Cannabinoid receptor 1 mediates high glucose-induced apoptosis via endoplasmic reticulum stress in primary cultured rat mesangial cells

Jae Cheong Lim, Seul Ki Lim, Min Jung Park, Gye Yeop Kim, Ho Jae Han, and Soo Hyun Park

Bio-Therapy Human Resources Center, Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Gwangju, Korea

Submitted 3 February 2010; accepted in final form 9 February 2011

Cannabinoid receptor 1 mediates high glucose-induced apoptosis via endoplasmic reticulum stress in primary cultured rat mesangial cells. Am J Physiol Renal Physiol 301: F179–F188, 2011. First published February 16, 2011; doi:10.1152/ajprenal.00032.2010.—The endocannabinoid system in animals and humans is involved in the onset of diverse diseases, including obesity and diabetic nephropathy, which is a major end-stage renal disease characterized by high glucose (HG)-induced apoptosis of mesangial cells. Endocannabinoids induce physiological and behavioral effects by activating two specific receptors, cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R). However, the pathophysiology of CB1R in diabetic nephropathy has not been elucidated. We investigated the effects of HG on CB1R expression and its signaling pathways in primary cultured rat mesangial cells. HG significantly increased CB1R mRNA and protein levels in a time-dependent manner and induced CB1R internalization. NF-κB and cPLA2 were involved in the HG-induced increase in CB1R levels. Using a CB1R antagonist (AM251) and CB1 siRNA transfection, we showed that HG-induced CB1R is linked to apoptosis. Specifically, HG inhibited the expression of GRP78, but induced increases in endoplasmic reticulum (ER) stress proteins, including phosphorylated (p)-protein kinase-like ER-associated kinase, p-eukaryotic initiation factor-2 (p-eIF2α), and p-activating transcription factor-4, and C/EBP homologous protein. In addition, HG increased the Bax/Bcl-2 ratio and increased the amounts of cleaved poly(ADP-ribose) polymerase and caspase-3. These apoptotic effects were prevented by AM251 and by the downregulation of CB1R expression by small interfering RNA. We propose a mechanism by which blockade of CB1R attenuates HG-induced apoptosis in rat mesangial cells. Our findings suggest that blockade of CB1R may be a potential therapy in diabetic nephropathy.

Address for reprint requests and other correspondence: S. H. Park, Bio-therapy Human Resources Center, Dept. of Veterinary Physiology, College of Veterinary Medicine, Chonnam National Univ., Kwangju 500-757, Korea (e-mail: parksh@chonnam.ac.kr).

http://www.ajprenal.org

1931-857X/11 Copyright © 2011 the American Physiological Society

DIABETIC NEPHROPATHY IS CHARACTERIZED by hyperglycemia-induced dysfunction of mesangial cells (21). In an environment of high glucose (HG), renal mesangial cells undergo cascades of deleterious reactions, including cell injury and extracellular matrix deposition, which lead to glomeruli dysfunction (1, 25, 49). Mesangial cell apoptosis promoted by HG contributes to the development of diabetic nephropathy (22, 40).

Endocannabinoids are endogenous lipid mediators with a wide range of biological effects, similar to those of marijuana. The endocannabinoid system (ECS) regulates synaptic plasticity, emotional responses, energy homeostasis, and glucose metabolism (44). Dysregulation of ECS is involved in the onset of obesity and diabetic nephropathy (12). Barutta et al. (5) have reported that cannabinoid receptor 1 (CB1R) was overexpressed within glomeruli in diabetic mice and CB1 blockade prevented diabetes-induced albuminuria. It has been also reported that the condition of HG increased the endogenous ligands of the ECS, N-arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) in vivo and in vitro (35, 36). They induce physiological and behavioral effects primarily by activating two specific receptors, CB1R and CB2R. Several investigators have demonstrated that CB1R is expressed in various peripheral tissues, including the kidneys, and have suggested that CB1R may be linked to the pathogenesis of diabetic nephropathy (11).

The accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) produces stress in the ER, which results in the activation of intracellular signal transduction pathways to restore normal ER function. This activation is called the unfolded protein response (UPR) (51). The ER stress response involves three major signaling pathways: the pancreatic double-stranded RNA-activated protein kinase-like ER-associated kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring ER-to-nucleus signal kinase 1 (IRE1) pathways (34, 51). When the stress cannot be resolved, the signaling pathways switch from prosurvival to proapoptosis (58). The PERK- eukaryotic initiation factor 2α (eIF2α) pathway induces activating transcription factor-4 (ATF4) during the ER stress response (32), and the ATF4 pathway induces the transcriptional activation of C/EBP homologous protein (CHOP) (46), which subsequently induces other proapoptotic genes, resulting in caspase activation (9, 15). Caspase is also activated by the IRE1 pathway (23). Caspase activation is the ultimate step in apoptosis (42). Accumulating evidence suggests that the ER plays an essential role in apoptosis (19) and may also be important in the development and pathogenesis of diabetic complications (2).

Based on these reports, we hypothesized that there is a close relationship between CB1R and the development of diabetic nephropathy in mesangial cells and that ER stress may be pivotal in linking CB1R and diabetic nephropathy. This study was conducted to investigate the effects of HG on CB1R and its related signal pathways in rat mesangial cells. We also examined whether HG-induced activation of CB1R is linked to ER stress and apoptosis in rat mesangial cells.

MATERIALS AND METHODS

Materials. DMEM and Ham’s nutrient mixture F-12 (DMEM/F-12) was purchased from Life Technologies (GIBCO BRL, Grand Island, NY). D-Glucose, CB1 small interfering (si) RNA, bisindolylmaleimide I, PDTC, and anti-mouse IgG were obtained from Sigma (St. Louis, MO). Phospho-eIF2α antibody, caspase-3 antibody, and anti-rabbit IgG were purchased from Cell Signaling Technology (Herts, UK). The β-actin antibody, GRP78 antibody, phospho-PERK antibody, CHOP antibody, Bcl-2 antibody, poly(ADP-ribose) poly-
merase (PARP) antibody, and rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ATF4 antibody was purchased from Spring Bioscience (Pleasanton, CA). Bax antibody was purchased from Chemicon (Temecula, CA). Mouse anti-GFP antibody was purchased from Invitrogen. AM251 and AA-COCF3 were purchased from Cayman Chemical (Ann Arbor, MI). LY294002 was purchased from Calbiochem (San Diego, CA). N-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) was purchased from Tocris Bioscience (Ellisville, MO). The CB1R L14 antibody was kindly provided by Dr. Ken Mackie (University of Washington, Seattle, WA). All other reagents were of the highest purity commercially available.

Cell cultures. Mesangial cells were obtained from glomeruli of 200-g male Sprague-Dawley rats. Glomeruli were isolated by progressive sieving of chopped renal cortex using 250-, 106-, and finally 200-μm stainless steel sieves (Newark Wire Cloth, Newark, NJ). The isolated glomeruli were cultured in DMEM (GIBCO BRL, Life Technologies) containing 5.6 mmol/l D-glucose, L-glutamine, and 110 mg/l sodium pyruvate, supplemented with sodium bicarbonate (3.7 g/l), HEPES (2.38 g/l), 1% penicillin-streptomycin (GIBCO BRL), and 10% FBS (GIBCO BRL). Primary mesangial cells were identified by positive immunofluorescence for the intermediate filaments desmin and vimentin but were negative for cytokeratin. Mesangial cells from passages 5–10 were used for all experiments and were cultured to 90% confluence. HEK293 cells were obtained from the Korean cell line bank. HEK293 cells were grown to confluence in DMEM/Ham’s F-12 (GIBCO BRL, obtained with 25 mM glucose) with 15 mM HEPES buffer, 5% FBS, 5 mM glucose, 0.35% additional sodium bicarbonate, and 1% penicillin-streptomycin. The cells were maintained at 37°C in 5% CO2 in a humidified cell culture incubator. The media were changed every other day. The Institutional Animal Care and Use Committee at Chonnam National University approved the protocols used in this study, and the animals were cared for in accordance with the Guidelines for Animal Experiments.

Cloning and DNA transfection. This procedure was performed as our previous study (28).

Immunofluorescence staining. This procedure was performed as our previous study (28).

