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Putative Nox2 inhibitors worsen homocysteine-induced impaired acetylcholine-mediated relaxation

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Abstract *Background and aim:* Increased homocysteine (Hcy) is associated with coronary artery disease (CAD). Hcy increases reactive oxygen species (ROS) via NADPH oxidases (Nox), reducing acetylcholine-mediated vasorelaxation. We aimed to determine if putative Nox2 inhibitors prevent Hcy-impaired acetylcholine-mediated vasorelaxation.
Methods and results: New Zealand White rabbit and wild-type (C57BL/6) and Nox2$^{-/-}$ (NOX) mice aortic rings were mounted in organ baths. Rabbit rings were incubated with either apocynin (10 μM), gp91ds-tat (GP, 1 μM) or PhoxI2 (1 μM) and mice rings GP (1 μM) only. Some rabbit rings were incubated with 3 mM Hcy, before pre-contraction, followed by dose–response relaxation to acetylcholine (ACh; 0.01μM-10μM). In rabbit rings treated with Hcy and GP, O_2^- donor pyrogallol (1 μM) or Akt activator SC79 (1 μM) was added 5 min before ACh. Mice rings were used to compare Nox2 deletion to normal acetylcholine-mediated relaxation.

In rabbits, Hcy reduced acetylcholine-mediated relaxation vs. control ($p < 0.0001$). Treatment + Hcy reduced relaxation compared with treatment alone ($p < 0.0001$). Pyrogallol and SC79 reversed the response of GP + Hcy ($p = 0.0001$). In mice, Nox2 deletion reduced acetylcholine-mediated vasorelaxation. Rabbit tissue analysis revealed that Hcy reduced eNOS phosphorylation at Thr^{495} and increased eNOS phosphorylation at Ser^{1177}; no further alteration at Thr^{495} was observed with GP. In contrast, GP prevented increased phosphorylation at Ser^{1177}.
Conclusions: Apocynin, GP and PhoxI2 worsens acetylcholine-mediated vascular relaxation in rabbit aorta, which is supported by results from mouse Nox2 deletion data. These inhibitors worsen Hcy-induced vascular dysfunction, suggesting that current putative Nox2 inhibitors might not be useful in treating HHcy.

Background

Cardiovascular disease (CVD) is a leading cause of mortality and morbidity [50], and impaired acetylcholine-mediated vasorelaxation is a classical risk for the development of CVD [22]. Hyperhomocysteinemia (HHcy) is associated with coronary artery disease (CAD) and atherosclerosis [59] via initiating impaired acetylcholine-

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mediated vasorelaxation [49,60]. Thus, restoration of endothelial function is a vital step in the prevention of CVD [43]. Endothelial function is measured by acetylcholine mediated vasorelaxation, and is reliant upon concomitant endothelial nitric oxide synthase (eNOS) phosphorylation of serine 1177 (Ser^{1177}) and dephosphorylation at threonine 495 (Thr^{495}) [45], resulting in nitric oxide (NO) release [57]. Impaired eNOS phosphorylation results in reduced acetylcholine mediated vasorelaxation, and the development of atherosclerosis, a key factor in CVD [18].

In the Heart Outcomes Prevention Evaluation 2 (HOPE2) study and the Vitamin Intervention for Stroke Prevention (VISP) study, Hcy levels were not reduced to the low risk level and indeed risk was still elevated for these patients [7,31]. Additional findings of the HOPE2 study found that overall risk of stroke was reduced but not the severity or disability [44]. Taken together, novel treatments for HHcy are necessary to reduce CVD.

Nox2 is a member of the nicotinamide adenosine diphosphate (NADPH) oxidases family (Nox1-5, Duox1, 2). These trans-membranous enzymes produce functional reactive oxygen species (ROS) essential for cell signalling and proper endothelial function [8,41]. Increased O_2^- has also been reported in HHcy and cell culture studies show that Hcy promotes cellular damage, presumably from ROS production via Nox2 [58]. Further, Hcy has been reported to stimulate the Nox2 subunit $p47^{phox}$ and increase apoptotic ROS production in rats [47].

