# Dietary Nitrate May Aid Prevention of Neovascular Age-related Macular Degeneration by Suppressing the Transcriptional Activity of Hypoxia-Inducible Factor-1 and Aiding Choroidal Perfusion

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## Abstract

Age-related macular degeneration (AMD) is associated with a reduction in choriocapillaris blood flow, and the magnitude of this reduction correlates with risk for neovascular AMD; reduced expression of nitric oxide synthase (NOS) in choroidal arteries may play a role in this phenomenon, and NOS expression in retinal pigmented epithelium (RPE) is also subnormal in AMD. Oxygen delivery to the RPE in AMD may also be compromised by structural modifications which increase oxygen diffusion distance. Hence, a relative hypoxia of RPE, by promoting activation of hypoxia-inducible factor (HIF) and hence increased synthesis of vascular endothelial growth factor (VEGF), may be key to the pathogenesis of neovascular AMD. In light of evidence that nitric oxide (NO), acting via cGMP and protein kinase G, can suppress the transcriptional activity of HIF-1, it is reasonable to suspect that measures which boost NO levels in submacular choroid and RPE may oppose the impact of hypoxia on neovascular AMD risk, both by suppressing HIF-1 activity and by promoting more effective choroidal perfusion. Dietary nitrate can boost endogenous NO production in hypoxic tissues, and it may not be coincidental that spinach and collard greens, lutein-rich foods found to have the most dramatically favorable impact on risk for neovascular AMD in case-control epidemiology, are also among the richest dietary sources of nitrate. If dietary nitrate can indeed aid control of neovascular AMD, its efficacy in this regard likely would be complemented by effective antioxidant measures and antagonism of IGF-I activity, which likewise could be expected to suppress RPE overexpression of VEGF.

### Retinal Hypoxia as a Pathogenic Factor in Age-Related Macular Degeneration

Numerous studies have concluded that blood flow through the choriocapillaris tends to be diminished in patients with age-related macular degeneration (AMD), and longitudinal studies have found that diminished choriocapillaris flow at baseline is associated with increased risk for development of neovascular AMD.[1-8] This diminution of choroidal perfusion may be traceable in part to reduced expression of the neural and endothelial isoforms of nitric oxide synthase (NOS) in choroidal arteries, as has been demonstrated recently in a case-control study of AMD.[9] This phenomenon is exacerbated in atrophic areas, as opposed to non-atrophic areas, in patients with geographic atrophy. Curiously, expression of neural NOS is also notably diminished in the retinal pigment epithelium (RPE) of AMD patients; this is the predominant isoform of NOS in these cells. Capillary dropout may also contribute to the observed diminution of choroidal blood flow.[8]

Steffanson and collegues have noted that increased diffusion distance, stemming from such factors as confluent drusen, thickening of Bruch's membrane, and sometimes detachment of the RPE, can also impair the efficiency of oxygen delivery to the retina and RPE, whereas the vitreoretinal adhesion, often

observed in AMD, can impair delivery of oxygen to the retina from the vitreous.[1] These authors therefore maintain that subnormal oxygenation of the retina and RPE may be a common feature of AMD that conceivably may contribute to its pathogenesis, and that is very likely to be a factor in the development of neovascular AMD. The role of retinal hypoxia in triggering choroidal neovascularization is well established in a number of syndromes; this neovascularization is thought to be mediated, in large part, by increased activity of hypoxia-inducible factor in the RPE, which promotes transcription of vascular endothelial growth factor (VEGF), the key angiogenic factor responsible for choroidal neovascularization.[10-14]

