Activated glycine receptors may decrease endosomal NADPH oxidase activity by opposing ClC-3-mediated efflux of chloride from endosomes

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ABSTRACT

Receptor-mediated activation of NADPH oxidase complexes commonly occurs in endosomes; the hydrogen peroxide produced by the dismutation of superoxide generated within the endosomes often functions to boost receptor function by reversibly inhibiting protein tyrosine phosphatases or by promoting formation of signaling complexes. NADPH oxidase-mediated formation of superoxide entails transfer of two electrons (provided by NADPH) from the cytosol to the endosomal lumen, where two molecules of superoxide are generated. This charge transfer must be balanced if NADPH oxidase activity is to be sustained. In many cells, this balance is achieved by ClC-3, a chloride-proton antiport which can extrude two chlorides from the endosome to balance the importation of two electrons. The efficiency of this chloride extrusion will evidently be contingent on the cytosolic chloride level. Pro-inflammatory hormones which stimulate NADPH oxidase activity in endosomes have been shown to promote chloride extrusion from the cell, thereby expediting endosomal chloride export. Conversely, high cytosolic chloride could potentially slow endosomal NADPH oxidase activity by impeding ClC-3-mediated chloride export. Glycine-activated, strychnine-inhibitable chloride channels, which boost intracellular chloride in cells which maintain intracellular chloride levels lower than that of plasma, have shown anti-inflammatory and anti-angiogenic activity in cell culture and rodent studies. It is proposed that many of these effects may be attributable to glycine-mediated suppression of endosomal NADPH oxidase activity. This model suggests that supplemental glycine may have utility for prevention and control of atherosclerosis, heart failure, angiogenesis associated with cancer or retinal disorders, and a range of inflammation-driven syndromes – including metabolic syndrome; and it might complement the suppression of NADPH oxidase activity achievable with phycocyanobilin-enriched spirulina extracts.

Chloride transport is a key determinant of endosomal NADPH oxidase activity

Ligand-receptor complexes which activate NADPH oxidase are often incorporated into endosomes; superoxide is then generated within the interior of the endosome [1,2]. Since endosomes are typically acidic, this superoxide assimilates a proton that neutralizes its charge, enabling it to diffuse through the endosomal membrane to the cytosol. Once there, the proton is shed; this superoxide is then likely to encounter superoxide dismutase affiliated with the external surface of the endosome, which converts it to hydrogen peroxide and molecular oxygen. This hydrogen peroxide may then be capable of influencing the activity and formation of receptor signaling complexes on the external surface of the endosome. This represents a simple strategy whereby membrane receptors incorporated into endosomes up-regulate their signaling by promoting generation of hydrogen peroxide in their immediate environment.

Superoxide formation within endosomes entails transfer of electrons from NADPH external to the endosome to molecular oxygen within the endosome. This charge transfer must be balanced if this process is to be sustained [3,4]. In some cells, this may be achieved straightforwardly by influx of protons via a proton channel. However, in other cells, the ClC-3 chloride channel mediates the required charge balancing [3,4]. NADPH oxidase imports into the endosome two electrons, derived from oxidation of NADPH, which react with molecular oxygen to generate two molecules of superoxide; these ultimately yield one molecule of hydrogen peroxide which can modulate endosomal signal transduction. To balance the importation of two electrons, ClC-3 can export two chloride atoms.

