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

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A B S T R A C T

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 antiporter 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 unimpeded, 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 nercrosis 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

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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 ClC-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 ClC-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 VEGR, 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-1B, that target activating tyrosine phosphorylations of the receptor [29–33]. In particular, PTP-1B reverses phosphorylation of Y-1175 [24]. Although the role of ClC-3 in regulating VEGFR2-mediated activation of NADPH oxidase activity in endothelial endosomes does not appear to have been studied, it is notable that ClC-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 ClC-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 ClC-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 ClC-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 ClC-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

strychnine [47].

The dysfunction of visceral adipocytes that drives metabolic syndrome entails increased production of pro-inflammatory hormones and cytokines, a suppression of adiponectin release, and a reduction in insulin responsiveness. Activation of MAP kinases – most notably JNK – and of NF-kappaB appears to mediate this dysfunction [48]. Adipocyte-specific knockout of JNK inhibits diet-induced induction of metabolic syndrome in mice [49]. This may reflect the fact that JNK, along with other MAP kinases, boosts activity of AP-1 transcription factors, which act in collaboration with NF-kappaB to promote transcription of genes coding for pro-inflammatory factors [50–52]. Moreover, JNK phosphorylates, and thereby inhibits, the PPARgamma transcription factor required for adiponectin expression [53,54]. And JNK promotes adipocyte insulin resistance by conferring a serine phosphorylation on insulin receptor substrate-1 (IRS-1) that blocks its interaction with the activated insulin receptor [55,56] (The kinase IKKbeta, immediately upstream from NF-kappaB activation, confers a similar phosphorylation on IRS-1 that promotes insulin resistance [57,58]). Oxidants also diminish the pro-lipolytic response of adipocytes to catecholamines – thereby increasing risk for weight gain – by translocating cAMP hydrolase to the surface of lipid droplets [59,60].

How hypertrophy of visceral adipocytes leads to activation of MAP kinases and NF-kappaB is still somewhat mysterious. Increased exposure to saturated free fatty acids – notably palmitate – has been shown to provoke this activation in vitro, at least in part by triggering endoplasmic reticulum (ER) stress [61]. Palmitate can also induce this activation via toll-like receptor-4 (TLR4) in a complex with fetuin [62]. Once activated macrophages are attracted to adipose tissue, their secretion of pro-inflammatory hormones – notably TNF-alpha and interleukin-1beta(IL-1β) – can provoke further activation of MAP kinases and NF-kappaB [63].

Importantly, palmitate, TNF-α and IL-1β all also amplify NADPH oxidase activity in adipocytes, and this appears to play an important role in mediating their signaling, as agents which inhibit NADPH oxidase activity suppress induction of metabolic syndrome in rodents [64–68]. Not only do oxidants up-regulate TLR-4, TNF-α, and IL-1β signaling, but they also can induce ER stress by provoking loss of ER calcium via inhibition of SERCA and activation of the IP3 receptor [69,70]. Notably, adipocytes from ClC-3-knockout mice are protected from palmitate-induced endoplasmic reticulum stress; moreover, when mice are rendered diabetic by a diet high in fat and sucrose, coupled with low-dose streptozotocin injections, ClC-3 knockout mice are resistant to subsequent onset of hyperlipidemia, hyperglycemia, and insulin resistance [71]. Hence, it is reasonable to speculate that endosomal NADPH oxidase activation is an upstream mediator of the MAP kinase and NF-kappaB activation that drives adipocyte dysfunction in metabolic syndrome, and that glycine can suppress this NADPH oxidase activity by increasing intracellular chloride.

Curiously, plasma glycine levels tend to be low in people with metabolic syndrome or type 2 diabetes, whereas they rise after corrective measures such as bariatric surgery or lifestyle improvement [72,73]. This suggests that metabolic syndrome somehow acts to lower plasma glycine – which in turn might be expected to reinforce metabolic syndrome. How metabolic syndrome might decrease plasma glycine remains obscure.

