a single immunoglobulin V-set fold structure, that enables it to interact with cell adhesion molecules, extracellular matrix proteins and intracellular scaffold proteins [55]. Proteomic studies have consistently implied a regulatory role of the β-subunits in the kinetics and cell surface density of sodium channels[52, 56].

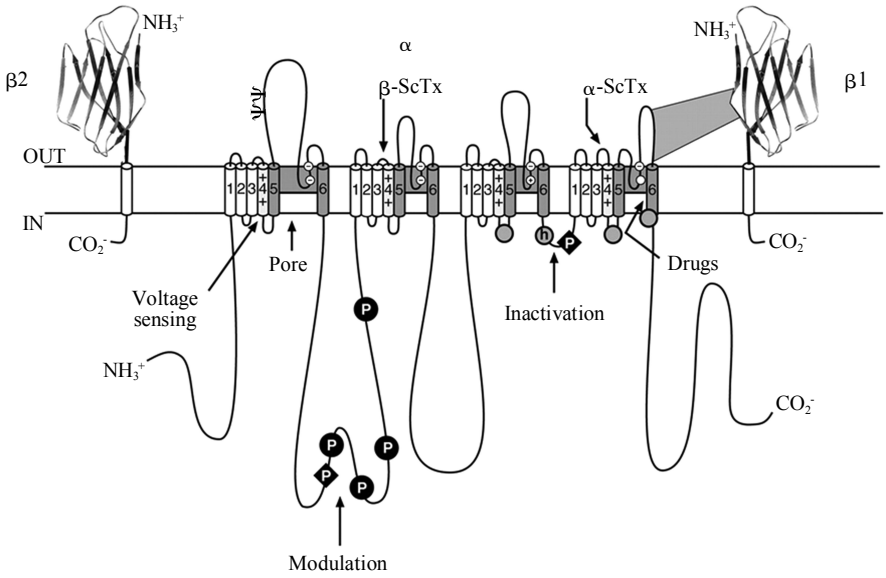

Fig. 2 Structural outline of voltage-gated sodium channels

The four repeat domains of the α-subunit is shown with trans-membrane spanning α-helices as cylinders, ψ indicates probable N-linked glycosylation sites. The α-subunits are shown as immunoglobulin-like folds. Adapted from [52].

Sodium currents are classified as either TTX-sensitive (Nav1.1- Nav1.7) or TTX-resistant (Nav1.8 and Nav1.9). Aberrations in the expression and function of these channels are thought to play a pivotal role in the states of inflammatory pain and painful DN. Waxman, *et al*. [51] (1999) showed that dysregulation of sodium channel gene transcription is contributing to hyperexcitability of dorsal root ganglion neurons, and thus inducing neuropathic pain of post axonal transaction. Furthermore inflammatory mediators are related to elevated voltage-gated sodium currents in sensory neurons, responsible for generation and propagation of action potentials [57–58]. Modulation of those currents could affect neuronal excitability. Increase in the expression of Nav1.3 and Nav1.7 and decrease in Nav1.6 and Nav1.8 is observed in diabetic rats. The level of expression of sodium channels Nav1.8 and Nav1.9 in small DRG neurons has been implicated to mediate the extent of painful diabetic neuropathy[43]. At the site of peripheral nerve damage (Figure 3), reactive carbonyls modify the sodium channels along the axons, and thus activate neural discharge. Dorsal-root ganglion projections from nociceptive neurons to spinal cord interneurons initiate excitation by release of glutamate, substance P and calcitonin gene-related protein (CGRP). Large diameter Aβ-fibers carry allodynia projections, while hyperalgesia is mediated particularly by C-fibers [59]. Central sensitization is fired by spontaneous activation of N-methyl-D-aspartate (NMDA) receptor in second-order neurons in the spinal cord, and subsequent increase in intracellular calcium and up regulated phosphorylation of intracellular proteins such as the NMDA receptors by protein kinases (PK). Chronic pain syndromes augment the opioid neuropeptide dynorphin, and thus also contribute to ectopic excitation in the second- order neurons *via* the NMDA receptors. Low input and down-regulation of γ-aminobutyric acid ($GABA_A$) receptors in that case evokes loss of the inhibitory mechanisms. Neuronal terminals from the dorsal-root ganglia -Aβ neurons express nociceptive transmitters in the dorsal horn, and thus trigger hyperalgesia and tactile allodynia [60]. Blocking of NMDA receptors by neramexane and memantine is putative treatment against mechanical hyperalgesia and allodynia in diabetic neuropathy[61].

It is well known that voltage gated sodium channel Nav1.8 is the major contributor of sodium currents underlying the depolarization phase of the action potential in cells in which it is located [62]. Increase in TTX-resistant sodium currents was paralleled by negative shifting of voltage-gated activation and steady-state inactivation [43] in DRGs from diabetic rats implicating functional alteration in Nav1.8 in the state of hyperglycaemia. Modification of arginine and lysine residues from the extracellular and intracellular domains of Nav1.8 by MG lead to increased TTX-resistant sodium currents in the state of diabetes observed by Hong *et al*. (2004) and Bierhaus *et al*. (2012), providing clear evidence for the involvement of the channel in the mechanism of diabetic neuropathy.

