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EC CARDIOLOGY

Review Article

HDFx and Magnesium in Combination Ameliorates Experimental Pulmonary Hypertension: Relevance to Treatment of Pulmonary Hypertension in Humans and Newborns and the Roles of Hypomagnesemia, Ceramides and PAF

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Received: December 16, 2019; **Published:** January 09, 2020

Abstract

Pulmonary arterial hypertension (PAH) not only causes major problems for the lungs, but the heart as well. In newborns, this often produces a syndrome of persistent pulmonary hypertension (PPHN) with mortalities approaching 80%. In order to better understand and treat PAH and PPHN, animal models have been employed for more than 50 years, yielding very important data and some new therapeutic approaches. Our group has been studying these diseases using monocrotaline (MCT)-induced PAH for more than 30 years. During these years, we have discovered a brand- new host-defense factor, HDFx, which displays remarkable anti-inflammatory properties along with the ability to accelerate wound healing. Numerous investigators have utilized magnesium (Mg) salts in the treatment of experimental PAH and PPHN. In this review, we present new findings on the successful use of combined therapy of HDFx and Mg in the treatment and prevention of MCT-induced PAH. We discuss our new findings on why hypomagnesemia, ceramides and platelet-activating factor (PAF) probably play important roles in the initiation of both experimental MCT-induced PAH and PPHN. We also review a number of studies which demonstrate some of the underlying mechanisms whereby this combined therapy is protective in MCT -induced PAH. We conclude that this new combined therapy might be successfully employed to treat clinical PAH and PPHN in newborns.

Keywords: *Monocrotaline; NF-kB; Proto-Oncogenes; Ceramide; Platelet-Activating Factor (PAF); Sphingolipids, Calcium*

Introduction

Pulmonary arterial hypertension (PAH) is considered a very deadly disease of the small blood vessels in the lung. In the youth, many such individuals presenting with PAH often die before their 18th birthdays [1,2]. PAH is characterized by mean pulmonary artery pressures of 25 mm Hg or greater. PAH has been classified into at least five different groups of patients on the basis of hemodynamic and clinical grounds [3]: group 1 (especially the idiopathic group-IPAH) is often the target of most clinical pathophysiological and clinical investigations. IPAH includes hereditary categories which often is found, genetically, in families and is characterized by a progressive pulmonary arteriopathy resulting in right-sided heart failure and death shortly after its diagnosis (i.e. usually within three years if untreated) [4]. In newborns, there is a syndrome of persistent pulmonary hypertension (PPHN) that is characterized by an increased pulmonary vascular

resistance, pulmonary arterial vasoconstriction, right-to-left shunt and severe hypoxemia without any good evidence of congenital heart disease. All forms of PAH found clinically are characterized by serious inflammatory reactions of the small blood vessels. PPHN is a very deadly disease in newborns, with mortalities often approaching 80%.

In order to better understand the pathophysiological origin of PAH and PPHN and to develop new drug treatments for the diseases, several animal models have been utilized, the most common being that developed in rats using seeds from the plant *Crotalaria spectabilis* which was originally discovered by Lalich and Ehrhart in 1962 [5] and later employed by Kay and Heath who first devised a method to measure right ventricular systolic pressure (RVSP) in rats [6]. Kay demonstrated that *C. spectabilis* seeds raised right ventricular systolic pressures from 62 to 112 mm Hg in rats administered MCT compared to control rat pressures of 22 to36 mm Hg. Kay and his co-workers also noted that several of the pulmonary arteries of many of the treated rats showed increases in pulmonary arterial medial thickness [7]. More recent studies using these monocrotaline (MCT) animal models demonstrate endothelial cell damage, in situ thrombosis, pulmonary edema, release of numerous cytokines and chemokines into the blood stream, and numerous types of inflammatory cells (mainly neutrophils, dendritic cells, macrophages, and lymphocytes) which infiltrate the lungs, mainly in the perivascular tissue areas [8,9]. Within weeks of these pathological events, the MCT-treated rats will die of progressive PAH. Although numerous investigators have intensely studied these pathologic events, the precise mechanisms of MCT PAH remain poorly understood [10]. Despite MCT not being identical to IPAH, many investigators feel this model in rats can serve as an *in vivo* model of severe PAH [11].

