Marinobufagenin causes endothelial cell monolayer hyperpermeability by altering apoptotic signaling

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Uddin MN, Horvat D, Childs EW, Puschett JB. Marinobufagenin causes endothelial cell monolayer hyperpermeability by altering apoptotic signaling. Am J Physiol Regul Integr Comp Physiol 296: R1726–R1734, 2009. First published April 22, 2009; doi:10.1152/ajpregu.90963.2008.—Marinobufagenin (MBG) is an endogenous mammalian cardiotonic steroid that is involved in the regulation of the membrane-bound sodium pump Na+/K+-ATPase. Increased plasma levels of MBG have been reported in patients with preeclampsia. MBG increases microvascular barrier permeability in an animal model of preeclampsia. However, the mechanism by which MBG impairs endothelial permeability is unknown. We utilized rat lung microvascular endothelial cells (RLMEC) to examine alterations in MBG-induced monolayer permeability and the effect of MBG on the phosphorylation status of ERK1/2, Jnk, and p38. Apoptosis was evaluated by examining alterations in caspases 3/7, 8, and 9 and annexin-V staining. We also examined the effect of MBG on the endothelial adherens junctions of the RLMEC monolayer. MBG inhibited the proliferation, and increased the monolayer permeability, of RLMEC. These actions of MBG were attenuated by ERK, p38, and pan caspase inhibition. MBG significantly decreased the phosphorylation of ERK1/2 and activated the phosphorylation of Jnk and p38. MBG also significantly increased the expression of caspases 3/7, 8, and 9, indicating the activation of apoptosis. MBG-induced apoptosis signaling was not observed in cells pretreated with a p38 inhibitor. MBG treatment induced the disruption of endothelial cell junctions. This effect was prevented by a pan caspase inhibitor. In conclusion, 1) MBG induced an impairment of RLMEC proliferation; 2) the bufodienolide also caused endothelial hyperpermeability; and 3) these effects of MBG were mediated by the downregulation of ERK1/2, the upregulation of Jnk and p38, by the activation of apoptosis, and by the disruption of endothelial cell junctions.

β-catenin; rat lung microvascular endothelial cells; MAP kinase; caspases;bufodienolide

Marinobufagenin (MBG) is an endogenous mammalian cardiotonic bufodienolide that has vasoconstrictive properties and is involved in the regulation of the membrane-bound sodium pump Na+/K+-ATPase (1, 13, 29, 36). Increased plasma levels of MBG have been demonstrated in volume expansion-mediated hypertension, including renal failure, primary aldosteronism, congestive heart failure, and preeclampsia (16, 23, 46). The latter disorder is a pregnancy syndrome, which includes hypertension, proteinuria, and, often, intrauterine growth restriction. Its incidence varies between 3 and 10% of all pregnancies (30, 31). In an animal model of preeclampsia (19), we have shown that the urinary excretion of MBG is elevated “prior” to the development of hypertension and proteinuria. These data suggest that MBG may play a pathophysiological role in preeclampsia (46), a syndrome that is characterized by shallow placentation (14, 32). MBG administration to normal pregnant animals has been shown to result in the development of hypertension and proteinuria (19). Furthermore, the provision of antibodies to MBG corrects the hypertension (12, 46). We have recently demonstrated that MBG impairs both the proliferation and growth-factor induced migration of first trimester cytotrophoblast (CTB) cells, which are critical for placental development (22, 43). We have recently demonstrated that MBG alters vascular permeability in a rat model of preeclampsia (41). We have also demonstrated that MBG impairs CTB cell function by the modulation of MAPK and stimulation of apoptosis (42). However, there have been no studies of the mechanism(s) by which MBG might alter vascular permeability.

Increased vascular permeability is an underlying pathophysiological event in preeclampsia (33, 34). Disrupted and enlarged intercellular junctions between endothelial cells have been observed in myometrial vessels in women with preeclampsia (37). A transmembrane adhesive protein, vascular endothelial (VE)-cadherin forms endothelial adherens junctions that are involved in the regulation of paracellular permeability in microvascular endothelium (7, 15, 20). Endothelial permeability is affected by many agonists (7, 20, 48). Increased endothelial monolayer permeability is associated with alterations in the junctional adhesion molecule VE-cadherin (48). Activation of MAPK signaling is known to play a critical role in the regulation of cellular proliferation and paracellular permeability in microvascular endothelium (4, 11, 15, 20, 27). Activated ERK1/2 controls cell proliferation and differentiation (4, 20). Stimulated Jnk and p38 pathways regulate cell proliferation, microvascular permeability, growth arrest, and apoptosis (4, 15, 20, 27). Several studies have implicated caspases as the molecular instigators of apoptosis (28, 38, 39). Recent studies have shown that the bufodienolide, bufalin, induces apoptosis in endometrial cells, which is associated with a downregulation of the cell cycle and the concomitant activation of Bax and caspase-9 (26).

