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# Potential Roles of Magnesium Deficiency in Inflammation and Atherogenesis: Importance and Cross-talk of Platelet-Activating Factor and Ceramide

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## Abstract

Epidemiologic studies in North America and Europe have shown that people consuming Western-type diets are low in magnesium (Mg) content (i.e., < 30 - 65% of the RDA for Mg); most such diets in the USA show that 60 - 80% of Americans are consuming only 185 - 235 mg/day of Mg. Low Mg content in areas of soft-water, and Mg-poor soil, is associated with high incidences of ischemic heart disease (IHD), coronary artery disease, hypertension, and sudden cardiac death (SCD). It is clear that the leading underlying cause of death worldwide is atherosclerosis. Importantly, both animal and human studies have shown an inverse relationship between dietary intake of Mg and atherosclerosis. The myocardial level of Mg has consistently been observed to be lower in subjects dying from IHD and SCD in soft-water areas than those in hard-water areas. Over the past 20 years, our laboratories, using several types of primary cultured vascular smooth muscle (VSM) cells, and myocardial cells, demonstrated that declining levels of extracellular Mg ($[Mg^{2+}]_0$) activated several enzymatic pathways to produce increases in cellular sphingolipids, particularly ceramides which are known to exert numerous types of cardiovascular manifestations including inflammatory effects ; the latter play important roles in atherogenesis and cardiovascular diseases. Approximately 20 years ago, we reported that low $[Mg^{2+}]_0$ caused formation of platelet-activating factor (PAF) as well as other types of PAF-like molecules and suggested that these molecules might be causative agents in low $Mg^{2+}$-induced IHD and SCD. Herein, we review results and data from our labs which strongly support roles for ceramides, PAF and PAF-like lipids in low $[Mg^{2+}]_0$-induced IHD and SCD.

**Keywords:** Sphingolipids; Ceramides; Ischemic heart disease; Sudden cardiac death; PAF; Vascular smooth muscle

## Introduction

Several epidemiologic studies in North America and Europe have shown that people consuming Western-type diets are low in magnesium (Mg) content (i.e., < 30 - 60% of the RDA for Mg [1-3]. Most such diets in the U.S.A. show that 60 - 80% of Americans are consuming only 185-235 mg Mg/day [4-6]. Low Mg content in drinking water found in areas of soft water and Mg-poor soil, is associated with high incidences of ischemic heart disease, severe atherosclerosis, coronary vasospasm, hypertension, hyperlipidemia, diabetes, and sudden cardiac death [4,7-14]. Both animal and human studies have shown an inverse relationship between dietary intake of Mg and atherosclerosis [4,13,15-19]. The myocardial level of Mg has consistently been observed to be lower in subjects dying from ischemic heart disease and sudden cardiac death in soft water areas [4,7,9,11,20,21]. Mg plays an essential role in more than 500 enzymatic reactions and is required for all energy-generating reactions and oxidative phosphorylation. Mg is a natural calcium (Ca) channel blocker on myocardial and vascular smooth muscle cells [4,16,17,22,23], which was first demonstrated by The Alturas [22,23], and is a natural statin in that it lowers blood cholesterol, LDL, and triglycerides, and lowers arterial blood pressure [4-6,15-18,24,25].

Using sensitive, specific $Mg^{2+}$-ion-selective electrodes, it has been shown that patients with hypertension, ischemic heart disease, cardiac failure, strokes, diabetes (types 1 and 2), gestational diabetes, renal disease-induced vascular changes (i.e., atherosclerosis and inflammation) exhibit significant depletion of serum/plasma and tissue levels of ionized, but not total, Mg [4,16,17,26-35]. Moreover, dietary deficiency of Mg, under very-controlled laboratory conditions, in rats and rabbits has been shown to cause vascular remodeling concomitant with hypertension and atherogenesis (i.e., arteriolar wall hypertrophy and alterations in arterial wall matrices) of unknown origin [4,6,15-17,36-39].