Several years ago, our laboratories found when MCT-treated rats were given daily(oral) doses of magnesium (Mg), PAH was ameliorated and RVSPs as well as right ventricular hypertrophy (RVH) were greatly reduced, and pulmonary arterial increases in medial thickness were diminished [12-14]; lung pathological alterations were also ameliorated. We also noted that infiltration of neutrophils, dendritic cells, macrophages, and lymphocytes into the perivascular spaces were attenuated markedly along with diminished release of cytokines and chemokines [unpublished findings]. Although the "cytokine storms" noted in MCT-induced PAH in rats are not identical to that seen in PAH, PPHN or IPAH in humans, the biochemical findings of the release of diverse cytokines and chemokines in MCT -induced PAH could provide potentially useful information if these pathological processes could be inhibited or ameliorated with new drugs or "biologics". It should be noted, here, that, to our, knowledge, none of the current therapies for either PAH or PPHN have been designed to prevent the 'cytokine storms" seen in these syndromes. In this vein, our laboratories have discovered a new anti-inflammatory biologic in rats, mice, rabbits, guinea-pigs, dogs, and subhuman primates that has multiple, unique characteristics which we have termed "HDFx" [15-17].

Unique characteristics of HDFx

Approximately 135 years ago, Elie Metchnikoff, the father of immunology, hypothesized that the body, under stressful conditions, would manufacture/release molecules that could stimulate various arms of the immune system and protect the host against major injuries, insults, and diseases [18]. Metchnikoff's early studies pointed to the importance of macrophages and phagocytic leukocytes to natural (later termed innate) resistance against pathogenic bacteria and viruses. Over the past 30 - 40 years, a vast body of information and studies have demonstrated a strong relationship between the functional (physiological) state of the microcirculation, macrophages and natural killer (NK) cells to host-defense and resistance to pathogens, trauma, circulatory shock, hemorrhages, infections and injuries of various types, and sepsis [15-17,19-26].

After thousands of experiments on rats, mice, and many other species of lab animals, we found that "HDFx" is protective (to varying degrees) against a variety of systemic bodily insults, ranging from hemorrhage, trauma, combined injuries, endotoxins, a variety of lethal bacteria (i.e. *E. coli, S. enteritidis, C. welchii,* among others), fungi (i.e. *A. fumigatus, C. albicans)* and centripetal forces, to septic shock [15-17,27-31]. More recent studies suggest that "HDFx" may be protective against certain hemorrhagic fever viruses, drug-resistant TB, sarcoidosis, and several flu virus strains [30-35]. A very unique attribute of "HDFx" is its ability to accelerate wound healing [17] and to protect against or ameliorate "cytokine storms" in animals that are made septic or administered several different gram-negative endotoxins [33,34]. "Cytokine storms" are readily observed in MCT-induced PAH and human PAH [10,36-38].

Mg and HDFx therapy and cytokine storms: Potential relationship to PPHN in newborns

Our recent studies seem to indicate that release of cytokines (e.g. TNF-alpha, IL-1a, IL-2, among others) and chemokines (e.g. various macrophage factors) in rats administered MCT are reduced markedly by pretreatment of these animals with "HDFx" [38]. When magnesium was added to the administration of "HDFx", there was a potentiation of the inhibition of release of cytokines and chemokines as well as an inhibition of the release and cellular entry of Ca^{2+} in rats given MCT [39,40]. We also noticed a marked reduction in the sticking of monocytes. Neutrophils and macrophages to the endothelial cell walls. To our knowledge, no other combined therapies can produce such unique actions against cytokine/chemokine release and subsequent inflammatory reactions in MCT-treated rats. Moreover, the combined therapy of Mg and "HDFx" drastically reduced the MCT-induced increases in pulmonary arterial blood pressures, RVH, RVSPs, pulmonary arterial medial thicknesses, infiltration of inflammatory cells across the endothelial cell walls, in situ thromboses, and PAH [39,40]. In addition, we found that measurement of serum ionized Mg levels found in untreated MCT-induced PAH was drastically reduced with the combined therapy [39,40].

It is of some interest to note here that, clinically, therapy with Mg in human PAH and PPHN has been shown to improve the downward pathological course of events in PAH and PPHN patients [1,41-47]. In addition, using PAH patients, our group was the first to report that a number of these patients exhibit deficits in blood levels of ionized, but not total, Mg levels [48,49], suggesting that Mg errors in metabolism may be playing an important role in the pathophysiology of PAH and IPAH. When studying newborn babies, our group found that all the normal infants (with normal APGAR scores) started out, early after birth, with elevated serum ionized Mg levels; these blood levels, over a period of weeks, gradually, came down towards those levels found in adult children [50,51].

