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BRAIN RESEARCH

Research Report

Advanced glycation end products induce moesin phosphorylation in murine brain endothelium

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ABSTRACT

Advanced glycation end products (AGEs) have been found to play an important role in the development of diabetes, and AGE levels are correlated with the severity of diabetic complications. We have demonstrated that moesin, a protein linker between actin filaments and the plasma membrane, undergoes phosphorylation of its threonine 558 residue by AGE stimulation in human dermal microvascular endothelial cells through activation of p38 and Rho kinase (ROCK) pathways. In this study, we observed *in situ* whether AGEs caused phosphorylation of vascular endothelial cells in the brains of AGE-stimulated mice. The animals were injected with AGE-modified mouse serum albumin (AGE-MSA) for 7 consecutive days. Immunohistochemistry was conducted to assess the phosphorylation of moesin in brain vessels. The level of moesin protein phosphorylation was also assessed in cerebral microvessels by western blotting. The effects of p38 and ROCK activation were determined by application of a p38 inhibitor (SB203580) and a ROCK inhibitor (Y27632) at 30 min before each AGE administration. The results showed specific expression of moesin in murine brain vascular endothelial cells. AGE treatment induced a significant increase of threonine 558 phosphorylation in moesin, while inhibition of p38 and ROCK remarkably attenuated the phosphorylation of moesin. The level of moesin protein phosphorylation was also increased in cerebral microvessels, along with an increased permeability of the blood–brain barrier, while inhibition of the p38 and ROCK attenuated these responses. These results demonstrate that AGEs cause the phosphorylation of moesin in murine brain microvascular endothelial cells, with p38 and ROCK being involved in this process.

1. Introduction

Diabetes mellitus is a major risk factor for cerebrovascular disease and the most common diabetic cerebrovascular complications are ischemic stroke and cerebral atrophy. Ischemic stroke is due to accelerated macrovascular atherosclerosis (Sonnen et al., 2009), while cerebral atrophy is known to result from microvascular lesions (Messier et al., 2004). The resulting alterations of cognitive function are a diabetes-related disability. Endothelial dysfunction and damage are believed to play an important role in the pathogenesis of these diabetic macrovascular and microvascular complications (Bakker et al., 2009; Huber, 2008). Disruption of the blood–brain barrier and an increase of brain vascular permeability

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are early changes during the development of diabetes in both animal models (Huber et al., 2006) and clinical observation (Hovsepyan et al., 2004). However, the pathological features and molecular pathogenesis of cerebrovascular complications related to diabetes are not fully understood.

Hyperglycemia and its related pathological alterations lead to the onset of vascular complications (Geraldes et al., 2009). There is growing evidence that advanced glycation end products (AGEs) produced by non-enzymatic reactions between reducing sugars and amino reactive groups of proteins and lipids are especially relevant to the processes of macrovascular and microvascular damages (Takeuchi and Yamagishi, 2009). It has been noticed that microvascular endothelial cells in the brain are more susceptible to AGE-induced inflammatory damage than aortic endothelial cells (Niiya et al., 2006). By binding to a specific receptor known as RAGE, AGEs trigger oxidative stress and the activation of the mitogen-activated protein kinases (MAPKs) and RhoA kinase (ROCK) signaling pathways in endothelial cells, leading to up-regulation of pro-inflammatory molecules and disruption of endothelial barrier function (Forbes et al., 2003; Guo et al., 2005). The activation of MAPK and ROCK signaling strengthens the construction of F-actin and opens inter-endothelial junctions (Mehta and Malik, 2006). It has been noted in numerous studies that the ezrin/radixin/moesin (ERM) family of plasma membrane–actin linking proteins plays an important role in mediating the response to kinase signals and F-actin (Niggli and Rossy, 2008). Moesin is regarded as the most important player in this endothelial response since it is the ERM dominantly expressed by endothelial cells (Berryman et al., 1993), as well as cerebrovascular endothelial cells (Hayashi et al., 1999; Johnson et al., 2002). Our previous study demonstrated that moesin is phosphorylated at threonine residue 558 by AGE-induced signaling, and this change plays an important role in modulating the endothelial cytoskeleton and barrier function in human microvascular endothelial cells (HMVECs) (Guo et al., 2009). We have already detected an increase of murine retinal microvascular permeability in AGE-stimulated mice (unpublished data). The purpose of the present study was to assess the changes of blood–brain barrier function after AGE stimulation and to explore the role of threonine phosphorylation in moesin in this response. The involvement of MAPK and ROCK in AGE-induced phosphorylation of moesin in brain microvascular endothelial cells was also assessed in this study.

