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Research Report

Hyperstimulation of macropinocytosis leads to lysosomal dysfunction during exposure to methamphetamine in SH-SY5Y cells

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ABSTRACT

Although various cytotoxic effects on neuronal cells caused by methamphetamine (METH) have been investigated, the cellular and molecular mechanisms of METH-induced neurotoxicity remain to be elucidated. We previously reported that METH-induced cytomorphological effects on retinoic acid (RA)-differentiated SH-SY5Y human neuroblastoma cells involved macropinocytosis, which is an actin-dependent endocytic pathway. We also noted that hyperstimulation of this process might play an important role in the cytotoxicity of METH in neuronal cells. In this study, we investigated the molecular mechanisms as well as subsequent outcomes of macropinocytosis during METH treatment. It was found that macropinosomes formed upon exposure to METH were colocalized with constitutively active GFP-Ras (G12V) and GFP-Rac1 (Q61L). Furthermore, both Ras inhibitor, farnesylthiosalicylic acid (FTS), and Rac1 inhibitor, EHT1864, significantly inhibited the formation of macropinosomes, suggesting the involvement of these molecules. The expressions of lysosome-associated membrane proteins (lamps) gradually increased by METH in a time-dependent manner. In contrast, the proteolytic activation of cathepsin L, a marker of lysosomal function, was markedly suppressed by METH. METH-induced dysfunction of lysosomal enzyme as well as cell death was significantly attenuated by nocodazole, a microtube interfering reagent that inhibits the transport of vesicles, including macropinosome, to lysosomes. Our results indicate that METH has cytotoxic effects, at least in part, by inhibiting normal lysosomal function through Ras- and Rac1-mediated macropinocytosis in RA-differentiated SH-SY5Y cells.

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1. Introduction

Macropinocytosis is a clathrin-independent endocytic pathway (Doherty and McMahon, 2009; Lim and Gleeson, 2011; Sandvig et al., 2011). Unlike other endocytic pathways, macropinocytosis occurs through the increase of actin polymerization at the cell surface (Kerr and Teasdale, 2009). The signal cascade of macropinocytosis involves Rho-family guanosine triphosphatases (GTPases) that trigger actin polymerization near the plasma membrane, leading to the formation of membrane ruffling

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Abbreviations: FITC, fluorescein isothiocyanate; FTS, farnesyl thiosalicylic acid; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain 3; METH, methamphetamine; MAPK, mitogen-activated protein kinase; PI, propidium iodide; PI3K, phosphoinositide-3 kinase; RA, retinoic acid; RFP, red fluorescent protein

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(Mercer and Helenius, 2009; Overmeyer and Maltese, 2011; Overmeyer et al., 2011; Sun et al., 2003; West et al., 2000). Among several GTPases, the activation of Ras and Rac1 is especially correlated with the induction of macropinocytosis (Bhanot et al., 2010; Mercer and Helenius, 2010; Overmeyer et al., 2011; Porat-Shliom et al., 2008). Research has indicated that activation of the phosphoinositide-3 kinase (PI3K) signaling pathway and the inactivation of ADP-ribosylation factor 6 (Arf6), one of the small GTPases, are also involved in macropinocytosis (Bhanot et al., 2010; Lindmo and Stenmark, 2006; McKay et al., 2011). Overmeyer et al. (2008) suggested that macropinosomes, which are formed at the plasma membrane upon overexpression of the Ras active form in glioblastoma cells, move further into the cytoplasm and these vacuoles are delivered to late endosomes. Vacuoles accumulated within the cytosol finally disrupt cell membrane integrity, resulting in a specified form of cell death called "methuosis" (Bhanot et al., 2010; Overmeyer et al., 2011); however, it remains unclear how hyperstimulation of macropinocytosis causes cytotoxicity during METH exposure in neuronal cells.

Lysosomes, which are acidic organelles, play essential roles in the degradation of intracellular proteins as well as foreign substrates that are incorporated into lysosomes through autophagy or endocytosis including phagocytosis (Ebrahim and Thilo, 2011). Lysosome-associated membrane proteins (lamps) are glycoproteins and components of the lysosomal membrane required for the maturation of autophagosomes or endosomes into lysosomes (Ebrahim and Thilo, 2011; Eskelinen, 2006; Huynh et al., 2007). The cathepsin proteases, which are lysosomal hydrolases, are major luminal lysosome proteins and are optimally activated in lysosomes at pH 4–5; therefore, cathepsins are used as indicators of lysosomal function (Collette et al., 2004; Fehrenbacher et al., 2008; Leu et al., 2009). Importantly, when the intracellular lysosomal pH gradient is changed and cathepsins leak into cytosol, lysosome-induced cell death pathways should be activated (Fehrenbacher et al., 2008).

