# Marinobufagenin and Cyclic Strain May Activate Endothelial NADPH Oxidase, Contributing to the Adverse Impact of Salty Diets on Vascular and Cerebral Health

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## Abstract

Limited but provocative ecologic epidemiology suggests that dietary salt may play a central role in the genesis of not only of stroke, but also dementia, including Alzheimer's disease. Impairment of nitric oxide bioactivity in the cerebral microvasculature is a likely mediator of this effect. Salted diets evoke increased adrenal secretion of the natriuretic steroid marinobufagenin (MBG), which promotes natriuresis via inhibition of renal tubular Na+/K+-ATPase; this effect is notably robust in salt-sensitive rodent strains in which other compensatory natiuretic mechanisms are subnormally efficient. MBG-mediated inhibition of sodium pumps in vascular smooth muscle likely plays a role in the hypertension induced by salty diets in these rodents. However, salt sensitivity in humans is associated with increased vascular mortality and ventricular hypertrophy independent of blood pressure; this suggests that MBG may be pathogenic via mechanisms unrelated to blood pressure control. Indeed, recent evidence indicates that MBG, via interaction with alpha1 isoforms of the sodium pump, can activate various intracellular signaling pathways at physiological concentrations too low to notably inhibit pump activity. An overview of current evidence suggests the hypothesis that MBG – as well as the cyclic strain induced by hypertension per se - may induce endothelial oxidative stress by activating NADPH oxidase. If so, this could rationalize the increase in vascular and systemic oxidative stress observed in salt-sensitive rodents fed salty diets, or in rodents infused with MBG; moreover, if this effect is a particularly prominent determinant of oxidative stress in cerebrovascular endothelium, it might help to explain the virtual absence of stroke and dementia in low-salt societies. As a corollary of this hypothesis, it can be predicted that spirulina-derived phycobilins, which appear to mimic the physiological role of bilirubin as an inhibitor of NAPDH oxidase complexes, may have potential for ameliorating the adverse health impacts of MBG and of salty diets. Potassium-rich diets are also likely to be protective in this regard, as they should suppress MBG production via their natriuretic impact, while their stimulatory effect on sodium pump activity may exert a hyperpolarizing effect on plasma membranes that suppresses NADPH oxidase activity.

### Salted Diets May Play an Obligate Role in Stroke and Dementia

Human societies consuming traditional diets very low in added salt have been found to be virtually free of essential hypertension, and the low-salt Melanesian culture on the island of Kitava appears to experience little if any stroke.[1, 2] Even more remarkably, senile dementia seems to be unknown on this island, even though a fair proportion of Kitavans live to old age; analogously, senile dementia was reported to be extremely rare in East Africans in the 1930s, when these people ate low-salt diets and were at very low risk for hypertension and stroke.[3] These findings are particularly intriguing in light of recent evidence that efficient nitric oxide production by the cerebral microvasculature may suppress brain production of

amyloid beta, thought to be a key mediator of Alzheimer's disease;[4] moreover, effective cerebrovascular nitric oxide bioactivity would be expected to prevent cerebral ischemia, suspected to play a cofactor role in Alzheimer's induction.[5] These phenomena may help to rationalize the growing epidemiology correlated Alzheimer's risk with increased cardiovascular risk factors.[4, 6] In aggregate, these findings suggest that lifelong consumption of a low-salt diet may reduce stroke and dementia risk by favorably influencing the function – notably, the protective nitric oxide bioactivity - of the cerebral microcirculation.

**Natriuretic Steroids May Mediate the Pathogenicity of Salty Diets**

In rodents, salty diets or saline infusion evoke the production of natriuretic steroids, structurally related to cardiotonic glycosides, which promote compensatory natriuresis by binding to and inactivating the transport activity of Na+/K+-ATPase (a.k.a. "sodium pump") in the renal tubules; the production of these steroids is amplified in "salt-sensitive" rodent strains prone to salt-induced hypertension.[7, 8] The chief blood-borne natriuretic steroid that has been observed under such conditions is marinobufagenin (MBG – first characterized in toad venom!), produced primarily in the adrenal cortex, although a transient increase in serum levels of oaubain is also seen. (Remarkably, volume expansion triggers an increase in *brain* ouabain production which in turn provokes the increase in adrenal MBG production.[7]) This increase in marinobufagenin is a mediator of the increase in blood pressure induced by salty diets in susceptible rodents, as demonstrated by the ability of infused anti-MBG antibodies to reverse this rise in blood pressure.[9] MBG-mediated inhibition of the sodium pump in vascular smooth muscle is suspected to be largely responsible for this rise in blood pressure; the resulting increase in intracellular sodium would be expected to boost intracellular free calcium by influencing sodium-calcium countertransport, and hence to promote contractile activity. Atrial natriuretic peptide (ANP), whose production is also evoked by salt-mediated volume expansion, acts on the kidney tubules to sensitize the sodium pump to inhibition by MBG, whereas in vascular smooth muscle it blunts this effect.[10] Salt-sensitive rodents are characterized by a blunted ANP response to volume expansion; hence, they tend to retain sodium more avidly, and their vascular smooth muscle is more sensitive to MBG-mediated inhibition of the sodium pump.[11] The extent to which these phenomena are relevant to humans consuming salty diets requires further clarification.