RNA isolation and RT-PCR. This procedure was performed as our previous study (28). The primers used were 5′-cttcactctcagaggagccgtggccatctcttgctcgaagtc-3′ (antisense) and 5′-GGTCCCAACACTGGATGTCCTTCTGAAGGGGAGC-3′ (sense), 5′-cttcactctcagaggagccgtggccatctcttgctcgaagtc-3′ (antisense) and 5′-GGTCCCAACACTGGATGTCCTTCTGAAGGGGAGC-3′ (sense), 5′-GTTTCCCCACACACTGGATGTCCTTCTGAAGGGGAGC-3′ (antisense) for GRP78, 5′-ATGAAGTCGATCCTAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGCAGGACAGAGATGGCCTTGAGCAGC
fluorescence microscope (Nikon, ECLIPSE TE300). Apoptotic cells were identified by nuclear condensation and fragmentation. The DAPI staining experiments were done in triplicate.

siRNA transfection. This procedure was performed as our previous study (28).

Statistical analysis. The results were expressed as means ± SE. All the experiments were analyzed by ANOVA. In some experiments, a comparison of the treatment means was made with the control using the Bonferroni-Dunn test. A P value <0.05 was considered significant.

RESULTS

Effects of HG on CB1R mRNA expression and protein levels. To examine the effects of HG on CB1R mRNA expression and protein levels, rat mesangial cells were exposed to 25 mM glucose for different time intervals (0–48 h). As shown in Fig. 1A, 25 mM glucose significantly upregulated CB1R mRNA expression over 1 h, with a maximum effect at 6 h after HG treatment. The mRNA levels then began to decline and were sustained to 48 h (Fig. 1A). Similarly, HG upregulated CB1R protein levels, which reached a maximum at 12 h, then declined, and was sustained to 48 h. The protein results were correlated with the mRNA results (Fig. 1B). To exclude the osmolar effects, cells were exposed to 25 mM mannitol or 25 mM l-glucose for 6 or 12 h, when they showed the highest effects of HG on mRNA or protein levels, and we excluded the osmolar effects on CB1R expression (Fig. 1C).

HG-induced CB1R internalization. CB1R has been previously reported to undergo agonist-induced or constitutive endocytic cycling between the plasma membrane and endosomes (20). This agonist-induced constitutive activity is inhibited by its antagonist, and it allows CB1R to remain in the plasma membrane (26, 38). To determine whether HG mediates CB1R endocytosis as well as CB1R upregulation, we monitored the HG-induced internalization of C-terminal green fluorescent protein (GFP)-tagged CB1R in transfected HEK293 cells. CB1R-GFP was localized at the cell surface of HEK293 cells (Fig. 2A–a); however, after HG treatment for 1 h, CB1R-GFP was endocytosed (Fig. 2A–b). HG-induced internalization of CB1R-GFP was prevented in the presence of 500 nM AM251, a CB1R agonist (Fig. 2A–c). To account for any effect of GFP, HEK293 cells were transfected with GFP alone under each condition (Fig. 2, A–d–A–f). Quantitative analysis of CB1R internalization showed a sevenfold increase in the cytosol/membrane fluorescence ratio after treatment with 25 mM glucose for 1 h, and this effect was significantly attenuated by AM251 treatment (Fig. 2B). To examine internalization in rat mesangial cells, cells under the same conditions as shown in Fig. 2A were separated into membrane and cytosolic fractions, and CB1R was detected by Western blotting. Treatment with 25 mM glucose caused a decrease in the CB1R protein level in the membrane fraction, and treatment with AM251 prevented this effect of HG. Conversely, treatment with 25 mM glucose significantly increased the CB1R protein level in the cytosolic fraction, and this increase was prevented by treatment with AM251 (Fig. 2C). Quantitative analysis of the cytosol/membrane fraction ratio gave results similar to those in Fig. 2B. HG treatment induced a twofold increase in the cytosol/membrane fraction ratio, which was prevented by AM251 treatment (Fig. 2D). These results suggest that HG stimulates the internalization and consequent activation of CB1R.