Methamphetamine (METH)-induced neurotoxicity is one of the most serious adverse events in acute and chronic abuses of this drug (Kita et al., 2008; Krasnova and Cadet, 2009). METH has been shown to cause neurotoxic effects by the release of dopamine (DA) and serotonin (5-HT) (Schmidt et al., 1985). It has also been indicated that METH-induced DA release and redistribution from synaptic vesicles to cytoplasmic compartments are mediated by dopamine transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2) (Kita et al., 2008; Krasnova and Cadet, 2009). In addition, reactive oxygen species (ROS), which are generated by the elevation of DA auto-oxidization, are associated with neurodegenerative damage by METH in various brain systems such as the striatum and hippocampus (Davidson et al., 2001; Kita et al., 2008; Krasnova and Cadet, 2009; Thrash et al., 2009). We previously showed that METH induces excessive cytoplasmic vacuolization, which was confirmed as macropinocytosis, on RA-differentiated SH-SY5Y human neuroblastoma cells (Nara et al., 2010). Although our previous results indicated that METH-induced SH-SY5Y cell death is non-apoptotic, as evidenced by the lack of caspase activation, we did not examine how the intracellular signaling mechanism by METH exposure causes macropinocytosis and subsequent cell death. Under normal conditions, some macropinosomes move back to the plasma membrane for recycling, while others are transported to late endosomes/lysosomes (Lim and Gleeson, 2011). It has been demonstrated that METH rapidly causes lysosomal alkalization by the collapse of acidic organelle pH, resulting in inhibition of the endosomal–lysosomal pathway in dendritic cells (Tallóczy et al., 2008). Thus, it is highly possible that the excessive trafficking of macropinosomes to lysosomes during METH exposure causes the dysfunction of endosomes/lysosomes, finally leading to cell death.

In this study, we further evaluated the mechanism of METH-induced cytotoxicity and found that hyperstimulation of macropinocytosis by METH was mediated by Ras and Rac1, since cytoplasmic vacuolization was significantly inhibited by both FTS (an inhibitor of Ras) and EHT1864 (an inhibitor of Rac1). Although the expression of lamps increased considerably in a time-dependent manner after METH treatment, the processing of cathepsin L to the active form was significantly decreased, indicating the dilation and dysfunction of lysosomes. Both inactivation of cathepsin L and cell death by METH were recovered by pretreatment with nocodazole and trehalose, an activator of lysosome biogenesis. These data show that hyperstimulation of macropinocytosis by METH causes lysosomal dysfunctional events, such as the enlargement of lysosomes and the impairment of cathepsin activity, and finally induces cell death in RA-differentiated SH-SY5Y cells.

2. Results

2.1. *METH induces macropinocytosis through the activation of Ras in RA-differentiated SH-SY5Y cells*

Macropinocytosis is known to involve Ras and Rho-family GTPases (Kumari et al., 2010; Lim and Gleeson, 2011; Overmeyer and Maltese, 2011). FTS, a Ras inhibitor, interferes with Ras function and blocks the formation of macropinosomes (Goldberg and Kloog, 2006; Haklai et al., 1998; Kloog et al., 1999); therefore, we first studied, by FTS pretreatment, whether Ras is involved in METH-induced hyperstimulation of macropinocytosis in RA-differentiated SH-SY5Y cells. The percentage of vacuolated cells during METH (7 mM) exposure was significantly decreased by pretreatment with 100 μM FTS (Fig. 1A). To evaluate whether FTS inhibits cellular vacuolization by inhibiting macropinocytosis, FTS-pretreated cells were incubated simultaneously with METH and 70 kDa FITC-dextran for 2 h. Macropinosomes, incorporated with FITC-dextran by METH exposure, were hardly observed in cells pretreated with FTS, suggesting that FTS suppresses METH-induced macropinocytosis (Fig. 1B). Parts of these macropinosomes were colocalized with GFP-Ras (G12V), the constitutively activated form of Ras, after exposure to METH (Figs. 1C–E). These results show that hyperstimulation of macropinosomes by METH treatment is dependent on Ras activity, since these vacuoles are colocalized with GFP-Ras (G12V) and mostly disappeared by FTS pretreatment.

2.2. *METH induces macropinocytosis through the activation of Rac1 in RA-differentiated SH-SY5Y cells*

We first found that METH-induced cytoplasmic vacuolization by macropinocytosis depends on Ras activity (Fig. 1). Bhanot

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