In humans, salt sensitivity is associated with a considerable increase in cardiovascular and overall mortality, *independent of blood pressure*.[12, 13] A portion of this increased risk may be attributable to insulin resistance syndrome, as the elevated insulin levels associated with this syndrome promote renal sodium retention and hence tend to amplify salt sensitivity.[14, 15] However, salt sensitivity also tends to reflect relative ineffectiveness of certain physiological strategies which promote natriuresis during volume overload, including pressure-natriuresis, baroreceptor-mediated reduction in renal sympathetic activity, and ANP production.[16] In salt-sensitive rodents, these mechanisms are indeed subnormal, so that increased production of MBG is required to achieve compensatory natriuresis. If salt sensitivity in humans likewise tends to be characterized by increased MBG production at a given high-normal salt intake – a credible proposition which still lacks clinical proof – it will be reasonable to suspect that elevated MBG, for reasons other than its impact on blood pressure, is a key mediator of the increased health risk associated with salt sensitivity. Also consistent with a pathogenic role for MBG is ecologic epidemiology pointing to salty diets as a risk factor for stroke – again, independent of blood pressure.[17] It is well known that East Asian societies whose traditional diets are exceptionally high in salt are at high

risk for stroke, even though their risks for coronary disease or metabolic syndrome are relatively low.[18] But if MBG is indeed a mediator of this risk, how does it do so?

A possible solution to this riddle may be offered by recent evidence that the Na+/K+-ATPase can act not only as an electrolyte transporter, but also as a transmembrane signal transducer.[19] In low nanomolar or sub-nanomolar concentrations too low to notably inhibit pump activity, natriuretic steroids act as ligands for the sodium pump that stimulate pro-inflammatory and fibrogenic intracellular signaling pathways associated with increased oxidative stress. Isoforms of Na+/K+-ATPase expressing the alpha1 chain may be particularly proficient in this regard. It is tempting to speculate that MBG-mediated signal transmission in vascular endothelium and other vascular tissues is a key mediator of the vascular complications evoked by salty diets in salt-sensitive individuals. Indeed, recent studies in rodents suggest that MBG mediates cardiomyopathy in rats with uremic kidney dysfunction, in part by boosting collagen production in cardiac fibroblasts; this phenomenon is independent of blood pressure.[20, 21] These findings may be pertinent to consistent clinical evidence that, at any given blood pressure, salty diets and salt sensitivity are associated with increased ventricular mass.[22, 23]

**How Might MBG Influence Cerebrovascular Endothelium?**

In light of the low risk for stroke and dementia observed in low-salt societies, it would be particular interest to examine the impact of MBG on cerebrovascular endothelium – in particular, on the NO bioactivity of this endothelium.

In various cell types, binding of natriuretic steroids to the alpha1 isoforms of the sodium pump has been shown to activate the non-receptor tyrosine kinase Src; indeed, this effect appears to be a central mediator of sodium pump-evoked signal transduction.[24, 25] Studies demonstrate that the SH2 and kinase domains of Src bind to the intracellular portion of the alpha1 chain of the sodium pump; in this conformation, the tyrosine kinase activity of Src is inhibited. However, extracellular binding of natriuretic steroids to the sodium pump causes a conformational change in the alpha1 chain, such that the kinase domain of Src no longer binds to the alpha1 chain – whereas the SH2 domain remains bound. As a result the tyrosine kinase activity of Src is strongly stimulated, as detected by a marked increase in Tyr418 autophosphorylation. When sodium pumps located in caveolae interact with natriuretic steroids, the resulting increase in caveolar Src activity leads to tyrosine phosphorylation of various other caveolar proteins, include caveolin-1 and the epidermal growth factor receptor (EGFR). This Src-mediated transactivation of EGFR has been shown to be responsible for many of the downstream signaling effects evoked by natriuretic steroids; in particular, the transactivated EGFR assembles a Shc/Grb2/Sos complex that stimulates the Ras/Raf/Erk1/s cascade, which in turn plays a role in the hypertrophic and fibrogenic effects of natriuretic steroids on certain types of vascular cells.

