Hypericin, a Naphthodianthrone Derivative, Prevents Methylglyoxal-Induced Human Endothelial Cell Dysfunction

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Abstract

Methylglyoxal (MGO) is a highly reactive metabolite of glucose which is known to cause damage and induce apoptosis in endothelial cells. Endothelial cell damage is implicated in the progression of diabetes-associated complications and atherosclerosis. Hypericin, a naphthodianthrone isolated from Hypericum perforatum L. (St. John's Wort), is a potent and selective inhibitor of protein kinase C and is reported to reduce neuropathic pain. In this work, we investigated the protective effect of hypericin on MGO-induced apoptosis in human umbilical vein endothelial cells (HUVECs). Hypericin showed significant anti-apoptotic activity in MGO-treated HUVECs. Pretreatment with hypericin significantly inhibited MGO-induced changes in cell morphology, cell death, and production of intracellular reactive oxygen species. Hypericin prevented MGO-induced apoptosis in HUVECs by increasing Bcl-2 expression and decreasing Bax expression. MGO was found to activate mitogen-activated protein kinases (MAPKs). Pretreatment with hypericin strongly inhibited the activation of MAPKs, including P38, JNK, and ERK1/2. Interestingly, hypericin also inhibited the formation of AGEs. These findings suggest that hypericin may be an effective regulator of MGO-induced apoptosis. In conclusion, hypericin downregulated the formation of AGEs and ameliorated MGO-induced dysfunction in human endothelial cells.

Key Words: Advanced glycation end products, Methylglyoxal, HUVECs, Hypericin, Apoptosis

INTRODUCTION

Hypericin, a powerful naturally occurring photosensitizer, is produced by Hypericum species. Hypericin, the most important active component in Hypericum perforatum L. (St. John's Wort), is a potent and selective inhibitor of protein kinase C and is reported to reduce neuropathic pain. In this work, we investigated the protective effect of hypericin on MGO-induced apoptosis in human umbilical vein endothelial cells (HUVECs). Hypericin showed significant anti-apoptotic activity in MGO-treated HUVECs. Pretreatment with hypericin significantly inhibited MGO-induced changes in cell morphology, cell death, and production of intracellular reactive oxygen species. Hypericin prevented MGO-induced apoptosis in HUVECs by increasing Bcl-2 expression and decreasing Bax expression. MGO was found to activate mitogen-activated protein kinases (MAPKs). Pretreatment with hypericin strongly inhibited the activation of MAPKs, including P38, JNK, and ERK1/2. Interestingly, hypericin also inhibited the formation of AGEs. These findings suggest that hypericin may be an effective regulator of MGO-induced apoptosis. In conclusion, hypericin downregulated the formation of AGEs and ameliorated MGO-induced dysfunction in human endothelial cells.
tective effects of several compounds from a library of natural compounds against MGO-induced damage. Hypericin was the most effective anti-MGO compound screened, so we investigated its role in MGO-related glucotoxicity. The aim of the present study was to determine the protective effects of hypericin against MGO-induced cell death in HUVECs and to assess whether hypericin inhibits the formation of AGEs; our results demonstrate that hypericin can regulate apoptosis-related signaling pathways, including those involving oxidative stress and MAPKs.

MATERIALS AND METHODS

Materials

MGO and 2',7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma (St. Louis, MO, USA). Hypericin was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Endothelial growth medium (EGM-2) was purchased from Lonza (Walkersville, MD, USA). p38, phospho-p38, ERK1/2, phospho-ERK1/2, JNK, and phospho-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). Bcl-2, Bax and p53 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The human umbilical vein endothelial cell line (HUVECs, Lot # 60319874) was purchased from the American Type Culture Collection (ATCC, VA, USA). HUVECs were cultured under standard cell culture conditions (37°C in a humidified incubator containing 5% CO₂) in EGM-2 supplemented with 4% FBS. The passage number of all the cells used was between 5 and 8.

Cell viability analysis and morphological examination

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HUVECs were seeded in 96-well plates at 1.0×10⁴ cells/well and incubated for 24 h at 37°C. The medium was then replaced with MGO at a final concentration of 0.1 mg/ml. This was followed by a 24 h treatment with MGO. MTT solution was added at 20 μl/well. The plates were incubated for 4 h at room temperature and exposed to the primary antibodies overnight at 4°C. The plates were then washed with PBS, and the formazan crystals were dissolved in 100 μl DMSO. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices, CA, USA).