Although increased vascular permeability is an important event in the pathogenesis of preeclampsia (34, 48), whether circulating factors elicit this endothelial barrier dysfunction is not known. In this study, we employed endothelial cells to determine whether MBG contributes to the increased vascular permeability in preeclampsia. Therefore, we examined the effects of MBG on ERK phosphorylation, as well as the proliferation and monolayer permeability of RLMEC. We evaluated the effects of MBG on ERK phosphorylation, as well as the proliferation and monolayer permeability of RLMEC. We evaluated the effects of MBG on ERK phosphorylation, as well as the proliferation and monolayer permeability of RLMEC. We evaluated the effects of MBG on ERK phosphorylation, as well as the proliferation and monolayer permeability of RLMEC.
addition, we examined whether or not microvascular permeability could be prevented by the inhibition of caspases and whether apoptosis of RLMEC could be prevented by the inhibition of p38 and Jnk. We hypothesized that MBG increases endothelial cell permeability and that it does so by disruption of endothelial cell junctions.

MATERIALS AND METHODS

Cell culture. RLMEC (VEC Technologies, Rensselaer, NY) were maintained on fibronectin-coated dishes in complete MCDB-3 media supplemented with 10% FBS and used at passages 3–7. Cells were incubated at 37°C, 5% CO₂, and 99% humidity (Isotemp CO₂ incubator, Fisher Scientific, Pittsburgh, PA). The RLMEC exhibited properties characteristic of all endothelial cells, such as typical cobblestone morphology and incorporation of acetylated low-density lipoprotein (7, 40).

Proliferation assay following MBG treatment. Cell proliferation was measured as described previously (9, 22, 24, 43). RLMEC were seeded onto 96-well plates in complete medium and allowed to adhere overnight at 37°C. Cells were then serum starved in PBS, and eight replicates were subsequently treated with 10% FBS/medium containing 0.5% FBS for 24 h, washed twice with 1x PBS, and cells were pretreated for 2 h with an ERK inhibitor (100 μM PD98059; Calbiochem-Novabiochem, San Diego, CA) before exposure to DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. Cells were incubated for 48 h with the respective treatments, at which time 10 μl of cell titer 96 was added to each well. Absorbance at 490 nm was determined by a microtiter plate reader (Microplate Spectrophotometer, Spectra Max 3400 pc; Molecular Devices, Sunnyvale, CA). Absorbance is directly correlated with the number of viable cells (24).

Cell viability assay. A cell viability assay was performed as described previously (43). The assay is based on the ability of living cells to convert a redox dye, resazurin into a fluorescent end product, resorufin. RLMEC were seeded onto 96-well plates in complete medium and allowed to adhere overnight at 37°C. Cells were then treated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. After incubation for 48 h with the respective treatments, 20 μl of Cell Titer-Blue reagent was added to each well. Absorbance at 530 nm was determined by a microtiter plate reader. The signal produced by conversion of resazurin to resorufin is directly proportional to the number of viable cells.

Monolayer permeability. The monolayer permeability study was performed by a method described previously (7). RLMEC were maintained on fibronectin-coated dishes in complete MCDB-3 media supplemented with 10% FBS. They were later grown as monolayers on Costar Transwell membranes (Corning, Corning, NY) for 48 h. One hour prior to the start of the experiment, the cells were exposed to fresh media without phenol-red dye. Cells were treated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 3 h. Some cells were pretreated for 2 h with an ERK inhibitor (100 μM PD98059, Calbiochem-Novabiochem, San Diego, CA), a p38 inhibitor (10 μM SB 202190, Sigma, St. Louis, MO), a Jnk inhibitor (20 μM SP 600125, Invitrogen, Carlsbad, CA), and a pan caspase inhibitor (20 μM Z-VAD-FMK, R&D Systems, Minneapolis, MN) before exposure to DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. FITC-albumin (5 mg/ml final concentration) was added to the luminal chamber for 60 min, and samples were removed from both luminal and abluminal chambers. The samples (100 μl) collected from the abluminal chamber were analyzed using a fluorometric plate reader (excitation 400 nm/emission 505 nm). The readings were converted with the use of a standard curve to albumin concentration. These concentrations were then used in the following equation to determine the permeability coefficient of albumin (Pₐ): Pₐ = ([A]/[L] × (1/A) × (V/[L])), where [A] is abluminal concentration, t is time in seconds, A is the area of the membrane in centimeters squared, V is the volume of the abluminal chamber, and [L] is luminal concentration.

