Methylglyoxal activates the human transient receptor potential ankyrin 1 channel

Susumu Ohkawara¹, Toshiko Tanaka-Kagawa², Yoko Furukawa² and Hideto Jinno²

¹Department of Pharmaceutical Science, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan
²Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagayaku, Tokyo 158-8501, Japan

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ABSTRACT — Methylglyoxal (MG) is an endogenous carbonyl compound that is produced in large quantity under hyperglycemic conditions, which are believed to contribute to the development of diabetic neuropathy. However, the mechanism by which this occurs and the molecular targets of MG are unclear. In the present study, we investigated the effect of MG on transient receptor potential ankyrin 1 (TRPA1) activation in human TRPA1-expressing HEK293 cells. MG activated TRPA1-expressing HEK293 cells, but failed to activate human capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1)-expressing HEK293 cells or mock-transfected HEK293 cells. MG also induced calcium (Ca²⁺) influx in a concentration-dependent manner, and the concentration-response curve indicates that the effect of MG has an EC₅₀ of 343.1 ± 17.3 μM. Interestingly, the time course in the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in human TRPA1-expressing HEK293 showed considerable differences in response to MG and cinnamaldehyde. Furthermore, we examined four endogenous carbonyl compounds, including MG, glyceraldehyde, glycolaldehyde, and glyoxal; only MG notably activated TRPA1-expressing HEK293 cells. These results may provide insight into the TRPA1-mediated effects of MG on diabetic neuropathy.

Key words: Methylglyoxal, TRPA1, Diabetic neuropathy

INTRODUCTION

Neuropathic pain is one of the most common complications of diabetes mellitus (Vinik et al., 2000). This pain is characterized by hyperesthesia, dysesthesia, hyperalgesia, paresthesia, and allodynia (Brown and Asbury, 1984), and it is difficult to treat with currently available therapeutic strategies (Jensen et al., 2006). Although diabetic neuropathic pain has often been reported to be associated with abnormal Ca²⁺ homeostasis (Fernyhough and Calcott, 2010), its underlying mechanisms have not been completely elucidated.

Transient receptor potential ankyrin 1 (TRPA1) is a Ca²⁺-permeable, nonselective cation channel in a subset of polymodal nociceptive neurons. It is activated by noxious cooling (< 17°C) (Story et al., 2003) and multiple pungent compounds including allyl isothiocyanate (Jordi et al., 2004; Bautista et al., 2005; Macpherson et al., 2005, 2007) and unsaturated aldehydes such as acrolein that are contained in cigarette smoke (Andrè et al., 2008, 2009; Simon and Liedtke, 2008). Functional studies, channel localization, and analysis of TRPA1-deficient mice indicate that the channel is the primary molecular site through which they activate the pain pathway (Kwan et al., 2006; Levine and Alessandri-Haber, 2007). In addition, recent studies demonstrated that TRPA1 contributes to the maintenance and development of diabetic hypersensitivity (Wei et al., 2009). Methylglyoxal (MG) is an endogenous carbonyl compound physiologically generated as an intermediate of glycolysis. MG accumulates during hyperglycemia (Beisswenger et al., 2001; Lapolla et al., 2003), and it has been suggested that this accumulation may contribute to the development of diabetic neuropathy. Indeed, several investigations have demonstrated that MG induces apoptosis in rat Schwann cells (Fukunaga et al., 2004), adrenaline secretion in the peripheral nervous systems (Davies et al., 1986), and an increase intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in the human neuroblastoma cells (Kuhla et al., 2006). Therefore, we hypothesized that TRPA1-chan-
nel activation by MG could be a cause of diabetic neuropathic pain.

The purpose of the present study was to examine whether TRPA1 acts as a molecular target for MG. To prove this, we examined the ability of MG to activate human TRPA1 by measuring [Ca^{2+}]_i in human TRPA1-expressing HEK293 cells.

