INVOLVEMENT OF THE BUFODIENOLIDES IN DISORDERS OF INFLAMMATION, BODY VOLUME, TISSUE INJURY, AND ISCHEMIA

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ABSTRACT

The bufodienolides are a group of circulating steroid hormones which, along with the cardenolides, compose the compounds referred to as the "cardiotonic steroids" or "cardiac glycosides." The most extensively studied of the bufodienolides is marinobufogenin (MBG). Recent evidence indicates that at least some of the effects of MBG are mediated by alterations in the MAPK signaling cascade. Previous investigations have largely been directed towards the involvement of the bufodienolides in changes in body volume and hypertension, including volume expansion-mediated essential hypertension, as well as the pregnancy-specific hypertensive disorder, preeclampsia. However, it is now clear that MBG and its antagonist, resibufogenin (RBG), are implicated in disorders characterized by the generation and maintenance of inflammation. Therefore, evidence has been presented in this review which implicates MBG in traumatic brain injury and the acute respiratory distress syndrome.

Furthermore, not only does RBG prevent preeclampsia in a rat model of the disturbance, but it is also effective in ameliorating the abnormalities in traumatic brain injury. Efforts are currently underway to determine if, as is the case in the latter two disorders, it is also effective in modifying the course of the disease in the acute respiratory distress syndrome. Thus, the investigative future seems bright with regard to the potential activity of these and other bufodienolides in medicine.

Key words: *Bufodienolides, preeclampsia, traumatic brain injury, acute respiratory distress syndrome*

BACKGROUND AND OBJECTIVES

The bufodienolides are a group of compounds which are members of a class of agents called the cardiotonic steroids, also referred to as "cardiac glycosides." Their chemical structures are similar to but different from the cardenolides (Figure 1). The cardenolides have a 5-membered lactone ring while the bufodienolides possess a 6-membered ring. Additionally, while there are similarities in their actions, there are also important differences. Both groups of compounds inhibit the ubiquitous enzyme, Na+/K+ ATPase. [1, 2] The bufodienolides act primarily upon the α1 isoform of the enzyme, the major isoform in the kidney which accounts for 90% of the action of the enzyme in that organ. However, the α2 and α3 isoforms represent the major form of the enzyme in skeletal muscle, nervous tissue, and the cardiac conduction system. [10, 33, 45, 46] The bufodienolides are synthesized largely in the adrenal gland, [10] but also in the placenta [22] and perhaps also in the brain. [29]

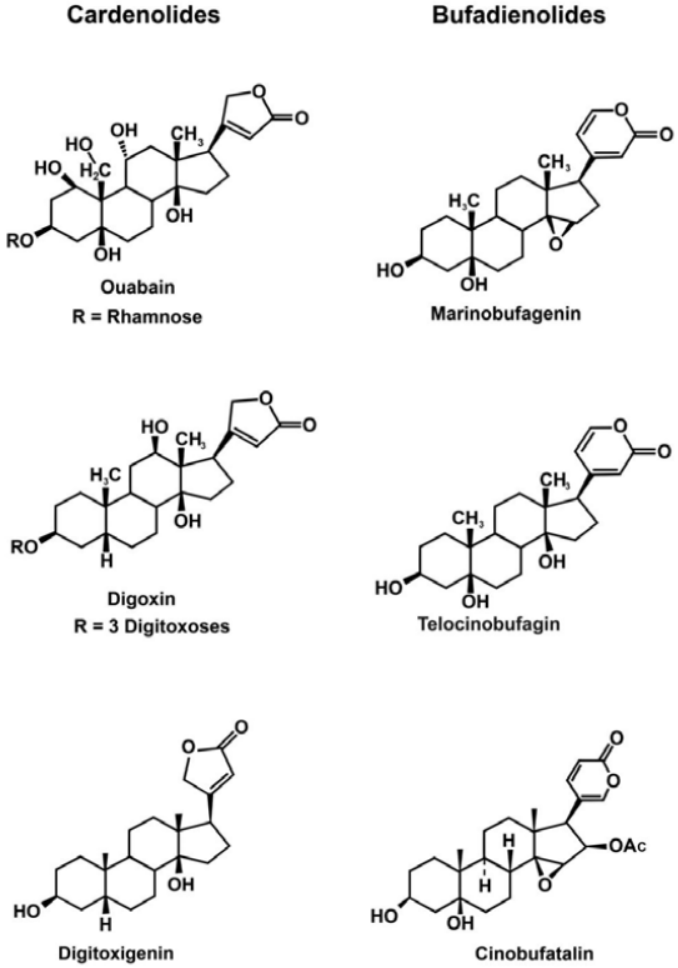

Figure 1 The cardiac glycosides ("cardiotonic steroids"). The cardenolides are shown on the left and the bufodienolides are on the right.