Fig. 1. A: inhibition of cell proliferation by marinobufagenin (MBG). Cell proliferation (ordinate) is expressed as the fold-change compared with basal. Cell proliferation was significantly inhibited in 1, 10, and 100 nM MBG-treated cells compared with DMSO-treated groups (*P < 0.001 for all groups). MBG-induced inhibition of rat lung microvascular endothelial cells (RLMEC) proliferation was significantly attenuated by the ERK inhibitor in a concentration of 100 μM (*P < 0.001 in each case). The 0.1 nM MBG treatment had no effect. The results are presented as the means ± SE (n = 5, 8 replicates each). B: MBG, at concentrations from 0.1 to 100 nM had no effect on RLMEC viability. Cell viability is expressed as the percentage of basal.

Fig. 2. The effect of MBG on the permeability of RLMEC monolayers. Plotted on the y-axis is the change in permeability coefficient of albumin (Pₐ) expressed as a percentage of the basal value. The 0.1, 10, and 100 nM MBG treatments significantly increased the monolayer permeability of RLMEC within 3 h of treatment (*P < 0.001 in each case). The 0.1 nM MBG treatment had no effect. Pretreatment with an ERK, a p38 or a pan caspase inhibitor significantly attenuated, but did not completely prevent, the MBG-induced hyperpermeability of the RLMEC (†P < 0.001 in each case).
Effect of MBG on ERK 1/2, Jnk, and p38 phosphorylation. The effect of MBG on ERK 1/2, Jnk and p38 phosphorylation was evaluated by the cellular activation of signaling (CASE) ELISA kit (SuperArray, Frederick, MD) (45). The kit includes a complete antibody-based detection system for colorimetric quantification of the relative amount of phosphorylated protein and total target protein. For the CASE assay, RLMEC were seeded into 96-well plates and were stimulated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 10, 30, 60, 120, or 240 min. Detection of total and phosphorylated protein expression was determined according to the manufacturer’s protocol.

Immunoprecipitation and Western blot analysis. RLMEC were treated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 60 min. After each treatment, total cellular protein was obtained by lysis buffer containing 50 mM Tris at pH 7.4, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.3 mM Na-orthovanadate, 50 mM NaF, 1 mM EDT, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Protein concentrations were determined by BCA reagent (Pierce, Rockford, IL). An equal amount of protein in each sample was separated by 12% SDS/PAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% nonfat milk in PBS and incubated with primary antibodies for each protein of interest for 2 h at 4°C. After washing with PBS, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h and washed again with PBS. Bands were visualized by the enhanced chemiluminescence kit (Amersham, Piscataway, NJ). Membranes were then exposed to autoradiography film and their intensity was quantified using ImageQuant software (Molecular Dynamics). Western blotting was repeated three times for each group with identical results. Statistical comparisons for multiple determinations were performed using one-way ANOVA with Tukey’s post hoc test. A P value of less than 0.05 was considered significant.

RESULTS

MBG inhibited RLMEC proliferation. As shown in Fig. 1A, MBG treatment significantly inhibited RLMEC proliferation compared with DMSO (vehicle)-treated cells (66%, 70%, and 70% inhibition by 1, 10, and 100 nM MBG, respectively; P < 0.001 for each). The 0.1 nM MBG treatment had no effect on RLMEC cell proliferation. MBG-induced inhibition of RLMEC proliferation was significantly attenuated by 100 μM ERK inhibitor (P < 0.001 for each) (Fig. 1A). These results indicate that MBG has an antiproliferative effect on RLMEC, which was attenuated by ERK inhibition. The antiproliferative capacity of MBG on the cells was not due to a cytotoxic effect of the steroid, as evaluated by cell viability assays (Fig. 1B). As shown in Fig. 1B, RLMEC viability was not affected by MBG treatment.