Detection of intracellular ROS

DCF-DA probe was used to measure the intracellular ROS scavenging activity of hypericin. Briefly, 2.0×10⁴ cells were seeded in a 12-well plate and incubated overnight at 37°C. After 24 h, cells were pre-incubated with hypericin for 30 min, followed by incubation with MGO for 1 h. The medium was then removed and the cells were washed with PBS. Then, medium containing 10 μM DCF-DA was added for 20 min at 37°C. After washing with PBS, cells were photographed using a Leica live-cell imaging system (NanoEnTek, Seoul, Korea). The fluorescence intensity was assessed using the computer software program Image J software (NIH, Bethesda, MD, USA) by background subtraction and converting images to 8-bit grayscale. The mean fluorescence intensity of the cells was expressed as mean ± S.D. Statistical analysis of results was performed using one-way ANOVA followed by Bonferroni’s test. A p-value<0.05 was considered statistically significant.

RESULTS

Effects of hypericin on MGO-induced apoptosis and ROS production

MGO-induced cell morphological changes in HUVECs after treatment with hypericin were observed. As shown in Fig. 1A, the percentage of apoptotic cells was increased (43%) in HUVEC incubated for 24 h in the EGM-2 media containing 400 μM MGO. In addition to decrease in cell number, typical morphological features such as shrinkage, fragmentation and rounding of the cell were observed at this time. But, treatment with hypericin rescued the morphological changes such as a loss of confluence and decreased the number of floating cell fragments induced by MGO. To determine the effect of hypericin on MGO-induced cytotoxicity in HUVECs, we performed an MTT assay. HUVEC cell viability was significantly reduced after MGO treatment, but hypericin treatment restored cell viability (Fig. 1B). Moreover, pretreatment with 0.5 μM hypericin increased cell viability.

It is known that MGO can increase intracellular ROS levels and may induce cell death. Therefore, we measured ROS formation in HUVECs following treatment with MGO. We also estimated the inhibitory effect of hypericin on ROS formation in HUVECs following treatment with MGO. We measured the
Fig. 1. The effect of hypericin on MGO-induced cytotoxicity and oxidative stress in HUVECs. (A) The representative photographs of MGO-treated HUVECs without (-) or with (+) hypericin. (a) control; (b) 400 μM MGO; (c) MGO+0.1 μM hypericin; (d) MGO+0.5 μM hypericin; (e) MGO+1 μM hypericin; (f) MGO+aminoguanidine. (B) The cell viability of HUVECs treated with MGO and hypericin. Cell viability was analyzed by the MTT assay. The percentage of cell viability is presented as mean ± SD of three independent experiments. (C) The protective effect of hypericin on MGO-induced ROS generation. HUVECs were pretreated with hypericin for 1 h and then treated with 400 μM MGO for 2 h. ROS generation was detected by staining with the fluorescent dye DCF-DA. (a) control; (b) 400 μM MGO; (c) MGO+hypericin; (d) MGO+aminoguanidine. (D) Fluorescent intensity was measured using Image J software. (E) The effect of hypericin on Bcl-2, Bax and p53 protein expression in MGO-treated HUVECs as assessed by Western blot. Cells were incubated without (-) or with (+) hypericin for 1 h and then treated with 400 μM MGO for 24 h. Tubulin was used as an internal control. (F) The relative band intensity of Bcl-2. (G) The relative band intensity of Bax. (H) The relative band intensity of p53. Bar values are presented as the mean ± SD of three independent experiments (**p<0.01 vs. control, ***p<0.001 vs. control, ##p<0.01 and ###p<0.001 vs. 400 μM MGO treatment only).
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antioxidative effect of hypericin using DCF-DA. As shown in Fig. 1C, we found that pretreatment with hypericin significantly reduced ROS generation in MGO treated HUVECs.