NF-κB and cytosolic PLA2 signaling pathways are involved in HG-induced CB1R activation. The elevation of plasma glucose was correlated with increased levels of glomerular cPLA2 in the early development of diabetes in OLEFT rats (16). In addition, Okamoto et al. (43) reported that PLA1/2 activity was required for hydrolyzing N-acylethanolamine or AEA, which was the first endo-
cannabinoid to be discovered. To identify the signaling pathways involved in HG-mediated CB1R activation, rat mesangial cells were treated with the NF-κB inhibitor PDTC (1 mM) or with arachidonyl trifluoromethyl ketone (AACOCF3), an inhibitor of the 85-kDa Ca2+-dependent and sn-2 arachidonyl-specific cytosolic PLA2 (cPLA2). High glucose upregulated phosphor-NF-κB and cPLA2. Results were normalized to the housekeeping gene β-actin. The example shown is representative of 4 experiments. *P < 0.05 vs. control. **P < 0.05 vs. 25 mM D-glucose.

CB1R antagonists attenuate the antiproliferative and apoptotic effects of HG. An MTT assay and DAPI staining were used to examine the involvement of CB1R in HG-induced apoptosis. Treatment with 25 mM glucose decreased cell proliferation, and this effect was blocked by treatment with AM251, in a concentration-dependent manner (Fig. 4A). Additionally, 25 mM glucose also induced apoptosis, and this was prevented by treatment with AM251, but not with DMSO (Fig. 4B). As expected, the treatment with 5 μM CB1 agonist ACEA decreased cell proliferation...
and induced apoptosis even more than the treatment with HG, but not with its vehicle; EtOH (Fig. 4, A and B). These results suggest that CB1R is involved in HG-induced apoptosis and that blockade of CB1R attenuates the effects of HG in rat mesangial cells.

CB1R antagonists prevent HG-mediated reduction of GRP78. To look for effects of HG on cellular GRP78 expression, we assessed GRP78 mRNA and protein levels in response to HG treatment for 0–24 h. GRP78 mRNA and protein levels were decreased in a time-dependent manner with HG treatment (Fig. 5A). The HG-mediated downregulation of GRP78 mRNA and protein was prevented by treatment with 500 nM AM251 (Fig. 5B). Thus CB1R appears to be involved in the HG-induced downregulation of GRP78.

CB1R antagonists prevent HG-mediated ER stress. To investigate the possible roles of HG and CB1R in the induction of ER stress, we assessed the levels of several ER stress markers after treatment with HG or a CB1R antagonist. As shown in Fig. 6A, HG induced the PERK-eIF2α-ATF4-CHOP signaling pathway, which is an ER stress response pathway. The phosphorylation of PERK, eIF2α, and ATF4 was increased for up to 24 h, in a time-dependent manner, and then gradually decreased by a small amount until 48 h. HG-induced CHOP also progressively increased in a time-dependent manner (Fig. 6A). We assessed whether a CB1R antagonist could reduce HG-induced ER stress at 24 h, which was the time when the signal molecule levels started to decrease. Treatment with 25 mM glucose stimulated p-PERK, p-eIF2α, p-ATF4, and CHOP, and these effects were blocked by pretreatment with AM251 (Fig. 6, B–E). Next, we assessed proapoptosis signal pathways under the same conditions, using an MTT assay and DAPI staining (as shown in Fig. 4), in the presence of 500 nM AM251. HG treatment also decreased the level of Bcl-2 and increased the level of Bax, which increased the Bax/Bcl-2 ratio, reflecting the activation of proapoptotic signaling pathways. Treatment with AM251 blocked the HG-induced decrease in Bcl-2, but did not block the HG-induced increase in Bax; thus, the Bax/Bcl-2 ratio decreased with AM251 (Fig. 7A). Treatment with HG also increased the cleaved forms of PARP and caspase-3, and this effect was blocked by AM251 treatment (Fig. 7, B and C). These results suggest that HG induces apoptosis via ER stress and that CB1R activation is involved in the HG-mediated effects.