The efficiency with which ClC-3 can export chloride to the cytosol will evidently be contingent on the cytosolic chloride level; if this level is low, export should be impeded, but a relatively high level could be expected to slow net endosomal chloride export, and thereby potentially slow endosomal NADPH oxidase activity. This may explain an intriguing study in which reductions in cytosolic chloride were shown to up-regulate tumor necrosis factor-alpha-mediated activation of NF-kappa B and pro-inflammatory signaling in endothelial cells [5]. Both tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) were found to provoke expulsion of intracellular chloride through the plasma membrane; the resultant loss of cytosolic chloride was required...
for subsequent efficient activation of NF-kappaB. A deficit in of cytosolic chloride could be expected to accelerate TNF-α-triggered NADPH oxidase activity by expediting export of chloride from endosomes. The resultant boost in hydrogen peroxide generation would then amplify TNF-α-mediated activation of NF-kappaB, key driver of the inflammatory response [2,6]. (More specifically, this hydrogen peroxide somehow enables incorporation of TRAF2 into the TNF-α signaling complex, a step critical to downstream signaling to NF-kappaB [2]). Consistent with this model, activation of NF-kappaB by either TNF-α or IL-1β in smooth muscle cells has been shown to be contingent on Nox1 and CIC-3 activity in endosomes [3].

**Glycine-activated chloride channels exert anti-inflammatory and anti-angiogenic effects**

These findings draw attention to the impact of cytosolic chloride regulation on endosomal NADPH oxidase activity. In this regard, many cells express glycine-activated, strychnine-inhibitable chloride channels which induce a hyperpolarizing chloride influx in cells in which cytosolic chloride concentration is lower than that of plasma. Stimulation of these receptors has been reported to exert anti-inflammatory effects in macrophages, Kupffer cells, and neutrophils, and to decrease platelet aggregation [7–11]. Inhibition of endosomal NADPH oxidase activity may play a role in these effects, as NADPH oxidase up-regulates inflammatory activity in phagocytes, and amplifies platelet aggregation [12–17]. Moreover, with respect to macrophages, there is evidence that CIC-3 knockdown suppresses the ability of lipopolysaccharide to activate NF-kappaB via toll-like receptor-4; such signaling is known to require NADPH oxidase activation. Glycine-mediated hyperpolarization of the plasma membrane might also contribute to some of these effects by suppressing calcium uptake through voltage-sensitive calcium channels.

Although the possibility that mast cells express glycine receptors has not been evaluated, it is notable that plasma glycine has been found to correlate inversely with risk for asthma. Moreover, NADPH oxidase has been shown to play a mediating role in mast cell activation.

**Explaining the anti-angiogenic activity of glycine**

Glycine-activated chloride channels are also expressed by vascular endothelial cells, and glycine-mediated activation of these channels opposes the pro-proliferative, pro-migratory effects of vascular endothelial growth factor (VEGF) [18–21]. This may explain why glycine feeding suppresses tumor-mediated angiogenesis and tumor growth in cancer-bearing mice – even though glycine exerts no direct effect on cancer cell proliferation, and only influences tumor growth when the tumor nodule becomes sufficiently large to require angiogenesis for growth [18,20–22].

The main pro-angiogenic receptor for VEGF, VEGFR2, undergoes endocytotic cycling, and possesses tyrosine kinase activity whether at the surface or in endosomes [23]. Tyrosine phosphorylation of Y-1175 in VEGFR2, of key importance to endothelial proliferation, occurs preferentially in early endosomes [24]. VEGF activates NADPH oxidase complexes in endothelial cells, and this activation is required for VEGF’s pro-angiogenic activity [25–28]. This likely reflects the fact that hydrogen peroxide generated in the microenvironment of VEGFR2 reversibly inhibits protein tyrosine kinases, such as PTP-B1, that target activating tyrosine phosphorylations of the receptor [29–33]. In particular, PTP-B1 reverses phosphorylation of Y-1175 [24]. Although the role of CIC-3 in regulating VEGFR2-mediated activation of NADPH oxidase activity in endothelial endosomes does not appear to have been studied, it is notable that CIC-3 is crucial to the endothelial activation of this complex by angiotensin II type 1 receptors [34,35]. Hence, it is reasonable to suspect that CIC-3 is expressed by endothelial endosomes and boosts the ability of VEGFR2 to activate NADPH oxidase in these endosomes.