Annexin-V staining. Annexin-V staining was performed by a method described previously (42, 44). RLMEC were incubated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 24 h. Other cells were pretreated for 2 h with a pan caspase inhibitor (10 μM SB 202190; Sigma) or a Jnk inhibitor (20 μM SP 600125; Invitrogen) before exposure to DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. One hundred microliters of Apo-ONE caspase 3, caspase 8, or caspase 9 (Promega, Madison, WI) reagent was added to each well. After a 1-h incubation at 37°C, the luminescence was measured by a luminometer (Fluoroskan Ascent FL, Thermo Labsystems, Franklin, MA), as described previously (21, 26, 28).

Effect of MBG on caspase 3/7, 8, and caspase 9 activities. The Apo-ONE homogeneous caspase 3/7, 8, and 9 assays were performed in 96-well formats after the RLMEC were incubated with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 3 h. Other cells were pretreated for 2 h with a p38 inhibitor (10 μM SB 202190; Sigma) or a Jnk inhibitor (20 μM SP 600125; Invitrogen) before exposure to DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. One hundred microliters of Apo-ONE caspase 3, caspase 8, or caspase 9 (Promega, Madison, WI) reagent was added to each well. After a 1-h incubation at 37°C, the luminescence was measured by a luminometer (Fluoroskan Ascent FL, Thermo Labsystems, Franklin, MA), as described previously (21, 26, 28).

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**MBG increased monolayer permeability.** RLMEC monolayer permeability was significantly increased (~2-fold) by 1, 10, and 100 nM MBG treatment for 3 h \( (P < 0.001, \text{in each case}) \) (Fig. 2). The 0.1 nM MBG treatment had no effect on the RLMEC monolayer permeability. To test whether the increased hyperpermeability was downstream of ERK, Jnk, p38, and caspases, RLMEC were pretreated with an ERK, a Jnk, a p38 or a pan caspase inhibitor before treatment with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG. The ERK, p38, and pan caspase inhibitors each significantly attenuated the hyperpermeability induced by 1, 10, or 100 nM MBG \( (P < 0.001, \text{in each case}) \), while the Jnk inhibitor did not do so.

**MBG downregulated ERK 1/2 phosphorylation.** To determine whether the decreased proliferation and increased monolayer permeability were due to reduced ERK1/2 activity, we measured ERK1/2 phosphorylation in RLMEC. As shown in Fig. 3A, a, 1, 10, and 100 nM MBG caused a significant decrease in the ratio of phosphorylated ERK1/2 to total ERK1/2 in RLMEC compared with controls [80%, 80%, and 72% decrease in phosphorylation at 10, 30, and 60 min, respectively; \( P < 0.001 \) for DMSO (vehicle) vs. 1, 10, or 100 nM MBG treatment]. Ratios of phosphoprotein to total protein at 2 h and 4 h time points were not statistically different compared with 1 h for all measures (data not shown). MBG had no effect on total ERK protein expression, demonstrating that the change in the ratio was due to increased phosphorylation.

To confirm the CASE data for the phosphorylation status of ERK1/2, we treated RLMEC with DMSO (vehicle) or 0.1, 1, 10, or 100 nM MBG for 60 min. Cell lysates were analyzed by Western blot analysis for phosphorylated and total ERK1/2. As shown in Fig. 3A, b, ERK1/2 phosphorylation was significantly downregulated by 1, 10, and 100 nM MBG \( [P < 0.001 \text{ for DMSO (vehicle) vs. 1, 10, or 100 nM MBG treatment}] \). The 0.1 nM MBG treatment had no effect.

**MBG increased phosphorylation of Jnk and p38.** A significant increase in the ratio of phosphorylated Jnk to total Jnk was observed in 1, 10, and 100 nM MBG-treated RLMEC compared with basal [50%, 56%, and 56% increase at 10, 30, and 60 min, respectively; \( P < 0.001 \) for DMSO (vehicle) vs. 1, 10, and 100 nM MBG treatment] (Fig. 3B, a). Similarly, 1, 10, and 100 nM MBG significantly increased the ratio of phosphorylated p38 to total p38 in RLMEC compared with basal [70%, 71%, and 71% increase at 10, 30, and 60 min, respectively; \( P < 0.001 \) for DMSO (vehicle) vs. 1, 10, and 100 nM MBG treatment] (Fig. 3C, a). Ratios of phosphoprotein to total protein at 2 h and 4 h time points were not statistically different compared with 1 h for all measures (data not shown). MBG had no effect on total Jnk and p38 protein expression demonstrating that the changes in the ratio were due to increased phosphorylation.