Approximately 40 years ago, Russell Ross and colleagues advanced the hypothesis that atherosclerosis is an inflammatory disease brought about by injury to the endothelial surfaces of blood vessels in the macro- and microcirculations [40]. Briefly, the hypothesis stated that different forms of injury will result in numerous dysfunctions in the homeostatic properties of the endothelium, e.g., increase in adhesiveness of leukocytes and/or platelets, alteration in the pro coagulant properties, formation/release of cytokines/ chemokine's and growth factors.

Usually, inflammation is defined as a response of microcirculatory blood vessels, and the tissues they perfuse, to infections and damaged tissues which bring cells and host-defense molecules to all the diverse sites where they are required, in order to eliminate/degrade the offending agents [41,42]. The mediators of the defense mechanisms include white blood cells, phagocytic leukocytes, antibodies, chemokines, adhesion molecules and complement proteins [40-42]. Most of these cells and molecules are recruited, when needed, from the blood itself. The inflammatory process brings these cells and molecules to the damaged or necrotic tissues. The absence of the normal inflammatory process would allow infections to continue unchecked, prevent wounds from healing, and result in festering sores/wounds. A typical inflammatory response develops in a sequential manner: recognition of the offending agent (s) by host cells and molecules; recruitment of leukocytes and plasma proteins; activation of leukocytes and certain plasma proteins to destroy and eliminate all offending substances; control and termination of the reaction (s); and finally repair of the damaged tissue (s).

During the normal inflammatory process, leukocytes migrate across the venous postcapillary walls through the endothelium due to increases in vascular permeability, low shear -rates, and move to the site (s) of injury via adhesion molecules and chemotaxis. The normal mediators for these processes to take place are: adhesion molecules; cytokines; and chemokines. Interestingly, all of these same mediators are needed for atherogenesis [40-42], and have been demonstrated recently to be formed, rapidly, in magnesium-deficient states [43-45].

## What happens if the inflammatory process is not curtailed or neutralized?-It starts the atherogenic process

If, however, the inflammatory response is not curtailed, or effectively neutralized, the inflammatory response will go-on and stimulate migration and proliferation of VSM cells which will become intermixed with the inflammatory cells and protein components to initiate and form an intermediate lesion (i.e., beginning of an atherosclerotic process). If these processes go-on unabated, the arterial walls will thicken and initially dilate to compensate, to a point. It is important to keep in mind, here, that release of various dilators, locally, including free $Mg^{2+}$ ions are powerful, direct dilators of all types of microcirculatory blood vessels [4,17,31,36,46-50]. After these events the arterial and arteriolar vessels undergo a remodeling process in which the normal contractile VSM cells are transformed into a new non-contractile phenotype (s) [40-42]. At every stage of this process, macrophages, monocytes, and T-lymphocytes are attracted to the endothelium lining the blood vessel walls [41,42]. Activation of these cells then would be expected to result in release of hydrolytic enzymes, cytokines, and growth factors, which have been shown to be released in states of magnesium deficiency [43-45]. These factors will sustain and perpetuate the atherogenic process forming, eventually, fibrous tissue and further enlargement of the lesion, which will overlie a core of lipid resulting in plaques and necrotic tissue. Using rabbits and rats, we have demonstrated that feeding the animals diets low in Mg (similar to the levels found in American diets) results in all of the hallmarks of atherosclerosis, i.e., inflammation followed by release of hydrolytic enzymes, release of cytokines, release of chemokines and growth factors involved in the early stages of the atherogenic process demonstrating lesions and plaques on the inner surfaces of the endothelial walls followed by invasion of VSM cells which become transformed phenotypes [4,16,17,43,45].

## Developing plaques in diets promoted by high cholesterol intakes or promoted by low Mg diets are virtually similar in experimental animals

In developing atherosclerosis, each plaque has a cap that retains cholesterol and exhibits inflammatory conditions inside the plaques which can dissolve the fibers, but, then, suddenly, the caps rupture, spilling cholesterol into the insides of the arteries which can promptly cause clots that could, eventually, completely block the flow of blood into the microcirculation of the surrounding tissues [40-42]. Using experimental rabbits, we have observed that these events clearly are similar in both the normal animal fed high cholesterol diets and those rabbits fed low Mg diets [15,17, unpublished findings]. Overall, we believe that such experimental findings lend considerable impetus to our hypothesis that diets low in Mg should be considered important risk factors in events resulting in inflammatory conditions leading to atherogenesis.