We therefore hypothesize that an increase in plasma glycine, by provoking an increase in cytosolic chloride levels in endothelial cells, diminishes the capacity of CIC-3 to extrude chloride molecules from endosomes, and thereby inhibits VEGFR2-mediated activation of NADPH oxidase, impairing its pro-angiogenic activity. This might explain the well-documented anti-angiogenic effects of boosting plasma glycine to the high physiological range. The possibility that supplemental glycine may have clinical utility as an anti-angiogenic agent for cancer control merits evaluation – albeit the concurrent effects of glycine on anti-cancer immune surveillance should be considered. The anti-angiogenic effects of glycine might also find application in prevention or treatment of the choroidal neovascularization associated with diabetic retinopathy and age-related macular degeneration. It will be of interest to determine whether retinal pigment epithelium expresses glycine receptors.

**Implications for atherogenesis**

The role of NADPH oxidase complex activation in promoting pro-inflammatory behavior of vascular endothelium is well documented; in particular, endothelial NADPH oxidase plays a mediating role in atherogenesis. It is reasonable to postulate that a high proportion of this NADPH oxidase activation occurs in endosomes, and is susceptible to modulation by cytosolic chloride level. If so, then we could expect elevated plasma glycine, via stimulation of glycine-activated chloride channels, to suppress endothelial inflammation by opposing endosomal NADPH oxidase activity. Moreover, the hyperpolarizing impact of glycine on endothelium might also promote vascular health by boosting calcium influx into endothelial cells, thereby enhancing the protective activity of the endothelial nitric oxide synthase [13]. It also seems not unlikely, given the documented impact of glycine on macrophages, that supplemental glycine could oppose atherogenesis and plaque instability via anti-inflammatory effects on intimal macrophages and foam cells [8,13,36]. Intriguingly, ApoE knock-out mice are substantially protected from atherosclerosis and foam cells formation when CIC-3 is also knocked out [37]. Hence, it is reasonable to propose that glycine supplementation might have anti-atherogenic potential – a proposition that has not yet been tested [13]. Intriguingly, recent prospective epidemiology has reported an inverse correlation between fasting plasma glycine level and risk for a myocardial infarct in patients with stable angina, after adjustment for traditional cardiovascular risk factors [38]. This association was found to be robust; hazard ratio for acute myocardial infarction for those in the top quintile of plasma glycine, as compared to those in the bottom quintile, was 0.71 (95% CI 0.54–0.94, p = 0.016); the trend was also highly significant (p = 0.012).

Glycine may also provide antioxidant protection to heart muscle. Cardiomyocytes have been shown to express functional glycine receptors, which oppose the pro-inflammatory effects of lipopolysaccharide on these cells [39]. Moreover, in mice subjected to cardiac pressure overload or angiotensin II administration, glycine supplementation lessens the ensuing cardiac hypertrophy [40]. In this regard, activation of NADPH oxidase in cardiomyocytes is known to play a role in the pathogenesis of ventricular hypertrophy and heart failure [41,42]. The role of CIC-3 in cardiac remodeling has not been assessed.

**Glycine vs. adipocyte dysfunction in metabolic syndrome**

Glycine can also work indirectly to protect vascular health by counteracting metabolic syndrome; this has been documented in rodents fed diets rich in fructose and/or fat [43]. Whereas glycine might be expected to exert anti-inflammatory effects on the macrophages that infiltrate hypertrophied adipose tissue, in vitro studies demonstrate direct effects of glycine on adipocytes; glycine suppresses the production of pro-inflammatory adipokines, while boosting that of adiponectin [44–47]. A recent study has provided evidence that adipocytes in fact express glycine receptors, the effects of which are inhibitable by
Once activated macrophages are attracted to adipose tissue, their se-
The PhCyB content of spirulina is about 0.6% by dry weight, and PhCyB's marked antioxidant activity may account for many of the favorable effects conferred by diets rich in spirulina or phycocyanin (the spirulina protein which carries PhCyB as a chromophore) in rodent studies [90,93-95].

Conflicts of interest

Mark McCarty is co-inventor and co-owner of a US patent covering nutraceutical uses of phycocyanobilin oligopeptides.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.01.012.

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