Pharmacological inhibitors that target enzymes which produce ROS are therefore an attractive therapeutic potential in CVD treatment. We hypothesised that apocynin, gp91ds-tat (GP) and PhoxI2, are potentially novel treatments to reduce the detrimental effects of Hcy on blood vessel disease.

Methods

All experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Ed. 2013). Apocynin, PhoxI2, pyrogallol, SC79, acetylcholine, homocysteine, and phenylephrine) were purchased from Sigma–Aldrich, (Merck), Darmstadt, Germany. Gp91ds-tat was purchased from Australian Biosearch, Perth, WA, Australia and U46619 (thromboxane analogue, Cayman Chemical) was purchased from Sapphire Biosciences, Sydney, NSW, Australia.

Rabbits

New Zealand rabbits (12 weeks, male, n = 15, VUAEC #12/019) were housed in separate cages and maintained at a constant temperature of approximately 23 °C and were provided water and food *ad libidum*. Whilst in our care, animals were fed a normal chow diet. Animals were anaesthetised (3 mg/kg xylazine + 2 mg/kg ketamine), exsanguinated and the abdominal aorta excised and flushed with cold oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$, 2.5, glucose 11.1, mM).

Mice

Wild-type (WT) and $Nox2^{-/-}$ (NOX) mice (12–14 weeks old, male, n = 8, VUAEC #14/014) [59]. Animals were housed in cages with a maximum of 5 mice per cage and maintained at a constant temperature of approximately 23 °C. Food and water were provided *ad libidum*. At sacrifice, mice were anaesthetised with isoflurane (4% O_2) followed by cervical dislocation.

The aortae were cleaned of fat and connective tissue, cut into rings (2–3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37 °C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). These rings were left to rest, unmounted, for 30 min. Rings were then mounted between two metal hooks attached to force displacement transducers, stretched to 2 g (rabbits) or 0.5 g (mice) and allowed to reach resting tension plateau. Rings were then re-stretched and allowed to reach resting tension plateau a second time (see Table 1).

Experimental protocol

Rings were incubated with the Nox2 inhibitors apocynin (10 μM [53]), gp91ds-tat (GP, 1 μM [39]) or PhoxI2 (1 μM [11]) for 30 min; NOX rings were not incubated with Nox2 inhibitors. Drugs were re-introduced into the baths immediately prior to the addition of 3 mM Hcy for 1 h where used, to compensate for possible drug metabolism. Control rings had neither Hcy nor Nox2 inhibitor added. To assess acetylcholine mediated relaxation (ACh; 0.01–10 μM [60]), rings were pre-contracted with either phenylephrine (rabbits; Phen; 0.1–0.3 μM [60]) or a thromboxane analogue (mice; thx; 0.3 μM [21]). After the contraction reached a plateau, a concentration–response curve to ACh-induced relaxation was obtained. Pyrogallol (1 μM) (an O_2^-donor) or SC79 (1 μM) (an Akt activator) were added 5 min (to induce phosphorylation) before the first ACh dose in rabbit rings treated with Hcy and GP [35].

Immunohistochemistry

Aortic rabbit rings were removed from the organ bath after the last dose of ACh and fixed in 4% paraformaldehyde for 24 h at RT and then maintained in 1x PBS at 4 °C, pH 7.3 for immunohistochemical detection of eNOS at Ser^{1177} and Thr^{495} (Envision kit system, monoclonal antibody (DAKO Corporation, Carpentaria, USA), eNOS antibody monoclonal IgG1, Transduction Laboratories, USA), as described previously [5]. Slides were prepared using established methods [34]. Images for all slides were taken with an Olympus microscope (x40 magnification) and the computer program Leica (Leica Microsystems GmbH, Wetzlar, Germany). For eNOS quantification, the endothelial layer was traced with the 'ribbon' tool (MCID Core; InterFocus Imaging, Linton, UK). The endothelial layer was quantified using MCID by setting the hue, saturation and intensity to detect the brown DAB reaction. The intensity and proportional area were recorded for all proteins and tracings

Table 1 EC_{50} for Rabbits and Mice.