CB1 siRNA blocks the effects of HG-induced ER stress. To confirm the involvement of CB1R in HG-induced apoptosis and ER stress, we used CB1 siRNA to knock down the level of CB1R mRNA. CB1 siRNA, but not control siRNA, downregulated HG-induced CB1R, in a concentration-dependent manner (Fig. 8A). Transfection with CB1 siRNA normalized the HG-induced level of GRP78 protein (Fig. 8B) and blocked the

![Fig. 5. CB1R antagonist AM251 prevented high glucose (25 mM D-glucose)-mediated GRP78 expression in mesangial cells. A: time course of GRP78 expression in 25 mM D-glucose. GRP78 mRNA and protein levels were reduced in 25 mM D-glucose in a time-dependent manner. B: effect of AM251 on 24-h high glucose-mediated GRP78 reduction. AM251 prevented high glucose-mediated GRP78 reduction. Results were normalized to β-actin. The example shown is representative of 4 experiments. *P < 0.05 vs. control. **P < 0.05 vs. 25 mM glucose.](Attachment)
HG-induced ER stress and apoptosis pathways (Fig. 8, C and D). Thus the blockade of CB1R at the mRNA level can reduce HG-induced ER stress.

DISCUSSION

In this study, we demonstrated for the first time that HG-induced CB1R activation is implicated in the development of diabetic nephropathy in vitro. The glucose concentrations used in this study may mimic in vivo conditions; glucose levels in nondiabetic animals are ~5.5 mM, and those in diabetic animals and patients are 20–30 mM (33). The HG concentration induces oxidative stress, glomerular hypertension, PKC activation, and transforming growth factor (TGF-β) in mesangial cells, which induces apoptosis of the cells and contributes to the progression of diabetic nephropathy (3, 4, 40). As expected, our results confirmed that HG increases apoptosis of mesangial cells. Our study extend the notion that HG-induced apoptosis is associated with CB1R expression.

The ECS is involved in the physiological regulation of many functions in the central nervous systems and peripheral organs, including energy homeostasis and glucose metabolism. Dysregulation of CB1 activity in peripheral tissues contributes to the development of diverse diseases such as fatty liver, adipose tissue lipotoxicity, and insulin resistance (12, 44). Matias et al. (35) have reported that the plasma levels of endocannabinoids are elevated in obesity and type 2 diabetes. They have also showed that the treatment of HG upregulated the levels of endogenous cannabinoid receptor ligands AEA and 2-AG in

Fig. 6. CB1R antagonist AM251 prevented high glucose (25 mM)-mediated endoplasmic reticulum (ER) stress by the protein kinase-like ER-associated kinase (PERK) pathway in mesangial cells. A: time course of 25 mM D-glucose-mediated PERK signaling pathway. Preincubating cells with AM251 (500 nM) in high glucose (25 mM) for 24 h inhibited phosphorylated (p)-PERK (B), p-eukaryotic initiation factor 2α (eIF2α; C), p-activating transcription factor-4 (ATF4; D), and C/EBP homologous protein (CHOP; E). ER stress was assessed for different ER stress markers as described in MATERIALS AND METHODS and were normalized to the β-actin. The example shown is representative of 3 experiments. *P < 0.05 vs. control. **P < 0.05 vs. 25 mM D-glucose.
RIN-m5F β cells (35) and suggested that significant elevations of the levels of either AEA and 2-AG in the kidney of high-fat diet-fed mice are involved in the development of overt obesity and hyperglycemia (36). Endocannabinoids may control energy metabolism via CB1R. Recent evidence demonstrates that CB1R antagonists have beneficial effects in obese patients with type 2 diabetes (55). In addition, the hippocampal densities of CB1R protein and specific CB1R binding sites are significantly increased in the animal model of streptozotocin-induced type 1 diabetes (13). Our recent report also demonstrated that palmitic acid induces CB1R activation in proximal tubule cells, suggesting that hyperlipidemia increases CB1R activation, which may be associated with the onset of diabetic nephropathy (28). Nonetheless, the involvement of CB1R activation in the hyperglycemia-induced onset of diabetic nephropathy was not evidenced. We hypothesized that HG also activated CB1R expression in mesangial cells. In accordance with these reports and our expectation, our study revealed that HG stimulated the levels of CB1R proteins in rat mesangial cells. Similar to other G protein-coupled receptors, CB1R is internalized following protracted agonist exposure (50), and internalized receptors are predominantly sorted into the recycling pathway for reactivation (62). To determine whether HG activates CB1R and upregulates its expression, we expressed C-terminal GFP-tagged CB1R in HEK293 cells, because the transfection efficiency in rat mesangial cells was too low to show the internalization. With HG treatment, CB1R was internalized by 1 h, and we assumed that the upregulated CB1R levels reflected its activation. Similar results were obtained in rat mesangial cells, by observing the membrane vs. cytosolic distribution of CB1R after HG treatment; therefore, our findings in HEK293 cells can be applied to rat mesangial cells. Our recent report also showed that palmitic acid induced CB1R internalization in proximal tubule cells (28). Thus we hypothesized that hyperglycemia as well as hyperlipidemia induces CB1R internalization. Additionally, it has been reported that CB1 agonists promote endocytosis from the plasma membrane, whereas CB1R antagonists inhibit constitutive endocytosis, resulting in sequestration of CB1Rs on the plasma membrane (26, 38). Similarly, in this study, the CB1R antagonist AM251 inhibited HG-mediated internalization in the HEK293 cells as well as in the rat mesangial cells. Although several studies have reported the relationship between the ECS and diabetes mellitus, none have described the role of CB1R activation in the hyperglycemia-induced development of diabetic nephropathy. Thus this study extends our understanding of the pathophysiological CB1R-associated mechanism that underlies diabetes mellitus in rat mesangial cells.