Src-mediated transactivation of EGFR is evoked by a number of other signaling mechanisms, and often is associated with activation of NADPH oxidase complexes. For example, in mesangial cells, cyclic strain somehow produces a rapid activation of caveolar Src, which quickly phosphorylates caveolin-1, and leads to a subsequent transactivation of EGFR that is dependent on this caveolin-1 phosphorylation.[26] A key downstream effect of EGFR transactivation in this system is a marked activation of caveolar NAPDH oxidase, associated with membrane translocation of Rac1, p47phox, and p67phox; these effects are blocked by AG1478, a potent inhibitor of the tyrosine kinase activity of EGFR. A key downstream

consequence of the resulting increase in oxidative stress is activation of RhoA, which in turn provokes the increased matrix production associated with glomerulosclerosis. These findings help to rationalize the adverse impact of chronically elevated glomerular capillary pressure on glomerular structure and function.

Studies with mesangial cells have not yet clarified how Src-mediated transactivation of EGFR induces activation of NADPH oxidase. However, recent studies examining the impact of protein-bound advanced glycation endproducts (AGEs) on human vascular endothelial cells may provide some insight in this regard.[27] Treatment of endothelial cells with AGE-modified bovine serum albumin leads to transactivation of EGFR associated with increased activation of PKCdelta and NADPH oxidase. Although this study did not examine the mechanism responsible for EGFR transactivation in this system, other studies have established that the activated RAGE receptor stimulates Src activity. The activation of PKCdelta observed during EGFR transactivation is associated with binding of PKCdelta to EGFR and the phosphorylation of Tyr-311 and Tyr-332 in PKCdelta. The EGFR inhibitor AG1478 inhibits this activation of PKCdelta, as well as the activation of NADPH oxidase; moreover, inhibition of PKCdelta with rottlerin blocks the activation of NADPH oxidase, These findings suggest that AGE-evoked transactivation of EGFR is upstream from PKCdelta activation, which in turn is upstream from NADPH oxidase activation. Indeed, transactivation of EGFR has been shown to provoke PKCdelta activation in other systems, and activation of NADPH oxidase by PKCdelta has likewise been demonstrated in other contexts; indeed, PKCdelta is well known to phophorylate and induce translocation of p47phox. Remarkably, exposure of endothelial cells to EGF fails to activate either PKCdelta or NADPH oxidase; conceivably, this reflects the fact that EGFR transactivation phophorylates tyrosine residues not influenced by the kinase activity of the receptor, which then serve as docking sites for PKCdelta.

**Hypothesis: MBG Activates Endothelial NAPDH Oxidase**

Interaction of natriuretic steroids with alpha1 isoforms of the sodium pump has been shown to evoke oxidative stress in cardiomyocytes, and in other cells is reported to induce activation of oxidant-sensitive enzymes such as the p38 and JNK MAP kinases.[28-30] Likewise, intravenous infusion of MBG in rats induces systemic oxidative stress.[20] Analogously, high-salt diets induce vascular, renal, and systemic oxidative stress in rats that are salt-sensitive, but not in those who are salt-resistant.[31-34] Although some evidence points to mitochondria as a source of oxidative stress in ouabain-treated cardiomyocytes,[35] little attention so far has been devoted to a possible role for NADPH oxidase in the oxidative stress evoked by natriuretic steroids. Yet a number of studies point to NADPH oxidase as a key source of oxidative stress evoked by salty diets in salt-sensitive rodents.[36-41] An overview of the evidence presented above suggests the following hypothesis:

Physiological concentrations of natriuretic steroids, via interaction with the alpha1 isoform of the sodium pump in the caveolae of endothelial cells, promote activation of caveolar NADPH oxidase, through a signaling pathway dependent on Src-mediated transactivation of EGFR. Phosphorylation of caveolin-1 may play an obligate role in this transactivation, and PKCdelta may be a key mediator of the stimulatory impact of transactivated EGFR on NADPH oxidase activity. Analogously, the increased cyclic strain associated with hypertension may activate NAPDH oxidase by a similar pathway. This hypothesis should be readily testable in cell culture studies.