Rabbits	Control	Apocynin	Apocynin + Hcy**	PhoxI2*	PhoxI2 + Hcy***
EC_{50}	−7.8	−7.1	−7.1	−7.6	−7.4
SEM	0.2	0.1	0.3	1.9	1.0

Rabbits	Hcy	GP	GP + Hcy***	GP + Pyrogallol*	GP + Hcy + Pyrogallol**	GP + Hcy + SC79
EC_{50}	−7.6	−7.0	−5.5	−6.9	−7.0	−7.1
SEM	1.0	0.1	15.2	0.2	0.2	0.2

Mice	WTChow	WTChow + GP	NOXChow
EC_{50}	−5.3	1.3	−6.8
SEM	5.1	446.3	0.4

There were significant differences in ACh response in rings compared with control. Significant differences between the control and treated groups indicate the reduced efficacy of ACh. All groups compared against control using one-way ANOVA followed by Dunnett's Multiple Comparisons Test. *$p = 0.01$; **$p = 0.001$; ***$p = 0.0002$. Results are presented as mean ± SEM.

were averaged and used for data analysis. All data points are arbitrary units and normalized to control as '1', described previously [5].

Statistical analysis

Isometric tension data were analysed using normal or two-way repeated measures ANOVA, measuring differences in response to drug dose between and within groups, followed by Sidak's Multiple Comparisons Test. GraphPad StatMate was used to calculate n, using an 80% Power to detect 30% difference between groups, for alpha <0.05. Control values for each vasorelaxation graph were all pooled. eNOS were analysed using ordinary one-way ANOVA followed by Tukey's Multiple Comparisons Test. An ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine significances for EC_{50}. All data were analysed using GraphPad Prism (version 7.01 for Windows, GraphPad Software, La Jolla, California USA). Data are represented as mean ± SEM. Significance was accepted at $p < 0.05$.

Results

Acetylcholine mediated vasorelaxation

Rabbits

Aortic acetylcholine mediated relaxation was significantly reduced by the addition of Hcy compared to control (68.5 ± 7.0% vs. 99.8 ± 0.53%, $p < 0.0001$; Fig. 1A–C). To investigate whether putative Nox2 inhibitors could restore this effect, three different pharmacological putative Nox2 inhibitors were used.

Apocynin worsened relaxation compared to control (93.1 ± 1.7% vs. 99.8 ± 0.53%, in the early doses ($p < 0.0001$, Fig. 1A). The combination of Hcy plus apocynin further reduced relaxation compared to apocynin (93 ± 1.4% vs. 37.2 ± 12.9%, p < 0.0001) and 3 mM Hcy alone.

GP reduced relaxation compared with control (83.4 ± 3.3 vs. 99.8 ± 0.53%, $p < 0.0001$, Fig. 1B). The addition of Hcy further reduced aortic relaxation compared with GP (43.5 ± 7.1% vs. 83.4 ± 3.3%, p < 0.0001, Fig. 1B) and 3 mM Hcy alone.

PhoxI2 reduced function compared with control (65.4 ± 6.5 vs. 99.8 ± 0.53%, $p < 0.0001$, Fig. 1C), similar to Hcy. Aortic relaxation was further reduced by the combination of PhoxI2 and Hcy compared with PhoxI2 alone (4.8 ± 28.8% vs. 65.4 ± 6.5, p < 0.0001, Fig. 1C) and 3 mM Hcy alone.

To investigate if O_2^- participated in changes to vascular relaxation, pyrogallol, an O_2^- donor, was added. The addition of pyrogallol inhibited the effect of GP in treated rings (71.2 ± 6.4% vs 43.5 ± 7.1%, $p < 0.01$; Fig. 1D). Secondly, to determine if O_2^- participated in signal transduction through the Pi3k/Akt pathway, as it is also involved in NO release (Auger 2010), we added SC79 (1 μM). SC79 negated the effects of GP in rings incubated with Hcy plus GP (68.9.4 ± 4.5% vs. 43.5 ± 7.1%, $p < 0.0001$, Fig. 1D).