Diverse signaling molecules can regulate CB1R expression. NF-κB is a target molecule in the treatment of diabetic nephropathy, especially in mesangial cells (8). In this study, we showed for the first time that the NF-κB activation is involved in HG-induced increases in CB1R mRNA and protein levels. Consistent with our results, Yang et al. (63) recently reported that HG stimulated NF-κB phosphorylation in human glomerular endothelial cells. Deb et al. (10) showed that an NF-κB inhibitor blocked HG-induced angiotensinogen expression in mesangial cells. Here, we provided additional evidence that NF-κB activation plays a pivotal role in HG-induced CB1R activation in rat mesangial cells. We also proved that cPLA2 is involved in HG-induced CB1R activation in rat mesangial cells. The enzyme cPLA2 has been shown to be responsible for arachidonic acid release and is involved in diverse diseases,
including diabetic nephropathy. Arachidonic acids released by mesangial cells in response to HG treatment can be metabolized through three different pathways: the cyclooxygenase, lipoxygenase, and cytochrome P-450 pathways. Many studies have reported that NF-κB and cPLA2 are downstream of the HG-mediated signal pathways and that cPLA2 is downstream of NF-κB (29, 31, 40), but this requires further study. It is interesting that the activation of PKC, phosphatidylinositol 3-kinase (PI3K), p38 MAPK, and JNK was not involved in HG-induced CB1R activation, based on our findings that pretreatment with bisindolylmaleimide I (a PKC inhibitor), LY294002 (a PI3K inhibitor), SB203580 (a p38 MAPK inhibitor), or SP200125 (a JNK inhibitor) did not block the HG-induced increase in CB1R protein levels (data not shown).

Recently, several lines of evidence have suggested a role for cannabinoids in the regulation of cell apoptosis (17, 18, 54). However, to our knowledge, there are no reports on the role of endocannabinoids in hyperglycemia-induced diabetic complications. This is the first report suggesting that the activation of CB1R by HG mediates mesangial cell apoptosis. GRP78, a prominent ER-resident chaperone, is required to maintain ER function during pathophysiological conditions (6, 56). Recently, we reported that GRP78 mRNA and protein were decreased in response to palmitic acid. These effects were restricted to the action of hyperlipidemia. In this study, we checked the effect of HG on GRP78 expression. HG significantly decreased GRP78 mRNA and protein levels. Wang et al. (60) reported similar results, showing that GRP78 was decreased over time under HG conditions in pancreatic β cells of streptozotocin-induced diabetic mice. Thus GRP78 may act as a protective signal against hyperglycemia, and reduced GRP78 expression may permit the activation of other signaling events such as ER stress and apoptosis. Decreased GRP78 expression induced by HG was reversed by pretreatment with AM251, suggesting that CB1R activation is an intermediate event in the HG-induced decrease in GRP78 mRNA and protein levels. Here, we provided further evidence that HG also decreased GRP78 mRNA and protein levels in mesangial cells.