MATERIALS AND METHODS

Preeclampsia

Preeclampsia is a disorder of pregnancy in which the patient develops hypertension and proteinuria after 20 weeks of gestation, accompanied by intrauterine growth restriction (IUGR). Evidence has accumulated which indicates that the most extensively studied of the bufodienolides; marinobufagenin (MBG) is an important factor in the institution and

maintenance of an inflammatory reaction that obtains in the pregnancy-specific disorder, preeclampsia (PE). [1, 2,5,13,14,18,31,4142,58] MBG is elevated early in the course of the development of a syndrome in a rat model [58] which demonstrates many of the characteristics of human PE. [39] The elevated excretion of MBG in the urine of these animals (Figure 3) **precedes** the development of hypertension and proteinuria. Furthermore, an antagonist of MBG called resibufogenin (RBG) (Figure 3) **prevents** the development of the rat syndrome if administered from early in the pregnancy. Not only are the development of hypertension and proteinuria prevented by RBG, but IUGR does not occur [23]. This model of PE, produced by overexpansion of the extracellular fluid volume (ECFV),[23] is also reproduced by the administration of MBG beginning in early pregnancy[57]. The initiation of the PE syndrome has been ascribed to MBG-induced baleful effects on both placentation (Figure 4) and on the failure of cytotrophoblast function. [27,55] Thus, the cytotrophoblasts do not remodel the vasculature of the uterus with the result that blood flow to the maternal-fetal unit is severely compromised. Ischemia of the uterus thereby results in the preeclamptic syndrome. [40] No doubt there are other contributory factors, including but not limited to immunologic and genetic [37] mechanisms, the release of reactive oxygen species, as well as other contributory processes. [36,53] These abnormal influences involve the effects of MBG to upregulate apoptosis of the endothelial cells. [54] These detrimental effects include the activation of the MAPK cascade. [54] In a recent rodent study, the elevation in MBG preceded alterations in vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) or the development of autoantibodies to the AT1 receptor. [3] Furthermore, as mentioned earlier in this discussion, the administration of RBG, the receptor specific antagonist of MBG, prevented these abnormalities, [23,42]. In a recent preliminary report, elevation in the excretion of MBG was noted in 16 of 19 (85%) preeclamptic patients [1]. These detrimental actions of MBG on the development of the fetus and upon blood flow to the maternal-fetal unit also appear to be related to activation of elements of the MAPK system, especially, p38. [54] In both the rodent model described above [56] and in human preeclamptic patients (Table 1) vascular leak is noted. Thus, preeclamptic patients are both excessively volume expanded but also hemoconcentrated [42] (Figure 5).

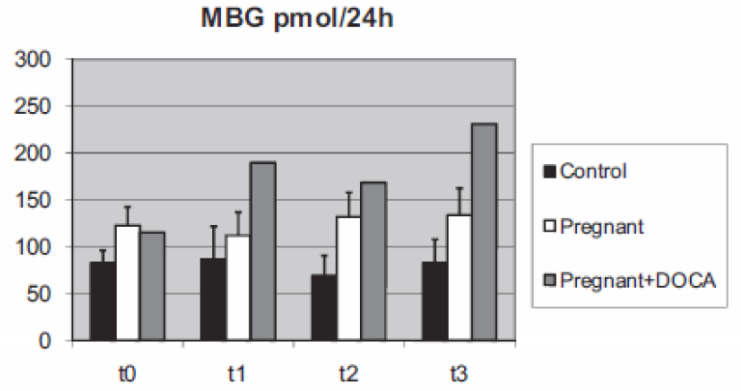

Figure 2 The excretion of marinobufagenin (MBG) in a rat model of preeclampsia (pregnant + DOCA), normal pregnant animals (pregnant), and control, nonpregnant rats (control).Time periods: t0 = baseline, t1 = 3 to 5 days of pregnancy,

t2 = 7 to10 days of pregnancy, and t3 = 18 to 21 days of pregnancy. MBG excretion was already increased at time t1 whereas hypertension and proteinuria had not yet occurred. MBG excretion remained elevated throughout pregnancy in the "preeclamptic" animals. (Reproduced from Vu H, Involvement of marinobufagenin in a rat model of human preeclampsia). Reproduced from the Am J Nephrol 2005;25:520–528 [42] with permission of the editors (S. Karger AG, Basel).

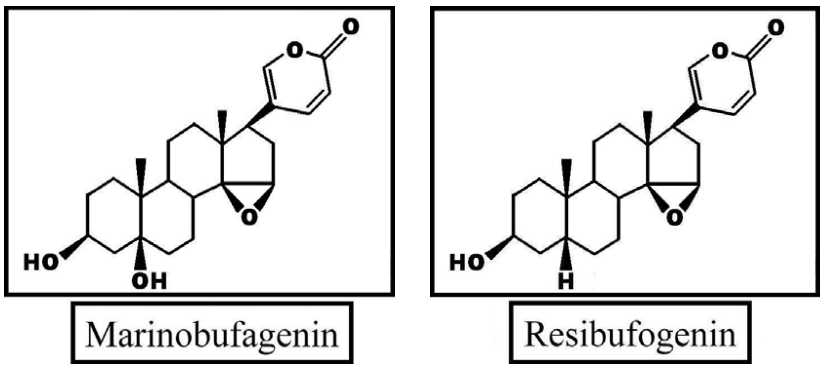

Figure 3 Chemical structures of marinobufagenin and resibufogenin.