Mice

To confirm the role of Nox inhibitors, NOX showed reduced vasorelaxation compared with WT (38.8 ± 5.8% vs. 73.7 ± 2.5%, $p < 0.0001$) but showed no difference compared with WT + GP (Fig. 2). However, WT + GP was significantly impaired compared with WT (42.4 ± 12.8% vs 73.7 ± 2.5%, $p < 0.0004$; Fig. 2).

eNOS phosphorylation at Thr^{495} and Ser^{1177}

Control phosphorylation of eNOS at Thr^{495} and Ser^{1177} were arbitrarily referenced as '1'. Hcy alone significantly reduced eNOS phosphorylation at Thr^{495} and significantly increased eNOS phosphorylation at Ser^{1177} compared to control (Fig. 3B). No further alteration at Thr^{495} was observed with GP. However, GP incubation prevented the increase in phosphorylation at Ser^{1177} (Fig. 3B).

Discussion

The present study provides direct evidence that apocynin, GP, and PhoxI2 worsens acetylcholine mediated vasorelaxation, and that these drugs further reduce the

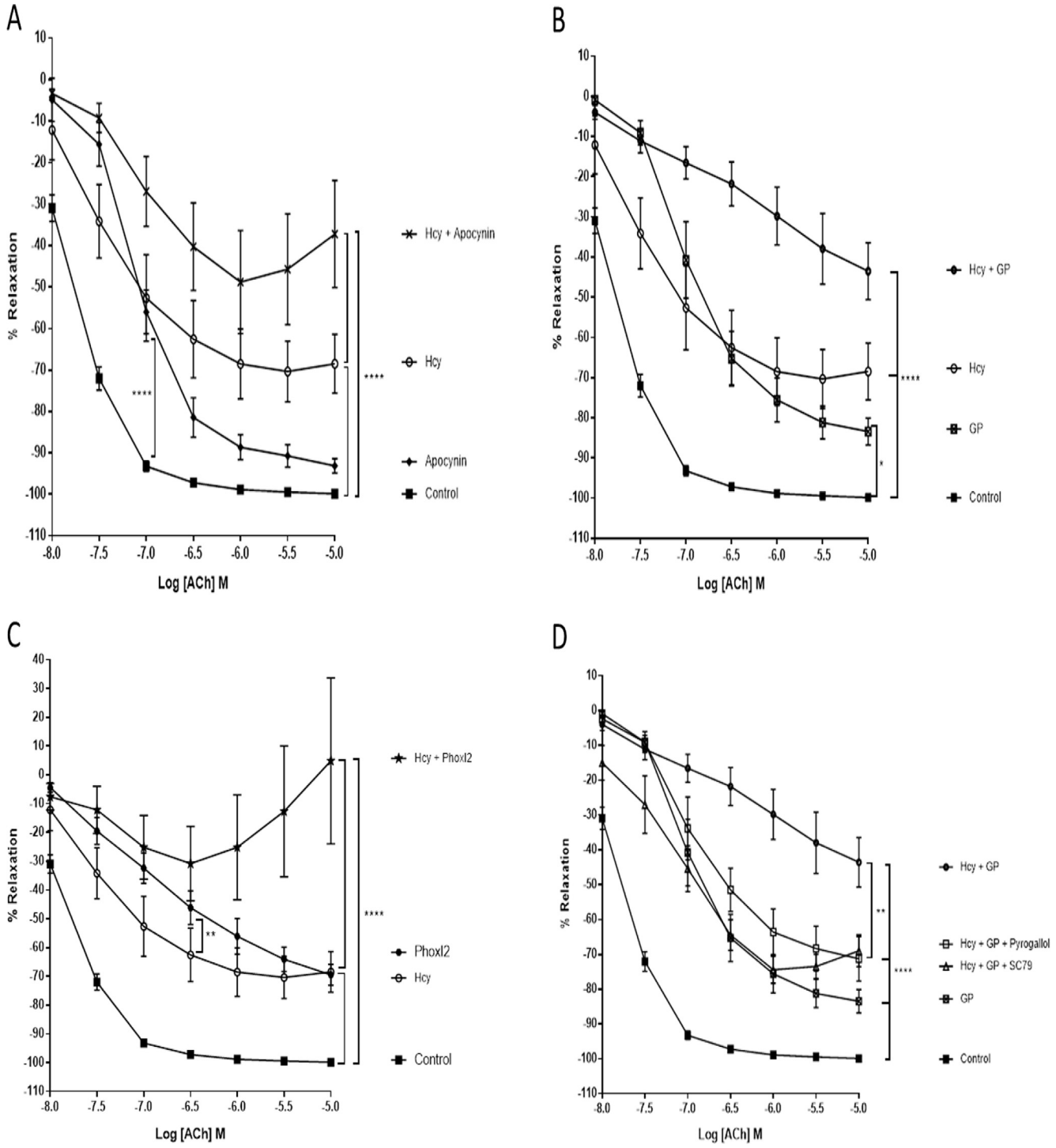

Figure 1 A) In control rabbit rings, incubation with 3 mM Hcy for 1 h significantly impaired function compared with control. Rings incubated with both Hcy and apocynin resulted in a severe impairment of relaxation, compared with control, Hcy treatment and apocynin treatment alone (****$p < 0.0001$). Results are mean ± SEM (n = 5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded. B) In control rabbit rings, GP significantly impaired function compared with control. Rings incubated with Hcy alone and in combination with GP resulted in a severe impairment of relaxation, compared with control and GP treatment alone (*$p < 0.05$, ****$p < 0.0001$). Results are mean ± SEM (n = 5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded. C) In control rabbit