**MATERIAL AND METHODS**

**Chemicals**

Cinnamaldehyde, MG, glyceraldehyde, glycolaldehyde, glyoxal, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

**Cloning of human TRPA1**

Oligo(dT)-primed cDNA was synthesized from 1 μg of the total RNA isolated from human dorsal root ganglion (Clontech, Mountain View, CA, USA) using the SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. An aliquot of the cDNA (2 μl) was then subjected to PCR amplification using Pfx DNA polymerase (Invitrogen) using the following primers, 5’-CACCATGAAGTGCAGCCTGAGGAAGA-3’ (N-terminal forward primer with CACC sequence) and 5’-CTAAGGCTCAAGATGGTGTGTTTTT-3’ (C-terminal reverse primer without a stop codon). The PCR products were then separated on a 0.8% agarose gel and the bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR products were subcloned into the pENTR™/d-TOPO vector (Invitrogen) and named hTRPA1-pENTR/d-TOPO. Six mutations identified by sequencing were corrected using the Quik-Change Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Next, hTRPA1-pENTR/d-TOPO was recombined with the pcDNA5/FRT mammalian expression vector (Invitrogen) using attL and attR reactions with Gateway™ LR Clonase™ enzyme mix (Invitrogen) and named hTRPA1-pcDNA5/FRT.

**Development of human TRPA1-HEK293 stable cell line**

HEK293 cells containing the FLP recombination site (Invitrogen) were cotransfected with hTRPA1-pcDNA5/FRT and pOG44 vectors (Invitrogen) using lipofectamine LTX (Invitrogen). Stable clones expressing TRPA1 were then selected using hygromycin B antibiotic selection and colonies were cultured to produce a large stock of TRPA1-expressing cells. TRPA1-protein expression was confirmed following previously published protocols (Ohkawara et al., 2010).

**Intracellular Ca^{2+} measurement using FlexStation**

Cells were plated at 80-90% confluence on a 96-well plate, poly-D-lysine black-walled, clear-bottomed plates (Griner bio-one, Frickenhausen, Germany) 24 hr before initiating the experiments. The cells were incubated for 1 hr at 37°C in Hank’s balanced salt solution (HBSS) buffer (HBSS with 20 mM HEPES buffer, pH 7.4) containing FLIPR® calcium 4 assay reagent (Molecular Devices, Sunnyvale, CA, USA) followed immediately by fluorescence measurement. Fluorescence was measured using FlexStation (excitation at 485 nm and emission at 525 nm, using a 515 nm cutoff) and SoftMax Pro 4.7.1 software (Molecular Devices). The test compounds were prepared in DMSO and added to the HBSS buffer (final DMSO concentration, 0.2%). EC_{50} values were determined using Prism 4 software (GraphPad Software, La Jolla, CA, USA).