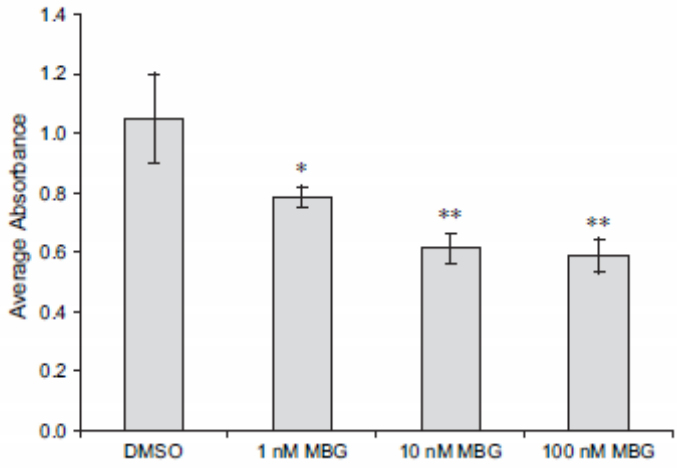

Figure 4a Inhibition of cell proliferation by MBG. Serum-starved SGHPL-4 cells were treated with DMSO (vehicle) or 1, 10 or 100 nM MBG in the presence of 10% FBS for 48 h at 37° C and cell proliferation was measured using the Cell Titer96 Aqueous Assay. Cell proliferation was significantly inhibited in MBG treated cells as compared to DMSO-treated groups (*$p < 0.05$, **$p < 0.001$). The mean was calculated from the average of 8 replicates per experimental condition and the results presented are the mean +/- SE from a representative experiment. The experiment was performed a total of 3 times. The human extravillous cytotrophoblast (CTB) cell line SGHPL-4 utilized in these studies was derived from first trimester extravillous tissue and was kindly provided by Dr. Guy Whitley, St. George's Hospital Medical School, London, U.K. These cells are well characterized and share many characteristics with isolated primary cells, including the expression of cytokeratin-7, HLA class I antigen, HLA-G, BC-1, CD-9, human chorionic gonadotropin and human placental lactogen.

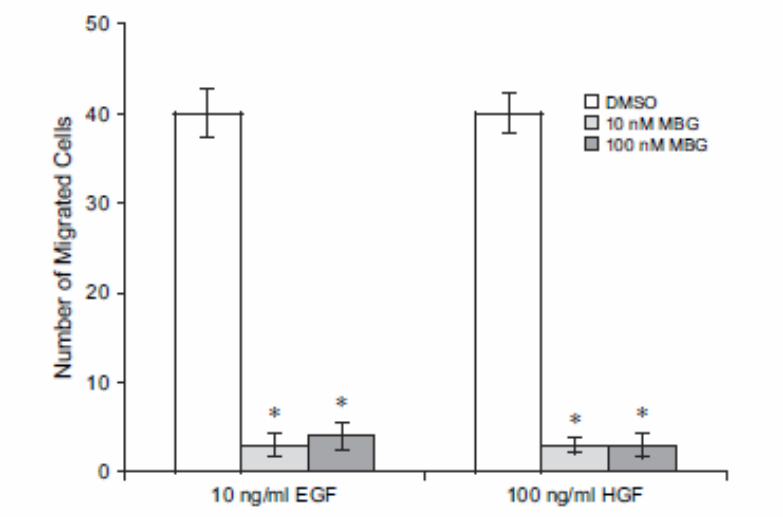

Figure 4b Inhibition of growth factor-induced chemotaxis by MBG. Serum starved SGHPL-4 cells were pretreated with DMSO, or 10 or 100 nM MBG for 2 h and subsequently added to the Boyden chamber that contained 10 ng/ml of EGF or 100 ng/ml of HGF. The chamber was incubated at 37° C to allow for cell migration, the cells that migrated through the porous membrane were stained, and the average number of migrating cells was calculated by counting stained cells from 6 fields of view (400 xs) per experimental condition. As expected, EGF and HGF stimulated CTB migration of DMSO (vehicle)-treated cells. However, MBG treatment significantly inhibited growth factor-induced migration of SGHPL-4 cells when compared to control cells ($p < 0.001$). The results presented are the mean +/- SE from a representative experiment and the experiment was performed a total of 4 times.