rings, the addition of PhoxI2 or Hcy significantly impaired function compared with control. Rings incubated with both Hcy and PhoxI2 resulted in a severe impairment of relaxation, compared with control, Hcy alone and PhoxI2 alone (**$p < 0.01$, ****$p < 0.0001$). There was no significant difference between treatment with Hcy and PhoxI2. Results are mean ± SEM (n = 5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded. D) The addition of pyrogallol and SC79 in rabbit rings incubated with Hcy and GP, resulted in an improvement in relaxation, compared with Hcy and Nox2 inhibition alone (****$p < 0.0001$). We also report an improvement in function between rings treated with Hcy + GP and Hcy + GP + Pyrogallol (**$p < 0.01$). Results are mean ± SEM (n = 5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.

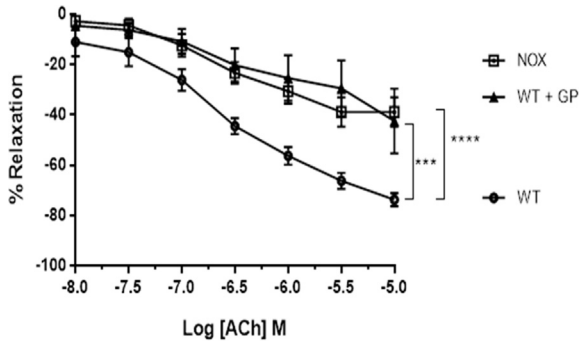

Figure 2 In mice, NOX (n = 8) and WT + GP (n = 4) significantly worsened relaxation vs WT (n = 8) (****$p < 0.0001$ and ***$p < 0.0004$, respectively). Interestingly, there was no difference in function between NOX and WT + GP. Results are mean ± SEM. All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been identified.

acetylcholine mediated vasorelaxation observed after Hcy incubation. Interestingly, NOX2 deletion in mice also show reduced acetylcholine mediated vasorelaxation. As well, histochemical analysis of rabbit aorta further revealed that incubation with Hcy decreased phosphorylation at Thr495 while increasing phosphorylation at Ser^{1177} (Fig. 3A), suggesting an underlying upregulation of eNOS to maintain homeostasis. In HHcy, GP reduced eNOS phosphorylation at Ser^{1177}, suggesting that GP can affect acetylcholine medicated vasorelaxation via this pathway.