2. Results

2.1. *Effect of AGE-MSA on immunohistochemical staining of moesin and threonine 558-phosphorylated moesin in the murine brain*

Cellular expression of moesin was immunohistochemically identified with an antibody against moesin or an antibody against 558 threonine-phosphorylated moesin. Immunohistochemistry of brain tissue showed that moesin was strongly and predominately expressed in murine brain microvascular endothelial cells (Fig. 1A, arrow). There was almost no phosphorylated moesin in control brain endothelial cells. Administration of AGE-MSA did not alter total moesin expression, but it significantly strengthened the staining of moesin phosphorylated at the 558 threonine residue in cerebrovascular endothelial cells (Fig. 1A, hollow arrow). Pretreatment with a p38 MAPK inhibitor (SB203580) or a ROCK inhibitor (Y27632) had no influence on total moesin expression, but markedly lessened AGE-MSA-induced moesin phosphorylation in the cerebrovascular endothelium (Fig. 1A). Immunofluorescence of murine brains revealed similar results, with very strong and specific expression of moesin in brain endothelial cells. AGE-MSA-induced phosphorylation of moesin was obviously detected with a fluorescent-labeled secondary antibody (Fig. 2A), while inhibition of the p38 MAPK or ROCK pathways attenuated phosphorylation of moesin at the 558 threonine residue. Quantitative analysis after immunohistochemical and immunofluorescent staining further confirmed these observations (Figs. 1B and 2B).

2.2. *Effect of AGE-MSA on the expression of moesin and threonine 558-phosphorylated moesin in murine brain microvessels*

Murine brain microvessels were isolated by dissection, and the expression of moesin and the amount of phosphorylated moesin in microvascular tissues were investigated. Since moesin is specifically expressed by vascular endothelial cells, the amount of moesin or phosphorylated moesin reflects the pattern of expression by cerebrovascular endothelial cells. The results demonstrated that expression of total moesin was not altered by either stimulation with AGE-MSA alone or by pretreating mice with SB203580 or Y27632 before AGE-MSA administration. The amount of 558 threonine-phosphorylated moesin in AGE-MSA-treated mice showed a significant increase (Fig. 3A, B), with quantitative analysis demonstrating that the density ratio of phospho-moesin to total moesin was increased remarkably ($p<0.05$ compared with control) (Fig. 3C, D). These results suggested that AGE-MSA treatment leads to phosphorylation of moesin in murine cerebral microvascular endothelial cells.

Prior administration of SB203580 or Y27632 attenuated the AGE-MSA-induced phosphorylation of moesin, with obvious decrease of the density of phospho-moesin/moesin for 10.11% or 26.47% respectively ($p<0.05$ compared with AGE-MSA treatment). However, inhibition of p38 or ROCK alone could not totally abolish AGE-MSA-induced phosphorylation of moesin, while the density ratio of phospho-moesin to total moesin was still higher than control ($p<0.05$ compared with control) (Fig. 3). These results indicated that both p38 and ROCK MAPK are involved in AGE-induced threonine phosphorylation of moesin. The inhibitory effect of SB203580 on p38 MAPK activation or Y27632 on ROCK activation was also confirmed by western blotting. The data showed that the activation of p38 MAPK or ROCK by AGE-MSA was significantly attenuated for 29.29% or 15.99%, respectively, by prior administration of SB203580 (Fig. 4A, C) or Y27632 (Fig. 4B, D) in AGE-MSA-treated mice.

2.3. *Effect of AGE-MSA on blood–brain barrier function*

The Evans blue leakage assay revealed that AGE-MSA treatment led to impairment of cerebral microvascular endothelial barrier function. The amount of Evans blue (EB) dye extravasation increased from 0.809±0.045 μg/g in control mice to 2.218±