## Causative events and pathways leading to inflammation-atherogenesis in MgD

What, however, are the cellular and molecular events leading to inflammatory events, release of hydrolytic enzymes, release of cytokines, chemokine's and growth factors, and transformation of contractile VSM cells to non-contractile VSM cells which behave as machines to synthesize and release these pro-atherogenic molecules? It has been shown by our group [4,25,43,51] and others [52-54] that ceramides, in increased levels, found in the atherogenic process[mixed in plaques; unpublished findings ], at least in rabbits, and stimulated in MgD states could be important causal and initiating agents [16,17].

Ceramides are sphingolipids known to be released as a consequence of sphingomyelinase (SMase) acting on sphingomyelin (SM), a component of all cell membranes, or as a consequence of the activation of serine palmitoyl transferase 1 and 2 (SPT 1 and SPT 2) (a de novo synthetic pathway) [55]. Ceramides are now thought to play important roles in fundamental processes such as inflammation, angiogenesis, membrane-receptor functions, cell proliferation, microcirculatory functions, cell adhesion, immunogenic responses, excitation-coupling events in smooth muscles, and cell death(i.e., apoptosis) [52-57]. SPT 1 and SPT 2 are the rate-limiting enzymes in the biosynthesis of sphingolipids [58]. More than 25 years ago, it was first demonstrated that SPT activity was increased in aortas of rabbits fed a high-cholesterol diet [59]. A short time after these latter studies were published , two of us showed that dietary deficiency of Mg , in levels found in Western diets, vastly increased atherosclerotic plaques in rabbits fed high-cholesterol diets, whereas high dietary levels of Mg inhibited plaque formations [15]. SPT is a heterodimer of 53-kDa SPT-1 and 63-kDa SPT 2 subunits [60,61], both of which are bound to the endoplasmic reticulum [62]. An upregulation of SPT activity has been hypothesized to play a role in apoptosis [63], cell death events taking place in atherogenesis [41,42,64 ].

Recently, we have reported that magnesium deficient diets given to rats for only 21 days results in an upregulation of SMase, SPT-1, and SPT-2 in a variety of cardiovascular tissues and cells as well as decreased levels of SM and phosphatidylcholine (PC) [65]. We also noted that MgD diets resulted in fragmentation of DNA [24], a release of cytochrome C [65], an increased expression of apoptotic protease factor-1 [65], and an activation of caspase-3 (needed for apoptosis) [24], hallmarks of atherogenesis [42]. When specific inhibitors of SMase and SPT (1 and 2) were utilized, in primary cell cultures of VSM

cells, exposed to low $Mg^{2+}$ environments, we noted an inhibition of formation and release of ceramides, inhibition of DNA fragmentation, inhibition of release of mitochondrial cytochrome C, reduced expression of apoptotic protease factor-1, and inhibition of activation of caspase-3 [65]. We believe, collectively, these new studies lend support to our hypothesis that generation and release of ceramides are pivotal molecules in the initiation of cellular and molecular events leading to inflammatory events and atherogenesis, at least in MgD states. Whether this hypothesis is causative in overall atherogenic events remains to be tested rigorously. Is there any direct evidence to implicate any of these events in the living microcirculation?