Next, we investigated whether MGO can affect expression of apoptosis related proteins such as Bcl-2, Bax and p53 in HUVECs. As shown in Fig. 1D, treatment with MGO decreased Bcl-2 protein expression, but enhanced protein expressions of Bax and p53 in HUVECs. Also, treatment with hypericin has downregulated the expression of Bax and p53 and upregulated the expression of Bcl-2. These data suggest that hypericin may prevent MGO-induced apoptosis in HUVECs.

Effects of hypericin on MAPK activation

Activation of MAPK plays an important role in MGO-induced apoptosis in various types of cells. In this study, we investigated three proteins in MAPK subfamilies, p38, ERK1/2, and JNK, in MGO-treated HUVECs, and evaluated the effect of hypericin on activation of MAPK cascades induced by MGO. As shown in Fig. 2, there was a significant increase in p38, ERK1/2, and JNK activation in MGO-treated cells, whereas pretreatment with hypericin prior to MGO stimulation reduced the activation of all three MAPKs.

Inhibitory effects of hypericin on the formation of AGEs

The AGEs formation assay was used to measure fluorescence at an excitation wavelength of 355 nm and an emission wavelength of 460 nm; aminoguanidine was used as a positive control. As shown in Table 1, we found that formation of AGEs was significantly increased in cells treated with BSA-MGO. Hypericin (10 μM) significantly attenuated the formation of AGEs. However, 1 μM hypericin did not produce a significant difference in AGEs formation.

**DISCUSSION**

MGO is one of the most significant reactive carbonyl species formed by the triose phosphate glycolytic intermediates of glucose metabolism (Lo et al., 2006). Elevated MGO levels have been reported in vascular endothelial cells cultured in high-glucose media (Mukohda et al., 2012). MGO is related to diabetic vascular complications, and it triggers cellular in-

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Table 1. Inhibitory effect of hypericin on AGE formation

| Sample            | AGEs Formation (%) |
|-------------------|--------------------|
| Control           | 12.24 ± 0.14       |
| MGO (400 μM)      | 100.00 ± 0.89***   |
| Hypericin (1 μM)  | 102.67 ± 0.11      |
| Hypericin (10 μM) | 80.06 ± 0.64#***   |
| Aminoguanidine (1 mM) | 28.82 ± 0.21#***  |

The percent of AGEs formation is presented as the mean ± SD of three independent experiments. ***p<0.001 vs. control, **p<0.01 and #***p<0.001 vs. 400 μM MGO treatment only.
journey and apoptosis in endothelial cells (Figarola et al., 2014). Recently, several natural compounds found in plants have been reported to be protective against MGO-induced cytotoxicity (Hu et al., 2013; Zhu et al., 2014). We screened a library of naturally occurring compounds for those that potentially ameliorated MGO-induced toxicity in HUVECs. In this study, we propose that hypericin, the most active ingredient in Hypericum perforatum, protects against MGO-induced apoptosis and oxidative damage. To evaluate the antiapoptotic effect of hypericin, we pretreated HUVECs with different concentrations (0.1-1 μM) of hypericin for 1 h followed by 400 μM MGO treatment for 24 h. We found that 0.5 μM hypericin protected against MGO-induced cell toxicity (Fig. 1B). Treatment with high concentrations of hypericin (more than 1 μM) had no effect on MGO-induced cell death (data not shown).

It is well known that intracellular ROS is generated in vascular endothelial cells in response to MGO treatment (Figarola et al., 2014). Antioxidant activity of Hypericum perforatum L. and its components have been reported (Gioli et al., 2009). Furthermore, Cakir et al. reported that hypericin has an antioxidative effect (Cakir et al., 2003). Therefore, we investigated whether hypericin ameliorates MGO-induced ROS generation. First, we found that ROS levels were significantly increased by treatment with 400 μM MGO. Also, MGO-induced ROS generation was significantly reduced by pretreatment with hypericin (Fig. 1C). It has been reported that the antioxidant activity of phenolic compounds is mainly governed by number of total hydroxyl groups (Weng and Wang, 2000). Since hypericin contains many hydroxyl groups, we expected hypericin to have antioxidant activity (Cakir et al., 2003). Namely, hypericin may be one of the antioxidants from Hypericum perforatum L.