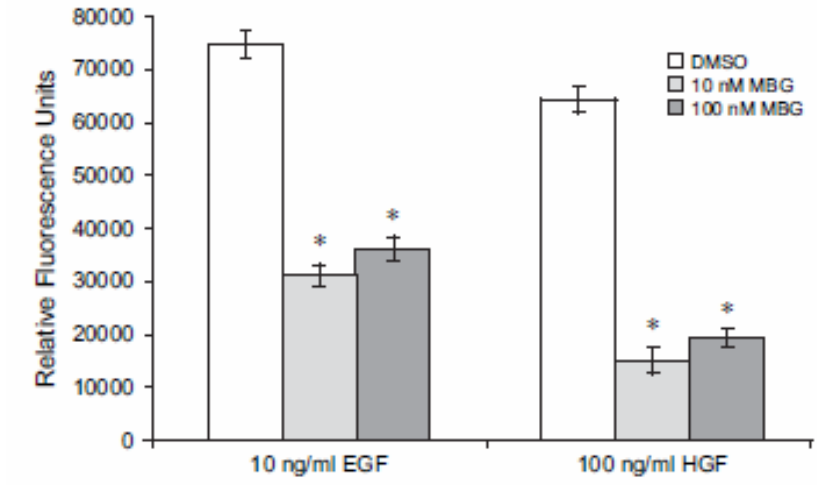

Figure 4c Inhibition of CTB invasion by MBG. SGHPL-4 cells were serum starved for 24 h and subsequently pretreated with DMSO (vehicle) or 10 or 100 nM MBG for 2 h at 37° C. While both EGF and HGF induced CTB invasion of DMSO treated control cells, MBG treatment significantly inhibited growth factor-induced CTB invasion ($p < 0.001$). The results presented are the mean +/- SE from a representative experiment and the mean was calculated from the average of 4 replicates per

experimental condition. The experiment was performed a total of 3 times. Reproduced with permission of the editors from LaMarca, H.L., et al, Marinobufagenin impairs first trimester cytotrophoblast differentiation, Placenta 27: 984-988, 2006

GROUP	AVERAGE HEMATOCRIT VALUES
Nonpregnant	42%
Normal pregnant	35%
Preeclamptic	39%

Table 1a Representative values of hematocrit in nonpregnant, normal pregnant and preeclamptic women

GROUP	AVERAGE HEMATOCRIT VALUES
Nonpregnant	44% †
Normal pregnant	34%
"Preeclamptic"	38%*

Table 1b Hematocrit values in experimental rat groups
† P< 0.05 vs. nonpregnant and normal pregnant
* P < 0.05 vs. normal pregnant and "preeclamptic"

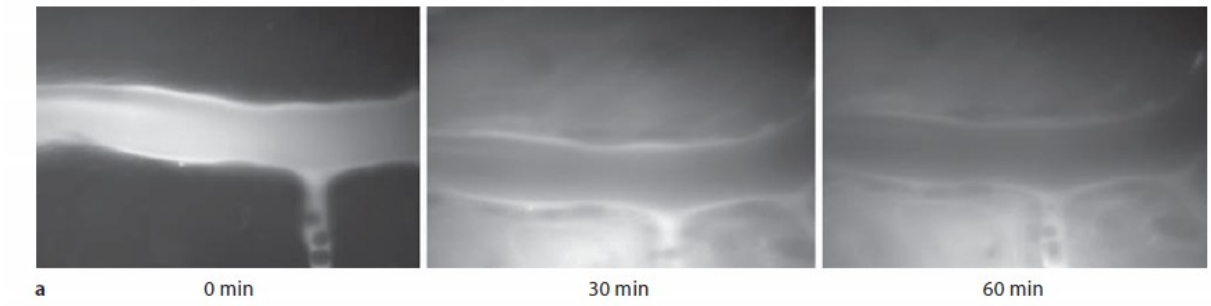

Figure 5a Representative study demonstrating the effect of MBG on vascular leakage in a single rat mesentery post capillary venule. Images shown were obtained prior to the injection (0 min) and at 30 and 60 min after the bolus injection of 200 nM MBG. FITC albumin extravasation into the extravascular space is virtually complete by 60 min after MBG injection. [6]

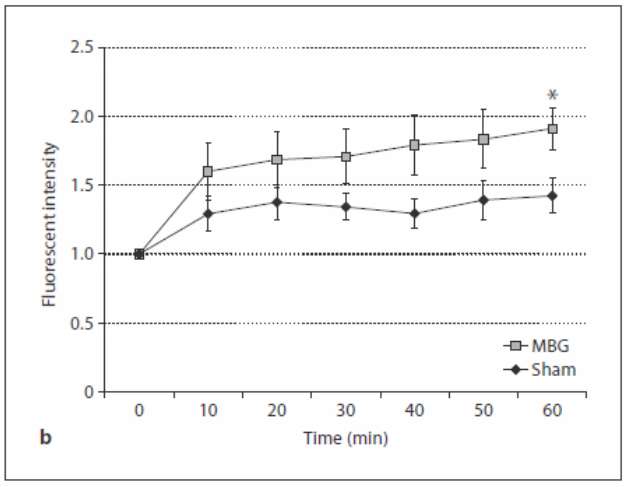

Figure 5b: Mean values for the effects of MBG on vascular leakage in mesenteric post capillary venules compared to rats infused with the vehicle (DMSO) only

(sham) at 10, 20, 30, 40, 50 and 60 min of observation. Vascular leakage is expressed as the change in fluorescent intensity inside the vessel compared to that outside the vessel. * $p < 0.05$; $n = 5$ for sham and $n = 7$ for MBG. (Data from references 54,56).