It is of interest to point out, here, that there are three forms of circulating (blood) Mg: ionized, total, and complexed. In this context, we found that the complexed Mg levels of Mg in the newborns were also vastly different from those found several weeks and months later; i.e. the concentrations of anions in the circulating newborn bloods exhibited significantly different levels of phosphates, acetates, etc. [unpublished findings]. However, when studying infants with either brain injury or PPHN, the serum ionized levels of Mg, early after birth, were lowered significantly when compared to normal infant births [51].

Using MCT-treated rats, we have also noted deficits in serum levels of ionized, but not total, Mg [39]. It is important to point out, here, that physiologically, ionized Mg is the biochemically-active Mg [52]. What is the mechanism (s) of protection with "HDFx" and Mg?

Insights into the mechanism (s) of protection against MCT induced by HDFx and Mg: Potential roles and cross-talk of NF-kB, and calcium in vascular remodeling

Next to potassium, Mg is the second most abundant intracellular cation and the fourth most abundant cation in the body. Mg is a co-factor for more than 500 cellular enzymes involved in cellular energy production, membrane functions such as hormone-receptor bindings, gating of Ca^{2+} channels, transmembrane fluxes of ions, regulation of adenylyl cyclases, numerous cell structural functions, excitation-contraction coupling of all types of muscle cells, stabilization of cell membranes, regulation of cell growth processes, regulation of cardiac and smooth muscle tone, neurotransmitter release, and metabolism of DNA, RNA, proteins, carbohydrates, and lipids [53-56]. Mg also plays important roles in programmed cell death processes (i.e. apoptosis, necroptosis, ferroptosis, etc.) [57-62]. Mg plays a pivotal role in control of neuronal activity, cardiac excitability and stability, neuromuscular transmission, vasomotor tone, blood flow and blood pressure [53-56]. A number of these characteristics appear to be, mechanistically, involved in the protective actions of Mg and the changes in pulmonary arterial remodeling that is seen in MCT-induced PAH [12-14,33,35,63]. As of today, hypomagnesemia has been reported to be as high as 65% in adult intensive care patients [61,64-66] and is the most deficient serum cation in newborns [67]. Mg sulfate has been extensively employed in premature human neonates with PPHN [42-47]. This is based on the fact that Mg is a good pulmonary arterial vasodilator, as it is on the peripheral microcirculation [45,67-70]. In addition, due to our reporting, almost 40 years ago, that a number of vasodilators, such as acetylcholine, bradykinin, and ATP relax pulmonary arterial blood vessels via the generation of nitric oxide (NO)

[71,72], which we found has an obligatory requirement for Mg^{2+} [73], this has served as the basis for utilization of inhaled NO and Mg sulfate in treatment of PPHN and PAH.

NF-kB is now known to be a prime regulator of growth processes, differentiation, cell migration, and cell death [74,75], all factors required for vascular remodeling in hypertension, PAH, and atherogenesis [76,77]. NF-kB is clearly a major transcription factor and a pleiotropic regulator of numerous genes involved in inflammatory processes and epigenetic phenomena [76,77]. NF-kB is currently thought to be a pivotal molecule in atherogenesis, hypertension, cardiac failure and stroke, thus being critical in vascular remodeling processes [78,79]. As of today, it is still not clear as to what factor(s) initiates expression of these molecular events. Our laboratory was the first to suggest and provide evidence for activation of NF-kB in the cardiovascular manifestations, particularly in atherogenesis and vascular remodeling, noted in Mg deficiency [60,61,78-80]. More recently, we have found evidence that both pulmonary arterial vascular smooth muscle cells (VSMC) and endothelial cells of rats given MCT demonstrate significant activation of NF-kB [unpublished studies]. We have also demonstrated that when VSMC were exposed to low concentrations of extracellular, ionized Mg ($[Mg^{2+}]_0$), a concentration-dependent upregulation of NF-kB took place; the lower the $[Mg^{2+}]_0$, the faster and greater the upregulation of NF-kB [unpublished studies]. A similar situation is seen in pulmonary endothelial cells [unpublished studies]. In addition, we have noted that monocrotaline- induced PAH in rats resulted in increased entry of calcium into the pulmonary arterial VSMc and endothelial cells as well as release of Ca^{2+} from intracellular stores, whereas oral administration of Mg ameliorated greatly the elevation and cellular entry of calcium [40]. Very recently, working with MCT-induced PAH in mice, Li., *et al.* reported that several inhibitors of NF-kB prevented PAH and RVH [81].