If this hypothesis is largely correct, it should help to rationalize the adverse impact of salted diets and of salt-evoked hypertension on the vascular health, as increased endothelial production of superoxide is well known to antagonize the protective bioactivity of vascular nitric oxide. Thus, superoxide, or oxidants derived therefrom, can directly quench nitric oxide (producing toxic peroxynitrite in the process), can promote uncoupling of NO synthase (which as a result becomes a further source of superoxide), and can impede the catabolism of ADMA, an endogenous competitive inhibitor of NO synthase.[42-46] There is considerable evidence that effective endothelial NO bioactivity plays a key role in the prevention of atherosclerosis, medial hypertrophy, ventricular hypertrophy, myocardial infarction and stroke, and suggestive evidence that it also provides protection from not only vascular dementia, but also Alzheimer's.

In any case, it may be of crucial importance to further characterize the impact of natriuretic steroids, particularly MBG, on oxidative stress and NO bioactivity of endothelial cells, particularly those of the cerebral microvasculature. The possibility that physiological concentrations of MBG might inhibit sodium pump activity in vascular endothelium has so far received little attention, and also deserves evaluation. Inhibition of endothelial sodium pump activity by MBG could be expected to promote membrane depolarization, which has been shown to provoke oxidative stress in endothelial cells, likely mediated by NADPH oxidase;[47, 48] this depolarization also could also be expected to lessen endothelial NO synthase activation, by diminishing calcium influx.[47] Hence the impact of MBG on both the signaling activity of endothelial sodium pumps, and on their pumping activity, requires study; each may be germane to the pathogenic effects of high-salt diets.

**Potassium and Phycocyanobilin as a Potential Antidotes to MBG**

High-potassium diets exert a natriuretic effect that could be predicted to decrease production of MBG;[49-51] although presently there do not yet appear to be any studies directly demonstrating this, Haddy and colleagues (pioneering proponents of the view that salt-evoked natriuretic factors mediate the hypertensive and pathogenic effects of salty diets[52]) have shown that potassium-rich diets decrease serum levels of a "digitalis-like substance" which not unlikely is MBG.[53] Moreover, the modest increase in serum potassium associated with high-potassium diets tends to hyperpolarize cellular plasma membranes by boosting the transport activity of sodium pumps, which are electrogenic. [47, 54, 55] (They extrude 3 atoms of Na for every 2 atoms of K they bring in, thereby rendering the interior surface of plasma membranes negatively charged relative to the exterior surface.) This effect is evidently antagonistic to the pump-inhibitory activity of MBG. Also, this hyperpolarizing effect has been shown to decrease endothelial superoxide production, likely by suppressing NADPH oxidase activity.[47, 54, 55] Hence, this effect would tend to neutralize the putative stimulatory impact of MBG on this NADPH oxidase activity (whether exerted by membrane depolarization or Src-mediated signaling activity). These considerations suggest that potassium-rich diets have the potential both to inhibit MBG production and to antagnonize the end-organ effects of the MBG that is secreted.

Consistent with this view, many studies show that high-potassium diets tend to antagonize the hypertensive, pro-oxidant, vasculotoxic effects of high-sodium diets in rodents.[40, 56-62] In humans, potassium-rich diets (such as the celebrated DASH diet) tend to lower elevated blood pressure; the effect is more notable in those with a relatively high sodium intake.[63, 64] As would be expected, salt sensitivity

tends to be blunted in people consuming diets that are relatively potassium rich.[16, 65] Potassium supplementation improves endothelial function in mildly hypertensive subjects, even when its impact on blood pressure is modest.[66] The dietary sodium/potassium ratio appears to be more meaningful than sodium intake as a determinant of vascular risk.[67-70] In the Rancho Bernardo prospective cohort study, a 400 mg/day greater intake of potassium was associated with a 40% lower risk for stroke, independent of blood pressure.[71] In a provocative long-term controlled study, in which residents of a Taiwanese retired veterans home were randomized to receive food seasoned either with regular salt or a 1:1 mixture of sodium and potassium chloride, the cardiovascular mortality rate was about 40% lower in the latter group during nearly four years of follow-up; measurement of urinary electrolytes suggested that daily sodium intake was about 27% lower in the mixed salt group, whereas potassium intake was about 76% higher.[72] Hence, a feasibly modest reduction in salt intake, coupled with a notable increase in potassium intake, may have great potential for improving vascular health. Dietary potassium intake can be increased by eating potassium-rich fruits and tubers, emphasizing whole foods as opposed to refined grains and added "empty calories" (sugars and oils), by consuming beverages enriched with potassium bicarbonate (which has a mild flavor in moderate concentrations), and by using potassium-enriched modified salts analogous to that used in the Taiwanese study.[66, 72-78]

In light of the above discussion, epidemiology examining risk for dementia as a function of dietary sodium/potassium ratio might prove very fruitful. It should be noted that the potassium intake of the dementia-free Kitavans (whose dietary staples are potassium-rich tropical yams) has been estimated to be about 8 grams daily, roughly triple the average potassium intake of Americans, whereas their salt intake (derived solely from cooking with sea water) is about one-fourth as high.[1-3] And when stroke and dementia were apparently rare in East Africans, their low-salt diets were largely unrefined and hence likely to be relatively potassium rich.