Mechanisms of action of MBG in preeclampsia

The sequence of events in the development of preeclampsia based upon the concepts related to the involvement of MBG are provided in figure 6. We hypothesize [43] that the following sequence of events occurs: 1) patients who develop PE have either an acquired or genetic [37] sodium transport tubular defect which manifests itself as an inability to excrete excess amounts of sodium. This defect is not exhibited or troublesome for the patient until and unless the dramatic increase in body volume related to sodium and water excess that occurs in pregnancy (Figure 7) causes this defect to become manifest. In a study performed in normal pregnant patients, patients with gestational hypertension and those with preeclampsia, this hypothesis was tested. The infusion of hypertonic saline to patients in these three categories demonstrated that preeclamptic patients were substantially more sodium-retentive than those in the other two groups. [7-9]

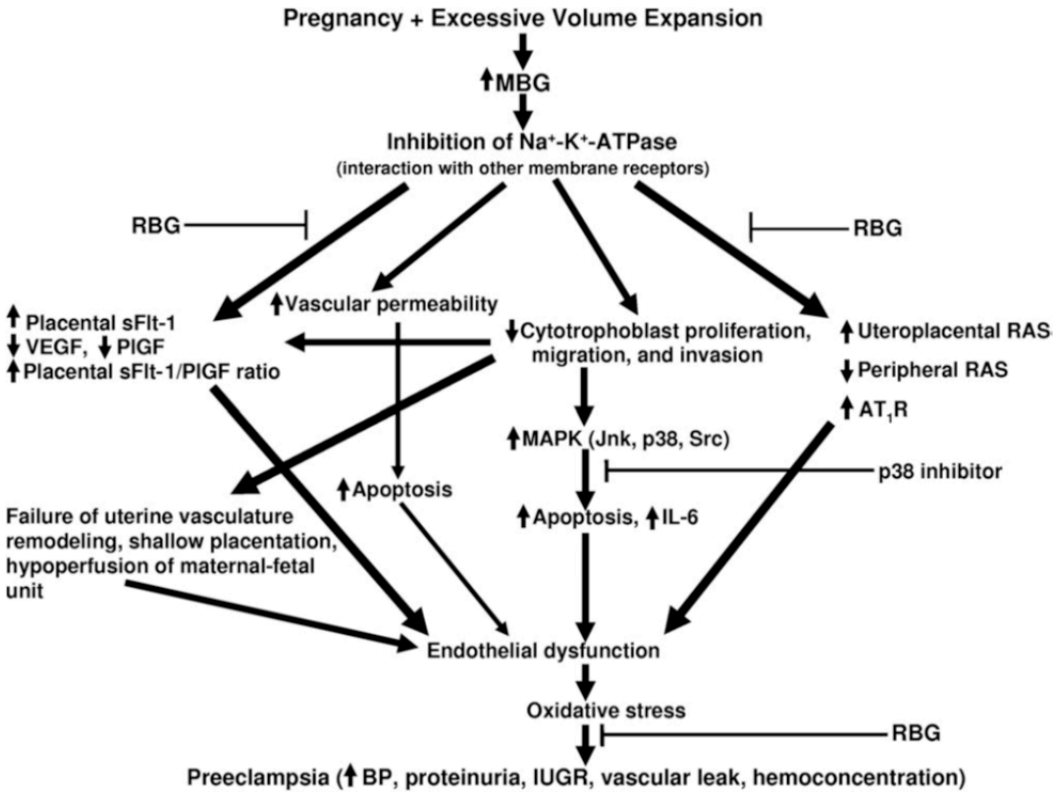

Figure 6 Proposed model of the involvement of increased marinobufagenin (MBG) levels in preeclampsia related to initial excessive volume expansion early in pregnancy. MBG causes defective placentation by interfering with cytotrophoblast function, resulting in a lack of vascular remodeling in the uterus. Consequently, hypoperfusion of the maternal-fetal unit eventuates. MBG also causes hypoxia and ischemia leading to an imbalance of pro- and antiangiogenic factors. Additionally, the bufodienolide causes increased vascular permeability

resulting in "leak" from the intravascular compartment and consequent hemoconcentration. Its actions also result in enhancement of the uteroplacental renin-angiotensin system (RAS) and angiotensin II type 1 (AT1) receptor function. The cytotrophoblast dysfunction is mediated by alterations in the MAPK (mitogen-activated protein kinase) system, which stimulates apoptosis causing disruption of endothelial cell layers. All of these abnormalities culminate in the production of endothelial cell dysfunction and oxidative stress. The actions of MBG can be prevented/ameliorated by resibufogenin (RBG). Inhibition of the activity of the MAPK p38 prevents MBG-induced enhancement of apoptosis. Abbreviations and definitions: BP, blood pressure; IL-6, interleukin 6; Na-K-ATPase, adenosine triphosphatase sodium-potassium pump; IUGR, intrauterine growth restriction; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor. From: Am J Kidney Disease, 56:359-370, (2010). Reproduced with permission of the editor.