Probable roles of synthesis/release of ceramides, PAF and proto-oncogenes in PAH-induced vascular remodeling

Approximately 20 years ago, we reported that lowering $[Mg^{2+}]_0$ in isolated VSMc and endothelial cells results in a rapid synthesis of ceramides and platelet-activating factor (PAF) [82,83] concomitant with an upregulation of five major sphingolipid enzymatic pathways responsible for formation of ceramide and sphingosine [82-87], prior to NF-kB upregulation [79,80,85-87]. In this context, we have found that pulmonary VSMC and endothelial cells obtained from rats given MCT (that developed PAH), demonstrate upregulated sphingolipid enzymes, as well as elevated levels of ceramide and PAF [unpublished findings]. Recently, Petrache., *et al.* found that hypoxia, cigarette-smoking, and endothelial mitochondrial lung damage produced increases in ceramide levels in the lung [88].

It should be recalled, here, that both ceramide and sphingosine are thought to play important roles in fundamental pathophysiological processes such as cell proliferation, membrane-receptor functions, angiogenesis, atherogenesis, immune inflammatory responses, cell adhesion and programmed cell death [89-92], all processes that take place in MCT-induced PAH. PAF is now known to play fundamental roles in regulation of the microcirculation, inflammation, cell adhesion, atherogenesis, cardiac regulation, and programmed cell death [77,85,93-96]. Interestingly, about 20 years ago, Caplan and colleagues found elevated levels of PAF in 13 newborns with PPHN which correlated with the severity of the disease [97]. As the clinical status of the newborns improved, the levels of PAF decreased.

It is our belief that MCT -induced PAH is dependent on production of a Mg deficiency and synthesis/release of sphingolipids and PAF. However, it is important to point out, here, that vascular remodeling, as takes place in MCT-induced-PAH is dependent on other key -signaling molecules such as proto-oncogenes [38-40]. Interestingly, we have found that the principal proto-oncogene families, c-fos and c-jun, which are key molecules in regulation of cell growth, differentiation, cell migration, and cell death, all important factors in vascular remodeling, which takes place in MCT-induced PAH, have been found by our group to be upregulated in Mg deficiency [78,86,87] and in pulmonary arterial smooth muscle as well as in endothelial cells of rats administered MCT [39,40].

In very recent experiments, we found that the use of several different inhibitors of PAF-receptors attenuated, greatly, the MCT -induced vascular remodeling normally seen in rats given MCT [98].

In other recent experiments, we have found that pretreatment of rats, given MCT, with HDFx exhibited, markedly- reduced inflammatory responses in the lungs (e.g. reduced release of cytokines and chemokines; reduced infiltration of lung tissue by leukocytes, macrophages, and dendritic cells; reduced thromboses; and reduced PAH) [38-40]. HDFx has an uncanny-ability to maintain vascular tone in all types of vascular beds under stressful bodily insults [15,16,28,32,34], including the pulmonary arterial tree, which most likely plays a major role in its protective role in MCT-induced PAH. In addition, and most importantly, we have found that HDFx reduces/prevents adhesion of macrophages, leukocytes, platelets, and dendritic cells to the inner walls of blood vessels (including pulmonary blood vessels), i.e. reduces sticking of these cell types, thus reducing, markedly, MCT-induced thromboses [15,16,28,32,34]. Lastly, HDFx's acceleration of wound healing cannot be overlooked in its overall potential therapeutic-effectiveness [17].

Conclusion

We believe our findings on Mg and HDFx should be helpful in understanding some of the pathophysiological mechanisms underlying MCT -induced PAH and may prove to be useful in aiding the treatment of human PAH and IPAH. Lastly, but not least, our new studies should be useful in future studies designed to counteract many of the pathophysiological manifestations of PAH and PPHN.

Acknowledgements

Some of the original studies mentioned in this report were supported, in part, by Research Grants from The National Institutes of Health (i.e. National Heart, Lung and Blood Institute) to B.M.A. and B.T.A. and unrestricted grants from several pharmaceutical companies (SANDOZ Pharmaceuticals, Bayer Pharmaceuticals, and CIBA GEIGY Corp.).

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