Alteration of the ratio of the expression of one or more members of the Bcl-2 protein family is significant in determining whether apoptosis occurs, because Bcl-2 family member proteins play critical roles in regulating the process of apoptosis (Murphy et al., 2000). p53 protein can regulate the function of mitochondria and oxidative stress (Lin and Beal, 2006). Also, p53 protein can directly activate Bax protein by translocating to mitochondria (Chipuk et al., 2004). In this study, exposure of HUVECs to 400 μM MGO decreased Bcl-2 and increased Bax and p53 expression in MGO-treated HUVECs. However, pretreatment with hypericin attenuated the increase in Bax and p53 proteins expression, and increased Bcl-2 protein expression (Fig. 1D). It is interesting that they found that potential effect of hypericin in HUVECs was strongly associated with positive regulation for p53 activation, suggesting a correlation with oxidative stress and apoptosis.

In endothelial cells, MGO may induce cytotoxicity via activation of several key molecules including ERK, JNK, and p38, which are involved in the MAPK signaling pathway (Akhand et al., 2001). MAPKs play a major role in the process of apoptosis and phosphorylate and regulate the activity of the Bcl-2 family in response to a variety of stresses (Liu et al., 2012). We confirmed that treatment with MGO activated phosphorylation of MAPKs and observed that hypericin downregulated activation of MAPKs in 400 μM MGO-treated HUVECs. These results show that hypericin modulates the MAPK signaling pathway, and may thus protect MGO-treated HUVECs from apoptosis.

To investigate the protective effect of hypericin against MGO-induced apoptosis, we performed annexin V-FITC/PI assay using flow cytometry. However, PI baseline was shifted to the necrosis zone, since hypericin is a red-colored naphthodianthrone derivative. So we could not get the reliable annexin V-FITC/PI assay data. However, the proteins expressions of Bcl-2, Bax, p53 and MAPKs by treatment of MGO were changed. Furthermore, many previously studies reported that MGO-mediated diabetic vascular complications might be due to apoptosis in endothelial cells (Phalitakul et al., 2013; Figarola et al., 2014; Lv et al., 2014). This indicates that MGO-induced cytotoxicity is prevented by incubation with hypericin in HUVECs, which seems to act by regulating cell apoptotic proteins.

MGO, a dicarbonyl compound, is more reactive than are reducing sugars such as glucose. This highly reactive compound is a major precursor in the formation of AGEs (Desai and Wu, 2007). One of the major AGEs adducts, Nε-(carboxymethyl)lysine, is formed from MGO (Thornalley, 1996). In endothelial cells, increased levels of AGEs cause oxidative stress, mitochondrial dysfunction, cellular dysfunction, and, ultimately, cell death (Li et al., 2011). That hypericin inhibits the formation of AGEs suggests that it can ameliorate AGEs-related endothelial cell dysfunction. Table 1 shows the percent inhibition of AGEs formation brought about by treatment with hypericin. After treatment with 10 μM hypericin and MGO with BSA, the formation of AGEs was decreased by 20% on 7 day. However, the potency of hypericin was less than that of aminoguanidine, which was used as a positive control. Hypericin is a red-colored anthraquinone-derived naphthodianthrone. AGEs formation is inhibited by phenolic acids carrying multiple hydroxyl groups (Xie and Chen, 2013). Phenolic compounds, such as caffeic acid and chlorogenic acid, exhibit a significant inhibitory effect on AGEs formation (Gugliucci et al., 2009). Likewise, anthraquinones with ortho or meta dihydroxyl groups seem to inhibit the formation of AGEs and aldose reductase (Yoo et al., 2010). Furthermore Luc Sero et al. reported that Hypericum perforatum L. decreased the AGEs formation (Sero et al., 2013). Therefore, we suggest that hypericin may be an active component from Hypericum perforatum L. for decrease of AGE formation. Given all of these observations, hypericin, a red-colored naphthodianthrone, may directly scavenge AGEs.

In conclusion, our results support the notion that hypericin protects against MGO-induced apoptosis in cultured HUVECs via scavenging of ROS and reducing the activation of cell apoptosis via the MAPK pathway. Therefore, our results suggest that hypericin may be a useful tool to prevent or reverse MGO-induced vascular damage.

ACKNOWLEDGMENTS

This research was supported by IPET (Korean Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry of Agriculture, Food and Rural Affairs (NO. 115045-3).

This research was supported by High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs (114006041HDO20).

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