There is recent evidence that the potent intracellular antioxidant activity of free bilirubin stems from its ability to inhibit NADPH oxidase complexes.[79-82] This may help to rationalize two recent prospective epidemiological studies correlating increased serum bilirubin levels with decreased risk for stroke;[83, 84] bilirubin also correlates inversely with prevalent carotid atheroma.[85, 86] Although bilirubin and its immediate precursor biliverdin are expensive to synthesize, the biliverdin derivative and homolog phycocyanobilin (PhyCB), the chief phytonutrient in spirulina, is rapidly converted within cells to a homolog of bilirubin that shares bilirubin's capacity to inhibit NADPH oxidase; this presumably explains why oral administration of spirulina (or of phycocyanin, the spirulina protein which contains PhyCB as a chromophore) shows profound anti-inflammatory and antioxidant activity in rodent studies.[87, 88] Hence, if MBG does indeed activate NADPH oxidase in endothelial cells, there is a reasonable prospect that this effect could be antagonized by ingestion of PhyCB. The potential implications for vascular health and prevention of dementia are evident.

Since PhyCB appears to cross the blood-brain barrier (as suggested by rodent studies in which oral administration of whole spirulina or of phycocyanin has exerted central neuroprotective effects), it may also have the potential to target oxidant production in glial cells and neurons, thought to play a mediating role in the pathology of Alzheimer's and other neurodegenerative disorders associated with oxidative stress and excitotoxicity.[89, 90] And activation of NADPH oxidase in the hypothalamus and other brain centers appears to play a mediating role in the sympathetic overactivity and blunting of the baroreceptor

reflex that commonly contribute to hypertension.[91, 92] Although there do not appear to be any published studies in which spirulina or phycocyanin have been evaluated in rodent models of hypertension, one of the few clinical trials to have studied spirulina in a dose likely to be physiologically significant (4.5 grams daily) observed significant reductions of blood pressure (systolic fell by 10 mm Hg, diastolic by 7 mm Hg) in healthy volunteers; this was however an open trial that requires replication.[93]

Salt-mediated activation of NADPH oxidase in brain parenchyma may also play a role in the impact of salty diets on dementia risk. In salt-sensitive spontaneously hypertensive rodents, very salty diets increase the NADPH oxidase activity of microglia, astrocytes, and neurons in the cortex and hippocampus, an effect associated with increased neuronal apoptosis.[94, 95] Oral administration of apocynin, a NADPH oxidase inhibitor, suppresses the neuronal apoptosis while also preventing the cerebrovascular remodeling and increased incidence of stroke evoked by salty diets in this rat strain. This increase in cerebrovascular oxidative stress is also prevented by the angiotensin receptor blocker valsartan, but not hydralazine. Other studies show that salty diets or i.c.v. infusion of sodium evoke synthesis of ouabain and aldosterone in the hypothalamus and pons of rats, which in turn act to increase brain angiotensin activity via up-regulation of angiotensin-converting enzyme.[96, 97] And a salt-evoked increase in brain ouabain production and an associated increase in angiotensin II activity in the brain, pituitary and adrenal cortex play a key role in triggering adrenocortical marinobufagenin production in salt-fed salt-sensitive rats.[98-100] These considerations suggest that salty diets may have the potential to up-regulate the oxidative stress in brain parenchyma thought to contribute to Alzheimer's progression, and that both phycobilins and angiotensin receptor blocker (ARB) drugs may be protective in this regard. Indeed, recent epidemiology indicates that patients treated with ARBs are less likely to develop dementia or Alzheimer's specifically than are patients using lisinopril or other cardiovascular medications.[101, 102] Moreover, in a transgenic mouse model of Alzheimer's disease, treatment with olmesartan alleviated cognitive dysfunction.[103] Phycobilins and ARB drugs may thus prove to have complementary potential for reducing dementia risk, by acting directly or indirectly to control NADPH oxidase-mediated oxidative stress in the brain parenchyma and cerebral microcirculation.

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