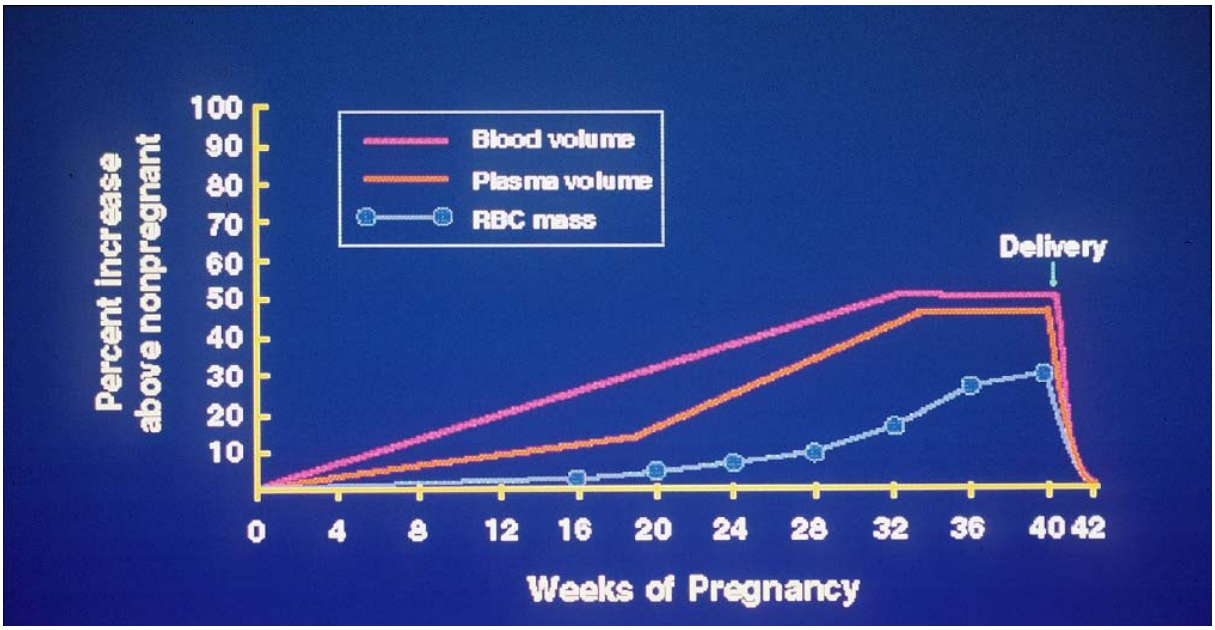

Figure 7 Alterations in blood and plasma volume and red cell mass during pregnancy. Reproduced from: Scott, D.E., Obstet Gynecol Annu, 1: 219, 1972, with permission of the editors.

Additionally, the fact that alterations in MBG secretion and release are sensitive to changes in body volume has been demonstrated previously. This is the case both in the situations of sodium surfeit as induced by volume expansion and in volume contraction related to sodium restriction. These alterations have been demonstrated in both human and animal experimentation. [1,2,7,8,9,25,43,57,58,60] Additionally, elevated levels of MBG result in toxic effects involving the function of the cytotrophoblasts, resulting in interference with normal placentation. [27,55,58] Studied i*n vitro,* MBG interfered with CTB proliferation, migration and invasion (Figure 4). [27,55] Thus, the normal process whereby the CTBs remodel the vasculature of the uterus is compromised resulting in narrowed vasculature. [58]

In addition to this interference in the process of placentation, MBG causes ischemia of the maternal fetal unit resulting from failure of the uterine vasculature to

become remodeled. Thus, the ischemia, itself, results in an enhancement of the production of MBG, causing a reinforcement of the ischemic process and perpetuating and reinforcing this untoward process. [58] Endothelial dysfunction, in turn, produces oxidative stress, [36] a process which is prevented by the administration of RBG in the rat model of PE. [53] Finally, MBG causes endothelial hyperpermeability by altering apoptotic signaling perpetuating vascular leak and adding to the ischemic insult. [56] This process results in injury to the "tight junctions" of the endothelial membrane. [24] In the latter studies, MBG down-regulated genes that are involved in membrane adhesion [24]. Interestingly, elevated levels of MBG in ischemia related to renal artery stenosis have recently been reported by Tian, et al. [52]