ER stress is involved in the dysfunctions associated with diabetes mellitus (27, 59). In the present study, we observed that HG increased the levels of ER stress-related proteins such as p-PERK, p-eIF2α, p-ATF4, and CHOP. PERK activation has been shown to result in downstream activation of eIF2α, ATF4, and CHOP (45, 46). Recently, it was reported that CHOP is activated in the diabetic kidney and induces apoptosis, which may contribute to the development of diabetic nephropathy (30). In the present study, HG-induced PERK activation was associated with the activation of eIF2α, ATF4, and CHOP. We also provided additional evidence for a link between CB1R activation and HG-induced ER stress. This hypothesis is supported by several recent reports showing that cannabinoids induced apoptosis through the stimulation of ER stress in glioma cells and pancreatic tumor cells, palmitic acid-induced CB1R activation is associated with ER stress in human proximal tubule cells, and hyperglycemia increased the activation of CB1R in diabetic patients (7, 52, 53).

ER stress can activate proapoptosis signal pathways. CHOP represses Bcl-2 gene expression and activates Bax, resulting in
cell death (37, 61). In the present study, HG decreased Bcl-2 and increased Bax in primary cultured rat mesangial cells; a CB1R blocker prevented the HG-induced decrease in Bcl-2, but did not block the HG-induced increase in Bax. These mechanisms remain to be elucidated. We also demonstrated that HG increased the levels of cleaved PARP and caspase-3, and that CB1R siRNA and a CB1R antagonist reversed these effects. This study provides the first evidence supporting a direct effect of endocannabinoids on the onset of hyperglycemia-induced diabetic nephropathy in vitro and provides additional evidence for a role of endocannabinoids in ER stress and diabetic nephropathy.

Recently, we reported that palmitic acid induces apoptosis via ER stress through CB1R in HK-2 cells, human renal proximal tubular cells (28). Hyperlipidemia and hyperglycemia are both pivotal factors in the development of diabetic nephropathy, but hyperlipidemia shows different mechanisms and phenomena compared with hyperglycemia. We used HG concentrations and palmitic acid to try and mimic the condition found in hyperglycemia and hyperlipidemia, respectively. Since HK-2 cells were grown in HG conditions, it was not possible to examine the pathophysiological effect of hyperglycemia in proximal tubule cells. Furthermore, we thought that a pathophysiological event in mesangial cells is somewhat different from that in proximal tubule cells, since some proteins are elevated in diabetic glomeruli but not in diabetic proximal tubule cells (41). However, our present study showed that HG also induces apoptosis via ER stress through CB1R in mesangial cells. Therefore, we speculated that two different insults (i.e., hyperglycemia and hyperlipidemia) in two different cell lines (i.e., mesangial cells and proximal tubule cells) induced the same cell death cascade, via CB1R activation, which may be associated with the development of diabetic nephropathy (27, 59).

Rimonabant, the first CB1R antagonist, has been widely used to treat obesity and type 2 diabetes mellitus. Treatment with rimonabant improves insulin sensitivity in diet-induced obese mice (39). However, several studies have reported the CB1R-mediated protective effect in neurons (14, 24, 48, 57). The pros and cons of the use of CB1R antagonists may be dependent upon cell and organ specificity. In this study, AM251, which is structurally very close to rimonabant but exhibits better binding affinity and selectivity for CB1R (47), was used as a CB1R antagonist. We provide additional evidence that AM251 has antiapoptotic effect against HG in rat mesangial cells.

Taken together, these studies strongly suggest that the blockade of CB1R may attenuate the progression of diabetic nephropathy. In summary, the present study demonstrates a hypothetical biochemical mechanism by which HG induces CB1R. Furthermore, the blockade of CB1R reduced the apoptosis induced by HG-mediated ER stress in rat mesangial cells. We demonstrated that CB1R is involved in the activation of ER stress in the pathophysiological condition of normal cells, as has been shown in cancer cells. These data provide a basis for potential therapeutic applications of these results in the treatment of diabetic nephropathy.

ACKNOWLEDGMENTS


We thank Dr. Ken Mackie (Indiana University, Dept. Psychology and Brain Science) for providing the CB1R L14 antibody.

GRANTS

This study was supported financially by a research grant from the Korean Science and Engineering Foundation (R01–2010-000082). The authors acknowledge a graduate fellowship provided by the Korean Ministry of Education and Human Resources Development through the Brain Korea 21 project.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


Downloaded from http://ajprenal.physiology.org/ by 10.220.32.47 on June 22, 2017