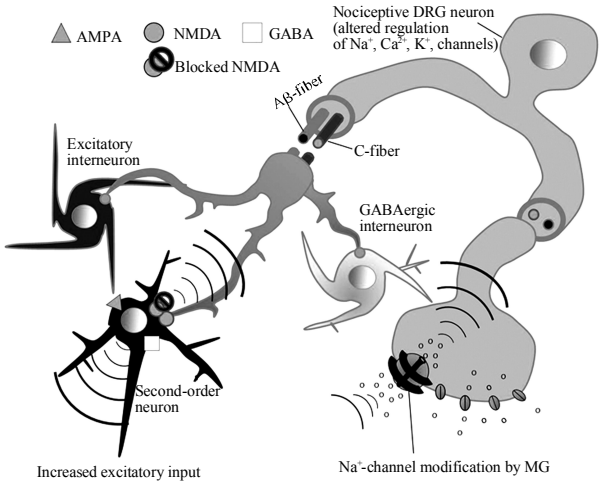

Fig. 3 Induction of pain in peripheral neuropathy

Sodium channels in the periphery nervous system (lower limb) are fired upon modification of methiylglyoxal (MG). Nociceptive neurons from dorsal-root ganglion project Aβ-fiber afferents to spinal cord interneurons *via* release of substance P, calcitonin gene-related, protein (CGRP), and glutamate. Second-order neurons are normally operating with glutamate and AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazole prioponic acid). However spontaneous sensation is induced through firing of the N-methyl-D-aspartate (NMDA) receptor. Therapeutic modification of NMDA receptors could prevent pain. Inhibition is mediated by γ-aminobutyric acid (GABA) receptors.

4.3 Transient receptor potential cation channel, member A1 (TRPA1) structure and function

The transient receptor potential ankyrin 1 channel (TRPA1) is highly conserved member of the large TRP family of ion channels. The TRPA1 function as a non-selective Ca^{2+} permeable cation channel with a large array of identified agonists [63–64]. Evidence that TRPA1 could be implicated in the pathogenesis of DN has already been provided as Wei *et al*. [65] (2009) showed that endogenously produced compounds from

the oxidative stress resulting from diabetes mellitus causes prolonged activation of the TRPA1 receptor. Additionally the authors showed that 10 mg/kg acute administration of the TRPA1 antagonist Chembridge-5861528 reduced pain-related behavior in STZ rats.

The only structural model of TRPA1 currently available is a 16Å electron microscopy resolution structure of amphipol-stabilized TRPA1 (EMD accession number EMD-5334). Using common bioinformatical techniques of the general topology of TRPA1 and its sequence, it has been shown that the TRPA1 covers approximately 55.7 kb on the human chromosome 8q13[66–67] with the encoded protein being close to 1100 amino acids long. The TRPA1 gene contains 27 exons and the expressed protein weighs 120～130 ku and is believed to contain six putative trans-membrane segments named S1～S6. The ion pore and selectivity filter is located between S5 and S6. The TRPA1 protein contains 18 ankyrin repeat domains of 33 amino acids in length, with an important EF motive responsible for intracellular calcium activation $[Ca^{2+}]_i$ located between ankyrin repeat domain 11 and 12[66, 68]. On the first extracellular loop between S1 and S2 two putative Asn-glycoslation sites are found, and on Asn855 is another important Asparagine associated with familial episodic pain syndrome[69].

TRPA1 is strongly expressed in the hippocampus and its expression has been linked with the activation of the cannabinoid receptor B1 during hippocampal formation[70]. The gene is also expressed in the nucleus supraopticus together with TRPV1 where they exists at presynaptic terminals and enhances glutamate release [71]. Evidence suggests that the regulation of TRPA1 expression is strongly controlled by the human tumor suppressor gene CYLD which is a ubiquitin hydrolase that binds to TRPA1 and de-ubiquinates the channels[72]. Due to the downstream pro-inflammatory response to TRPA1 activation, the gene is considered a promising target for developing new analgesics in treatment of DN, but the differences between human and rodent TRPA1 causing often unpredictable, and sometimes opposite, responses to pharmacological ligands has hindered development of new therapeutics[73–74].

TRPA1 has a remakable promiscuity in agonists that can activate it with activation by electrophilic compounds resulting in large inward Ca^{2+} currents. In contrast, the constitutive open TRPA1 channel shows outwardly rectifying currents, just as observed with activation by non-electrophilic compounds[74].

It has also been recently shown that MG can act as an agonist of the transient receptor potential channel A1 (TRPA1)[75]. The authors found that MG activated TRPA1 receptor channels in DRG neurons by modification of intracellular N-terminal lysine and cysteine residues. The MG-stimulated release of the pro-inflammatory calcitonin gene-related peptide CGRP was dependent on expression of TRPA1. As intradermal injection of MG in human skin is strongly noxious, causing irritation, burning pain and general nociceptor activation, and it has been shown that MG activates the TRPA1 channel, evidence for the role of MG and TRPA1 as important targets for treating DN pain is increasing.

5 Conclusion

Major advances in our understanding to approach the mechanisms of diabetic neuropathy have been made. It is now widely recognized that researchers and physicians must aim to counteract multiple pathophysiological mechanisms in order to improve treatment of DN. Particular attention to detoxification of reactive dicarbonyl species and their modification of proteins may be the key to address neurodegeneration and pain.

In the future, novel therapeutic targeting at the level of pain receptors, and voltage-gated sodium channels are likely to reduce significantly the incidence of DN.

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