Traumatic Brain Injury

According to a recent report in the Journal of the American Medical Association (May 14, 2014), between 2006 and 2010 the total number of visits to emergency departments in a nationwide sample of hospitals grew by 3.6 percent. However, during that same period, the number of visits by patients seeking treatment for a traumatic brain injury (TBI) grew by 29.1 percent. It has been suggested that at least part of the increment in TBI visits has to do with the wide publicity related to high profile injuries to NFL players. Recent reports have emphasized that although brain scans in civilian patients with TBI are generally inconclusive in determining the degree of injury, a recent study has focused on the volume of the hippocampus as a potentially important finding. [48] The advent of multiple reports and investigations of military head trauma have also led to increased emphasis on the diagnosis, prognosis and management of brain injury. [30] The employment of the technique of diffusion tensor imaging to evaluate the severity of cerebral edema has also been postulated as a potential mechanism to evaluate compartmental brain edema to determine the degree of injury. [26] Interestingly, evaluation of the future course of brain trauma victims utilizing physical examination, imaging and laboratory studies often does not coincide with tests of neurocognitive function. [49] Consensus for the diagnosis and treatment of concussion in sports based on recommendations of three separate groups of experts has been published. [59] It includes discussions by: 1) the American Medical Society for Sports Medicine, [19] 2) the American Academy of Neurology, and 3) the Zurich Concussion Working Group. [35] A comparison and discussion of the similarities has been published along with areas identified by each group that remain uncertain. Causes of inaccurate early assessment of neurologic severity in head injury have also been reported [50]. Attempts have recently been made to develop reliable biomarkers that might have predictive properties as well as the ability to guide therapeutic effort. [4,28,51] One of the mechanisms by which brain function is altered by head trauma is the breach of the blood/brain barrier (BBB). This event may lead either to general or localized brain edema. [20,21] Because of their finding that MBG may cause an increase in the permeability of the endothelial membrane, resulting in vascular leak, [24,53,56] the author and his colleagues determined that breach of the BBB might play an important role in the edema of TBI. Furthermore, we reasoned that MBG could play an important role in the generation of this membrane abnormality. Accordingly, we

studied MBG excretion in a rat model of mild-moderate TBI. We utilized a model developed by Marmarou and his colleagues called Impact Accelerated Injury (IAI). [17,47] The latter method involves the following procedures: a circular incision is made in the skull, a metal plate is placed into the defect resulting from the removal of the portion of the skull excised, and the dura and skin are replaced over the deficit. A weight is then dropped on the skull from a pre-specified height in the area of the metal plate. Utilizing this model and following the imposition of the brain contusion, the animals were allowed to recover from the anesthesia. They were then placed in metabolic cages and a 24-hour urine was obtained for the determination of MBG (TBI group). In a second group of animals (shams) surgery was performed on the skull but no brain contusion was performed. In a third group, one hour after the brain trauma, a bolus of RBG was injected intraperitoneally and a 24-hour urine was then obtained (TBI plus RBG). The results of these studies on MBG excretion and brain histology are provided in table 2 and figure 8, respectively. [47] The experiments revealed the following: 1) MBG was elevated in the animals which were recipients of the brain contusion. 2) The administration of RBG resulted in a return of the MBG excretion values to those obtained in the sham animals (Table 2). 3) The brain contusion resulted in abnormalities in the histology demonstrated in the examination of the brain slices (Figure 8). Studes are currently underway in the author's laboratory, in conjuction with colleagues in neurosurgery, neuroscience and radiology to examine this issue in patients with mild TBI. Initial preliminary results (Puschett, J.B. et al, unpublished observations) indicate that MBG is elevated in human brain trauma as was the case in the animal model. [47]

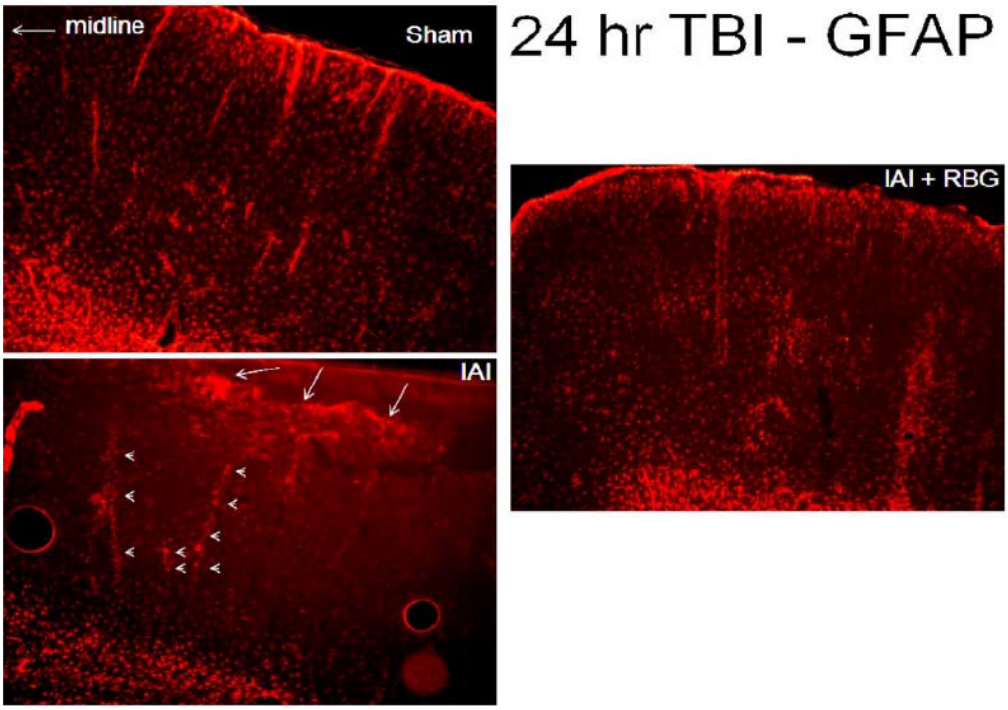

Figure 8 Low and high magnification micrographs of GFAP (glial fibrillary acidic protein) immuno-fluorescence captured using a laser-scanning confocal microscope. GFAP antibody labels the intermediate filaments of most astrocytes