Glycine supplementation as a practical lifestyle strategy

Since the K_m for glycine of glycine-activated chloride channels is near the physiological fasting level, it follows that ample supplemental intakes of glycine, administered several times daily, can be expected to sustain increased activation of these channels [74,75]. Fortunately, glycine is inexpensive, highly and rapidly soluble, and has a pleasant, mildly sweet flavor [75]. Moreover, intakes as high as 31 g daily have proved safe [76]. It is therefore ideal for incorporation into functional foods and beverages. A teaspoon of glycine added to a cup of coffee or tea can be employed as a healthful substitute for sugar. Rodent studies, as well as pilot clinical studies, suggest that glycine supplementation may: exert favorable effects on metabolic syndrome; help to ward off diabetic complications; moderate the adverse metabolic effects of high-fructose diets; provide protection from alcoholic or non-alcoholic liver disorders; help to prevent cardiac hypertrophy; and even aid effective sleep [43,75]. While many of these effects may reflect stimulation of glycine-activated chloride channels, glycine also can complement supplemental N-acetylcysteine in boosting glutathione synthesis, can boost oxidant-scavenging activity in hepatocytes via its conversion to pyruvate (a direct scavenger of hydrogen peroxide), and can oppose formation of age-advanced glycation end-products [75]. Collagen is extremely rich in glycine, and chondrocyte collagen production in vitro increases as the medium concentration of glycine is boosted through the high-physiological range; hence, it has been suggested that high-glycine diet might aid maintenance of cartilage integrity [77].

In light of the versatile heath protection which glycine supplementation may afford, it should be of particular interest to determine whether glycine can indeed inhibit endosomal NADPH activity in cells that express glycine-activated chloride channels; this could be readily addressed in cell culture studies. This hypothesis also predicts that ClC-3 knockdown or pre-inhibition will eliminate or depress the inhibitory impact of glycine on inflammation and oxidant production. While it appears that little research activity to date has examined glycine's impact on NADPH oxidase activity per se, it is notable that, in humans with metabolic syndrome supplemented with 15 g glycine per day (5 g thrice daily), plasma markers of oxidative stress declined by 25% relative to placebo [78].

An additional possibility is that glycine supplementation might help to control NADPH oxidase activity by enhancing bilirubin production. Intracellular unconjugated bilirubin functions physiologically as a potent inhibitor of certain NADPH oxidase complexes, in low nanomolar concentrations; the isoform specificity of this effect has not yet been fully defined [79–82]. This likely explains much of the profound antioxidant benefit of heme oxygenase induction [82]. This enzyme cleaves heme to generate carbon monoxide, free iron, and biliverdin; the latter is rapidly reduced to bilirubin by biliverdin reductase. Increased heme levels drive increased transcription of the heme oxygenase-1 (HO-1) gene, and also enhance availability of its substrate [83]. Glycine is a substrate for the rate-limiting enzyme in heme production, delta-aminolevulinic acid (ALA) synthase; the other substrate required, succinyl-CoA, is an intermediate of the Krebs cycle. This enzyme's K_m for glycine is about 2.6 mM, about an order of magnitude higher than plasma concentrations of glycine [84]. The level of glycine in human erythrocytes has been reported to be in the range of 200–500 μM, and the mitochondrial glycine importer SLC25A38 has a K_m of 750 μM [85,86]. Hence, glycine's intracellular level seems likely to be sub-saturating for ALA synthesis. 8 molecules of ALA –and hence 8 molecules of glycine - are required for the synthesis of one heme molecule. It is therefore reasonable to speculate – though direct proof may currently be lacking – that glycine supplementation may exert an antioxidant effect by boosting heme and bilirubin synthesis. This effect would likely be of modest magnitude, as ALA synthase expression is subject to post-transcriptional feed-back inhibition by heme [87]. Moreover, concurrent iron supplementation likely would be required, to optimize the availability of intramitochondrial ferrous iron needed for heme synthesis [88]. Concurrent supplementation with ALA and iron has been shown to boost HO-1 expression in mice and in humans [88,89].

If an inhibitory impact of glycine on NADPH oxidase activity can be confirmed, its utility in this regard might be complementary to that of phycocyanobilin (PhyCB), which functions as as a light-absorbing chromatophore in cyanobacteria and many blue-green algae. PhyCB is biosythesized from, and is almost identical in structure to, biliverdin, and has been found to mimic the NADPH oxidase inhibitory activity of biliverdin and bilirubin [90,91]. PhyCB might also promote *in situ* generation of biliverdin/bilirubin by boosting HO-1 expression [92].

The PhyCB content of spirulina is about 0.6% by dry weight, and PhyCB'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 PhyCB 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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