**RESULTS AND DISCUSSION**

We first examined the ability of MG to activate TRPA1 using FlexStation-based calcium flux assay. MG caused an increase in [Ca^{2+}]_i, in human TRPA1-expressing HEK293 cells (Fig. 1B), but not in human TRPV1-expressing cells (Fig. 1C) or mock-transfected HEK293 cells (Fig. 1D). These results indicate that an increase in [Ca^{2+}]_i by MG was mediated by TRPA1. The TRPA1 agonist cinnamaldehyde also increased [Ca^{2+}]_i, to a similar extent (Fig. 1A). Interestingly, the time course in [Ca^{2+}]_i in human TRPA1-expressing HEK293 cells shows marked differences in response to MG and cinnamaldehyde. The initial slope of the fluorescence change for MG is less steep than that for cinnamaldehyde. Furthermore, the maximal intensities induced by MG were approximately 20% higher than those induced by cinnamaldehyde (Figs. 1A and B). These kinetics are consistent with those previously reported for heterologously expressed TRPV1 channels (Tóth et al., 2005). For example, the response to capsaicin is rapid and diminishes after reaching an initial maximum. In contrast, the response to resiniferatoxin is gradual. This response pattern is consistent with the sustained channel opening regulated by resiniferatoxin (Winter et al., 1990; Liu and Simon, 1996), which contributes to channel desensitization and/or potent irritation and cytotoxicity. Although correlations between Ca^{2+} response pattern-mediated TRPA1 and physiological functions have not been reported, TRPA1 activation by MG may be an important mechanism in painful diabetic neuropathy.
Fig. 1. Effect of MG on $[\text{Ca}^{2+}]_i$ in human TRPA1-expressing HEK293 cells. Time course of $[\text{Ca}^{2+}]_i$ in human TRPA1- (A,B), human TRPV1-(C) expressing HEK 293 cells, or in mock-transfected(D) HEK293 cells stimulated by 1 mM cinnamaldehyde (A) and 1 mM MG (A-C). Test compounds were added at 30-s time points, and the fluorescence was monitored using a FlexStation. RFU represents the relative fluorescence units of the calcium 4 assay reagent. The fluorescent traces are shown in the averages of three wells.

Fig. 2. Concentration-dependence of MG to elevate $[\text{Ca}^{2+}]_i$ in human TRPA1-expressing HEK293 cells. (A) Comparison of time courses of fluorescence changes induced by different concentrations of MG. (B) Concentration-response relationships for MG-induced $[\text{Ca}^{2+}]_i$ changes in human TRPA1-expressing HEK293 cells. RFU represents the relative fluorescence units of the calcium 4 assay reagent. Data are the mean ± S.E. of at least three separate experiments. Solid lines were fitted to the Hill equation.
Next, we investigated the concentration-dependence of MG on human TRPA1-expressing HEK293 cells. As shown in Fig. 2A, MG induced a concentration-dependent increase in [Ca$^{2+}$], in human TRPA1-expressing HEK293 cells. The concentration-response curve indicates that the effect of MG has an EC$_{50}$ value of 343.1 ± 17.3 μM (mean ± S.E. for three experiments) (Fig. 2B). It should be mentioned that the concentration of MG in diabetic patients is below the EC$_{50}$ value for MG to activate TRPA1 (Beisswenger et al., 2001). However, the intracellular concentration of MG varies widely and is generally higher than that in the plasma. For example, 300 μM of MG was found in Chinese hamster ovary cells (Chaplen et al., 1998). Thus, our results may be physiologically relevant.

In addition to MG, the hyperglycemic conditions may lead to the production of other endogenous carbonyl compounds. To ascertain whether the activation of TRPA1 is confined to MG or widespread, we examined four endogenous carbonyl compounds, including MG, glyceraldehyde, glycolaldehyde, and glyoxal. Of the four endogenous carbonyl compounds, only MG caused notable [Ca$^{2+}$] increases in human TRPA1-expressing HEK293 cells (Fig. 3A). Glyceraldehyde appeared to be a very weak activator (EC$_{50}$ > 1 mM) (Fig. 3B), whereas glycolaldehyde and glyoxal did not have an effect on human TRPA1-expressing HEK293 cells (Figs. 3C, D). This result indicates that only certain endogenous carbonyl compounds have a stimulatory effect on TRPA1.

Several TRPA1 ligands have been shown to activate TRPA1 through the covalent modification of intracellular cysteine and lysine residues in the N-terminal region of the ion channel (Hinman et al., 2006; Macpherson et al., 2007). MG also modifies arginine,
lysin, and cysteine residues in proteins (Lo et al., 1994). Therefore, the TRPA1 activation by MG may be caused by the modification of lysine and/or cysteine residues.

In summary, we identified TRPA1 as a novel molecular target of MG; however, its precise mechanism is not yet understood. Further detailed studies regarding the activation of TRPA1 and its molecular mechanism may lead to the development of new therapeutic strategies for painful diabetic neuropathy.

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REFERENCES