**Nitric Oxide Opposes the Transcriptional Activity of HIF-1**

The relative deficit of NOS activity in the submacular choroid and in RPE noted in AMD is of particular interest in light of evidence that nitric oxide (NO), acting via cGMP and protein kinase G (PKG), can suppress the transcriptional activity of HIF-1. In both vascular smooth muscle cells and in a colorectal cancer cell line, NO-cGMP-PKG have been shown to suppress the hypoxia-evoked transcription of the VEGF gene and other HIF-1-inducible genes; this phenomenon is not associated with any alteration of the expression or nuclear localization of the HIF-1 heterodimer, but rather reflects interference with HIF-1's ability to bind to its response elements.[15, 16] Hence, possibly via direct phosphorylation, PKG activity has the potential to block the transcriptional activity of HIF-1. This may rationalize a report indicating that, in immortalized human RPE cells, the NO donor sodium nitroprusside blocks the hypoxia-evoked increase in expression of VEGF mRNA; this effect of sodium nitroprusside was abolished by a guanylate cyclase inhibitor, and hence presumably was mediated by cGMP.[17] This phenomenon was likewise demonstrated in endothelial and fibroblast cell cultures. Arguably, the ability of NO to oppose the chemoresistance and invasive behavior of hypoxic cancer cells may stem largely from inhibition of HIF-1 activity.[18]

(Studies employing often supraphysiologically high levels of NO have shown that NO has the potential to either boost or inhibit the transcriptional activity of HIF-1 by affecting its proteasomal catabolism;[19-21] these results are not likely to be germane to the low-NO environment of the choroid and RPE in AMD patients.)

These findings suggest that relative hypoxia may contribute to the increased production of VEGF by RPE that drives neovasularization in wet AMD, and further suggest that measures which boost the diminished NO levels in RPE and submacular choroid may be able to inhibit this contribution of hypoxia to neovascular AMD pathogenesis both by suppressing the transcriptional activity of HIF-1, and also likely by improving choroidal perfusion – thereby alleviating retinal hypoxia.

**Dietary Nitrate vs. Neovascular AMD?**

One of the most exciting recent developments in cardiovascular physiology is the discovery that dietary nitrate can give rise to increased plasma levels of nitrite, which in turn can be converted to NO by interaction with certain heme proteins.[22-24] This conversion of nitrite to NO is most avid in hypoxic tissues, since oxygen binding will be less likely to impede this catalytic activity of heme proteins. Hence, nitrate-rich diets can promote NO evolution – independent of NOS activity – somewhat selectively in the hypoxic tissues most in need of NO's vasodilatory activity.[25] The ability of nitrate to boost plasma nitrite levels reflects the fact that absorbed dietary nitrate can be secreted into saliva and reduced by oral bacteria

to nitrite; this nitrite can then be reabsorbed. (Curiously, the use of antibacterial mouthwashes has been shown to impede the protection afforded by dietary nitrate.[26])

It is therefore reasonable to speculate that increased dietary nitrate might have the potential to mitigate the contribution of retinal hypoxia to the pathogenesis of neovascular AMD. In this regard, it is pertinent to recall that, in the pioneering case-control epidemiological study which first linked the xanthophyll carotenoids lutein and zeaxanthin to diminished risk for neovascular AMD, subjects who reported consumption of at least half-a-cup of spinach or collard greens 5 or more times weekly had an odds ratio of 0.12 for neovascular AMD (one case versus eleven controls).[27] The trend for lower risk of AMD with increasing intake of spinach and collard greens was highly statistically significant ($p<.001$). Whereas spinach and collard greens are well known to be excellent sources of lutein, they are also among the richest sources of dietary nitrate.[28] While the number of subjects in this study consuming high amounts of these vegetables is too low for any definitive conclusions to be drawn, it can be noted that the multivariate-adjusted odds ratio for neovascular AMD in the highest quintile for lutein/zeaxanthin consumption was 0.43 – impressive, but less impressive than the 0.12 odds ratio observed in heavy consumers of spinach and collard greens. Hence, it is tempting to speculate that an increased nitrate intake may have contributed to the exceptionally low risk for neovascular AMD among the subjects making heavy use of spinach and/or collard greens.