To confirm the CASE data for the phosphorylation status of Jnk and p38, we treated RLMEC with DMSO (vehicle) or 0.1, 1, 10, or 10 nM MBG for 60 min. After each treatment, cell lysates were analyzed by Western blot for phosphorylated and total Jnk and p38. As shown in Fig. 3B, b and 3C, b, Jnk and p38 phosphorylation were significantly upregulated by 1, 10, and 100 nM MBG \( (P < 0.001 \text{ for DMSO vs. 1, 10, and 100 nM MBG treatment}, \text{in each case}) \). The 0.1 nM MBG treatment had no effect on either Jnk or p38 phosphorylation.

**Activation of apoptosis by MBG in RLMEC cells was attenuated by p38 inhibition.** Apoptosis was evaluated by caspase 3/7, 8 and 9 activation and Annexin-V staining. As shown in Figs. 4, A–C, 1, 10, and 100 nM MBG significantly (~2.0-fold) activated caspase 3/7, 8, and 9 activities, respectively, in RLMEC compared with basal at 3 h \( [P < 0.001 \text{ for DMSO (vehicle) vs. 1, 10, or 100 nM MBG treatment}] \). The 0.1 nM MBG treatment had no effect on the activation of caspase 3/7, 8, or 9 activities (Fig. 4, A–C). Cells pretreated with the p38 inhibitor had no effect on MBG-induced caspase activation.
inhibitor prevented the activation of caspase 3/7, 8, and 9 activities ($P < 0.001$), while the Jnk inhibitor did not alter MBG-induced activation of the caspases (Figs. 4, A–C). Staining with annexin-V revealed apoptotic cells in the RLMEC treated with 1, 10, and 100 nM MBG. However, 0.1 nM MBG had no such effect. Contrarily, apoptosis was not observed in cells treated with either p38 or Jnk inhibitors alone. These data indicate that apoptosis was activated by the upregulation of the p38 pathway (Fig. 5).

**MBG-induced disruption of endothelial cell adherens junctions was attenuated by a pan caspase inhibitor.** RLMEC treated with DMSO showed strong and continuous $\beta$-catenin immunofluorescence at the endothelial cell-cell junctions (Fig. 6), indicating an intact cell barrier. Treatment with 1, 10, and 100 nM MBG induced disruption of endothelial cell junctions, as evidenced by irregular and scattered $\beta$-catenin fluorescence (Fig. 6). Cells treated with 0.1 nM MBG did not demonstrate this effect (Fig. 6). Cells pretreated with a pan caspase inhibitor prior to 1, 10, or 100 nM MBG demonstrated attenuated $\beta$-catenin immunofluorescence at the endothelial cell-cell junctions (Fig. 6). The cells treated with the pan caspase inhibitor alone did not show any disruption of the endothelial cell barrier (Fig. 6).

**DISCUSSION**

Preeclampsia is a hypertensive disorder which develops de novo in women after 20 wk of gestation (30, 31). In normal
pregnancy, expansion of the extracellular fluid volume appears to be distributed rather evenly between the intravascular and interstitial compartments (5). In preeclampsia, however, the hematocrit rises above that seen in normal pregnancy. Although the patient is volume expanded, the fluid seems to be redistributed more extensively to the interstitial compartment than is seen in normal pregnancy (5, 18). Accordingly, the preeclamptic patient has been suspected of demonstrating increased vascular permeability or “capillary leak” syndrome. A rat model that has many of the phenotypic characteristics of human preeclampsia has been developed in our laboratory (19). Recently, we demonstrated an alteration in vascular permeability in this model (41). We also reported that MBG is involved in the development of the vascular leakage (41).