in the brain. These micrographs depict areas of cortex slightly lateral from the midline. The pial surface is on the top of the images and midline is to the left of all images. In the sham animals (top left figure), a normal distribution of GFAP-labeled astrocytes is seen, including their endfeet which surround the neurovasculature. At 24 hours after an impact acceleration injury (IAI) (bottom left figure), a noticeable scar is observed (arrows) in layers 2 and 3 of cortex. There is also a patchy loss of GFAP-labeled astrocytes throughout the cortex and the vasculature (arrowheads) appears damaged. It should be noted that many of the remaining astrocytes appear hypertrophied. The loss of GFAP-immunoreactivity and the appearance of astrocyte hypertrophy are indicative of a neuroinflammatory response to injury. As can be seen in the third image (the figure on the right), most of these alterations are ameliorated if the animals are treated with resibufogenin (RBG) at 1 hour after IAI (IAI+RBG). A glial scar was not observed in the IAI+RBG animals. The RBG treatment also reduces the decrease in GFAP immunoreactivity and a greater proportion of GFAP-labeled astrocytes have a normal, rather than hypertrophied appearance. (Data from Shapiro, L., et al. [47])

Group	Sham	TBI	TBI + RBG
n	10	10	10
values	592.8 ± 79.3	1340.9 ± 308.5	475.3 ± 56.1

Table 2
Urinary marinobufagenin (MBG) excretion in sham and traumatic brain injured (TBI)* animals

P values:

sham vs. TBI = 0.04 TBI vs. TBI + RBG = 0.02 sham vs. TBI + RBG = > 0.05

**Abbreviations*:*

sham = sham operated animals; TBI = rats subjected to impact acceleration injury; RBG = resibufogenin; n = number of animals in each group.

Values are reported as mean ± SE in pg/mg creatinine.

Acute Respiratory Distress Syndrome (ARDS)

The acute respiratory distress syndrome (ARDS) frequently complicates serious illnesses. The latter may include sepsis, trauma, shock and severe pneumonia as well as other disturbances. The syndrome consists in the development of vascular leak in the lungs with the result that gas exchange is severely compromised. Both hypoxia and hypercapnea, often severe, are commonly present and the advent of this complication leads to mortality in from 40-52 percent of ARDS patients. [38] Beyond the original lung damage leading to this condition, it has been determined that repetitive overstretching or collapse of the lung with mechanical ventilation can itself cause local tissue injury and systemic inflammation, both of which contribute to multi organ failure and death. [15] High-frequency, low volume oscillatory

ventilation does reduce secondary lung damage but mortality is not improved. [32,33,61] Additionally, survivors of ARDS face the possibility of lung complications once they leave the ICU and hospital.

Given the fact that vascular leak of the lung endothelial membrane is a defining abnormality in ARDS, the author and his colleagues are studying two aspects of this disorder: 1) Is MBG involved in the pathogenesis of the lung lesion?, and 2) If so, could the damage be ameliorated by administering RBG either as a prophylactic method or at least early in the disorder, once ARDS is clinically evident? Preliminary studies have been performed in collaboration with physicians at the Memorial Hermann Hospital (UT Health) in Houston, Texas, in ICU patients who have been diagnosed with ARDS according to the Berlin criteria. [16,44] In the initial sample, 84 percent of 19 patients with ARDS were determined to have elevated levels of MBG when compared with non-ARDS ICU patients. [11] An investigation is also underway currently in an animal model of ARDS to assess the hypotheses that not only is MBG elevated in the syndrome, but also that RBG can successfully ameliorate, prevent and/or treat this disorder.

RESULTS AND DISCUSSION

In this review, data implicating the involvement of the bufodienolide, MBG, in the etiology of three important disturbances: preeclampsia, traumatic brain injury, and the acute respiratory distress syndrome, have been summarized and presented. In addition, instances in which the antagonist of MBG, RBG, may have value in either the prevention and/or treatment of these disorders has been provided. Should additional studies prove to verify these data, it would become possible not only to utilize MBG as a diagnostic biomarker, but to enable the use of its antagonist, RBG, as either a preventative or treatment technique. In none of the syndromes discussed is there currently an **early** diagnostic aid, and therefore, no successful preventative or therapeutic technique. Thus, MBG and RBG may represent the first useful diagnostic/therapeutic match, representing an example of "personalized medicine." Until efforts of the kind outlined in this review are available, all three of these illnesses will remain examples of substantial unmet medical need.

ACKNOWLEDGMENTS

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