### Direct *in-vivo* evidence on the microcirculation to implicate ceramides

Using open - and closed- window chambers [66-69], implanted in the cerebral cortex of rats and mice, and in-situ studies on omental tissue of rabbits [69], given MgD diets for 21 days, as well direct microcirculatory studies in the skin and skeletal muscles of MgD rats and mice, we found increased numbers of white blood cells (including monocytes, phagocytic leukocytes and lymphocytes) on the endothelial surfaces of microcirculatory blood vessels using high-resolution TV microscopy [69]. This was followed by increased permeability's of venular post capillaries to where, in some animals, white blood cells traversed the walls into the surrounding tissues, true signs of inflammation [69]. Although we have demonstrated that low Mg diets increase cellular free $Ca^{2+}$ in all types of cardiovascular muscle and endothelial cells, we believed some other fundamental molecule (s) in addition to changes in ceramides and $Ca^{2+}$ must perforce be operative in the low MgD -induced atherogenesis. We felt some lipid-like fast-reacting molecule which could be generated, rapidly, and easily penetrate cell membranes is probably also involved in these inflammatory-atherogenic events.

### Is the mysterious intermediary molecule possibly related to platelet-activating factor?

Platelet-activating factor (PAF) is known to play major roles in both inflammatory responses and atherogenesis [70-72]. A variety of the circulating blood -formed elements (e.g., polymorph nuclear leukocytes, platelets, basophils, and macrophages) and endothelial cells can elaborate PAF [71,72]. We have recently demonstrated that cerebral, aortic and coronary VSM cells can also elaborate and release PAF [69]. There are some reports that both PAF and ceramides may result in transformation of VSM cells from one phenotype to another, as is typical in the atherosclerotic process [53,54,72,73]. In addition, like we see in MgD, PAF produces vasoconstriction of blood vessels and a variety of VSM types [for recent review, see 69], as do several of the ceramides [69,74,75]. A number of investigators employing intravital microscopy techniques, similar to those used by our laboratories [76-80], have demonstrated that PAF increased the number of white blood cells in the microvessels concomitant with intense vasoconstriction-spasms with increasing concentrations of the putative lipid mediator (i.e., PAF), less leukocyte rolling, and increased adherence of the leukocytes to the endothelial surfaces with increases in vascular-capillary permeability [78-80]. Using open and closed chambers implanted in rodent cerebral cortex and skeletal muscles, as mentioned above, we have observed similar phenomena [69]. Further, we have reported that a variety of ceramides produce similar microcirculatory actions in rodent cerebral, cutaneous and skeletal microvascular tissues, including increased permeability of the postcapillary venular walls, the major sites of inflammatory reactions [75]. Collectively, these *in-vivo* microcirculatory findings strongly support the hypothesis that both PAF and ceramides induce similar, true inflammatory responses in diverse vascular beds in diverse mammalian species.

Using the above reports and experimental findings, in a large number of *in-vivo* studies, from our laboratories , and others mentioned above, we hypothesize that since MgD results in most of the attributes of early inflammatory responses- atherogenesis, including the synthesis/ release of PAF and ceramides, PAF and ceramides most likely are important , if not critical , contributing mediators released/ synthesized early in cardiovascular tissues , and blood formed elements, to initiate inflammation and the atherogenic process.

## Future Considerations

Since we have demonstrated in both rats and rabbits fed low Mg diets that increased levels of both ceramides and PAF are found, in situ , in all chambers of the heart , aortae and coronary blood vessels, and these manifestations were associated with increased plaques, elevated serum cholesterol , elevated trigycerides , elevated ceramides, and increased generation of PAF [4,15-17,24,25,43,45,51,65,69,81], it is highly unlikely that these *in-vivo* manifestations are merely epiphenomena. However, in order to solidify our hypothesis, regarding inflammation and atherogenesis (induced by low dietary Mg), one could utilize PAF knock-out or knock-down rats and mice subjected to low dietary Mg. Such PAF knock-out animals should result in reductions in expression of many of the downstream molecules and their pathways, e.g., decreased levels of the ceramide -generating enzymes, decreased ceramide levels, reduction in DNA fragmentation, reduced expression of apoptotic protease factor-1, reduction in levels of caspase-3, reduction in elevated levels of cholesterol and triglycerides, and reduced levels of plaques on carotid and coronary arteries, etc. Only time will tell whether these suggested experiments will prove to validate our hypothesis.

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