To determine the cellular and molecular mechanisms underlying the process of altered vascular permeability, we examined the effect of MBG on RLMEC. We demonstrated that MBG treatment inhibited the proliferative capacity of RLMEC, similar to its effects on CTB cell proliferation (22, 43). We also demonstrated that MBG increased the monolayer permeability of RLMEC. We endeavored to directly examine the intrinsic apoptotic-signaling cascade as a potential contributor to MBG-induced hyperpermeability. Therefore, following exposure to MBG, we blocked caspase activation and demonstrated a marked attenuation of microvascular hyperpermeability (Fig. 2). Hemorrhagic shock following trauma has been shown by several investigators to activate mediators of apoptosis, including caspases (7, 10, 49). In our study, we found that MBG induced RLMEC apoptosis. This was demonstrated by the activation of caspases 3/7, 8, and 9 (Fig. 4, A–C) and also by positive annexin-V staining (Fig. 5). These effects of MBG were similar to those reported previously in CTB cells (42).

MBG-induced downregulation of ERK 1/2 suggests that this cardiotonic steroid leads to a decrease in cell cycle progression.
In ovarian endometrial cyst stromal cells bufalin downregulates the expression of cyclin A, which is important for DNA replication (26). Thus, MBG may inhibit RLMEC proliferation and increase monolayer permeability by modulating ERK1/2 activation, as well as by activation of apoptosis. In a recent study, we demonstrated that MBG-induced impairment of CTB cell function was associated with decreased ERK1/2 activity (43). Furthermore, it has been reported that inhibition of ERK activity is associated with apoptosis and the activation of Jnk and p38 phosphorylation (2, 8). To determine whether MBG-induced apoptosis might contribute to RLMEC dysfunction, we examined the effects of MBG on Jnk and p38 activity in RLMEC. We found that MBG upregulated Jnk and p38 phosphorylation. To examine the involvement of p38 in the activation of apoptosis, we blocked MBG-induced apoptotic signaling with a p38 inhibitor. We demonstrated that a marked attenuation of apoptotic signaling resulted. Jnk inhibition had no such effect. Similar findings were reported by Chuan et al. (8) in CL13 human lung adenocarcinoma cells by treatment with cadmium. Apoptotic signaling cascades eventually lead to a common effector phase of apoptosis characterized by the activation of caspases and p38 or Jnk signaling pathways (2, 8, 35).

Our results show that the disruption of β-catenin/endothelial cell adherens junctions occurs after treatment of cells with MBG (Fig. 6). These results support the view that endothelial cell hyperpermeability occurs because of the activation of caspase-3 and subsequent cleavage of β-catenin resulting in cell detachment. Caspase-3 cleaves a variety of cell adhesion proteins (39). The caspase-3-dependent cleavage of β-catenin occurs during apoptosis (3, 17, 25). Our results also demonstrate that the MBG-induced disruption of β-catenin is attenuated by a pan caspase inhibitor (Fig. 6). These observations are in agreement with previous studies (3, 17, 25). Our results support the involvement of caspases in the cleavage of endothelial cell adherens junction protein β-catenin in the MBG-induced hyperpermeability of RLMEC monolayers (Fig. 6). Previously, Childs et al. (7) have demonstrated that hemorrhagic shock serum induces disruption of endothelial cell adherens junctions.

In Fig. 7 is presented a schematic diagram of the mechanisms involved in MBG-induced RLMEC monolayer hyperpermeability.

**Perspectives and Significance**

Multiple etiologic factors have been proposed in the etiology of preeclampsia (30, 31). We have proposed that MBG plays a role, perhaps a major one, in the development of leak from the vascular tree into the interstitium in preeclampsia (41). To test this hypothesis in vitro, we utilized RLMEC to determine whether alterations in monolayer permeability occurred as the result of treatment of these cells with MBG. We also examined changes in MBG-induced cellular signaling. We found that MBG downregulated phosphorylation of ERK1/2 but upregulated phosphorylation of p38. Furthermore, MBG-induced hyperpermeability of RLMEC monolayers and apoptotic signaling. The latter effect was attenuated by p38, but not Jnk inhibition. In addition, MBG caused disruption of endothelial cell junction integrity, which was attenuated by a pan caspase inhibitor. This communication provides the first evidence that MBG increases vascular permeability in endothelial cells. Furthermore, these data provide confirmatory evidence for the potential importance of MBG as an etiologic factor in the alteration in vascular permeability seen in our rat model of human preeclampsia. These observations may have important implications with regard to the involvement of MBG in the causation of an abnormal vascular leak in preeclampsia and perhaps, other syndromes characterized by “capillary leak.”

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**REFERENCES**

6. Childs EW, Tharakan B, Byrne N, Tinsley JH, Hunter FA, Smythe WR. Angiopoietin-1 inhibits intrinsic apoptotic signaling and vascular...