In light of these considerations, it may be worthwhile to attempt further epidemiology evaluating the association of dietary nitrate with risk for AMD, and especially neovascular AMD. The impact of dietary nitrate on rodent models of hypoxia-driven choroidal neovascularization could also be assessed. And perhaps it would be prudent to encourage patients at risk for AMD to obtain at least a portion of their daily xanthophyll carotenoid intake from vegetables such as spinach, collard greens, and kale that are rich both in these carotenoids and in nitrate.

**Complementary Strategies for Controlling VEGF**

While HIF-1 is clearly a key regulator of VEGF transcription, the VEGF promoter can also bind a number of other transcription factors capable of boosting VEGF transcription, including most notably Sp1 and AP-1.[29] Oxidative stress, which characteristically afflicts RPE cells in AMD and is thought to play a key pathogenic role in this regard,[30, 31] has the potential to activate each of these factors. Oxidative stress can stimulate HIF-1 activity by opposing its proteasomal degradation, by promoting HIF-1alpha transcription via NF-kappaB activation, and also potentially by boosting activation of Erk, which induces a phosphorylation of HIF-1 that enhances its transactivational activity.[32-34] Erk also phosphorylates Sp1, stimulating its binding to the VEGF promoter and amplifying its transactivational activity.[35] And oxidative stress can promote AP-1 activity via both Erk and JNK activation.[36, 37] As expected, oxidative stress has been shown to stimulate RPE production of VEGF.[38-40] Hence, measures which effectively control oxidative stress in RPE cells can be expected to complement the utility of NO for moderating VEGF synthesis. Moreover, oxidatively-stressed RPE cells make lesser amount of pigmented epithelium-derived factor (PEDF), which opposes the pro-angiogenic impact of VEGF on the choroidal vasculature.[41] The balance between RPE production of VEGF and PEDF is thought to be a critical determinant of risk for choroidal neovascularization, and oxidative stress shifts this balance in favor of VEGF.

Growth factor activity – most notably IGF-I activity – can also stimulate VEGF transcription by boosting activity of HIF-1, Sp1, and AP-1. Via the PI3K/Akt/mTOR/eIF4E pathway, IGF-I stimulates translation

of HIF-1alpha mRNA; via the Ras/Raf/Erk pathway it promotes a phosphorylation that enhances HIF-1's transactivational activity.[34, 42, 43] Like oxidative stress, it can enhance the activity of Sp1 and AP-1 via Erk-mediated phosphorylation. And IGF-I can also amplify translation of VEGF mRNA, via Akt-mTOR-eIF4E;[44, 45] up-regulation of this signaling pathway by oxidative stress can exert a similar effect. [46] Not surprisingly, IGF-I has been shown to boost VEGF production in RPE cells.[47-49] Although the RPE in situ is exposed to IGF-I of autocrine and paracrine origin,[48, 50] it seems likely that plasma levels of free IGF-I will be a meaningful determinant of the IGF-I activity experienced by RPE cells; indeed, growth hormone and systemic IGF-I appears to play a key role in the promotion of diabetic proliferative retinopathy.[51-53] The bioactivity of plasma IGF-I can be modulated by diet and lifestyle strategies: vegan diets of moderate protein content can decrease plasma IGF-I levels by about 30%, and measures which keep diurnal insulin levels fairly low (such as low-insulin-response meals, low saturate-unsaturate ratio, and exercise training) can oppose IGF-I bioactivity by boosting hepatic synthesis of IGFBP-1.[54-62] And pharmaceuticals which inhibit the IGF-I receptor – such as monoclonal antibodies, and small molecule inhibitors such as picropodophyllin – may eventually be applied in the control of neovascular AMD and other proliferative retinopathies.[63]

These considerations thus suggest that, if dietary nitrate does indeed prove useful for prevention of neovascular AMD, concurrent effective antioxidant therapy and measures which oppose IGF-I activity should enable even better control of the RPE's VEGF production.

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