Nox2 has been implicated as a vasoconstrictor molecule in cardiomyopathy and its inhibition has been shown to improve vascular contractility and reduce hypertension [23]. More recently, in a type 2 Diabetes Mellitus (T2DM) mice model, pan-Nox inhibition was shown to reduce oxidative stress and improve renal nephropathy, as well as improve insulin sensitivity, indicating that Nox2-generated ROS may have effects beyond signalling, but also in maintaining function in disease [12]. Uncovering the role of Nox2-induced vasoconstriction in pathologies is therefore important, as there is supposition that increases in Nox2-induced ROS is related to a reduced function in disease; indeed certain agents can increase the risk of damage from Nox2-derived ROS. For example, it has been shown that the first-line cancer treatment, doxorubicin, increases production of myocardial ROS, likely via Nox2 [33]. The authors of this study showed that in $Nox2^{-/-}$ mice, those treated with doxorubicin had reduced O_2^- levels and cardiomyocyte dysfunction, a mechanism attributed to Nox2-derived products [33]. Given the role of Nox2 in generating functional ROS, there has been speculation that it contributes to increased vascular oxidative stress and damage in pathological conditions [3,4,15,28]. The rabbit model showed increased acetylcholine mediated relaxation in the presence of Hcy (Fig. 1A–C). The drugs employed in this study all act on Nox2 assembly differently: apocynin is a small molecule Nox2 inhibitor that blocks the migration of $p47^{phox}$ to the membrane [37], GP is a chimeric 18 amino-acid sequence peptide which blocks assembly of $p47^{phox}$ and $gp91^{phox}$ [37] and PhoxI2, another small molecule inhibitor, exerts its inhibitory action on Nox2 by binding to $p67^{phox}$, disrupting the binding of $p67^{phox}$ to Rac [11,26]; they are also structurally different from each other (Fig. 4). Interestingly, despite differences in structure and function, apocynin, GP and PhoxI2 all impaired function in both healthy and HHcy vessels, in both rabbits and (GP) in mice.

Thus, here we report that these drugs might not be suitable to impair the detrimental effects of homocysteine on blood vessel function, and suggest further development of novel Nox2 inhibitors for this purpose. Low risk individuals report plasma Hcy between 6 and 9 μmol/L, risk is increased >10 μmol/L, with levels reported as high as 300 μmol/L in chronic kidney disease (CKD) [8,9]. We showed reduced vascular function in NOX mice (Fig. 2), which could be due to failure of genetic compensatory mechanisms. For example, in an eNOS mouse knock-out, it was recently reported that vasodilatory responses in the ophthalmic artery were kept stable by a compensatory mechanism of endothelium-derived hyperpolarising factors, specifically lipoxygenase and with some participation from calcium-controlled K+ channels, which are essential for vasodilation [32]. Additionally, gene deletion has resulted in significant upregulation of "non-essential" genes to perform the same or similar function [10], suggesting that compensation is an essential function. Additionally, there was no difference in response between the WT GP treated and NOX mice, which suggests that the effect of GP on blood vessels is similar to the genetic deletion of Nox2 in this model. This indicates a possible specificity of GP for Nox2 (Fig. 2). The major finding in this study is that Nox2 inhibition worsens Hcy-induced dysfunction. The failure of apocynin, GP and PhoxI2 to improve Hcy-induced dysfunction is surprising, given that it has been widely reported that acetylcholine mediated vasorelaxation has been restored with these drugs [16], indeed as have genetic knockout models of Nox2 [41].

SC79 is a recently described specific intracellular Akt-activator which has been shown to suppress neuronal ischaemic excitotoxicity [27]. Activation of the Akt pathway is essential for endothelial function [38]; it's role as an eNOS and NO modulator is well-reported [6,55]. For Akt to function as a mediator of endothelial-dependent vasodilation, Nox2 assembly and activation is essential [2]. Additionally, ROS in the cardiovascular system can activate Akt [1], although the exact impact on vasodilation remains unclear. SC79 has been shown to have other effects. It has been reported to reverse doxycycline-mediated anti-histamine effects in mast cells [51], improved myelination in damaged neuronal cells [13] and in human T regulatory cells, SC79 activated Akt to rescue Foxp3-induced Glut1 repression [42]. Here we have shown that SC79 reduced the effect of Hcy + GP and improved vasodilation (Fig. 1D). SC79 elicits its effect by directly binding to the Akt PH domain and inducing a favourable conformation, thus improving downstream signalling [27] and eNOS-stimulated NO release [52]. Crucially, SC79 allows for Akt activation in the cytosol, avoiding the need for targeting Akt on the membrane [13].

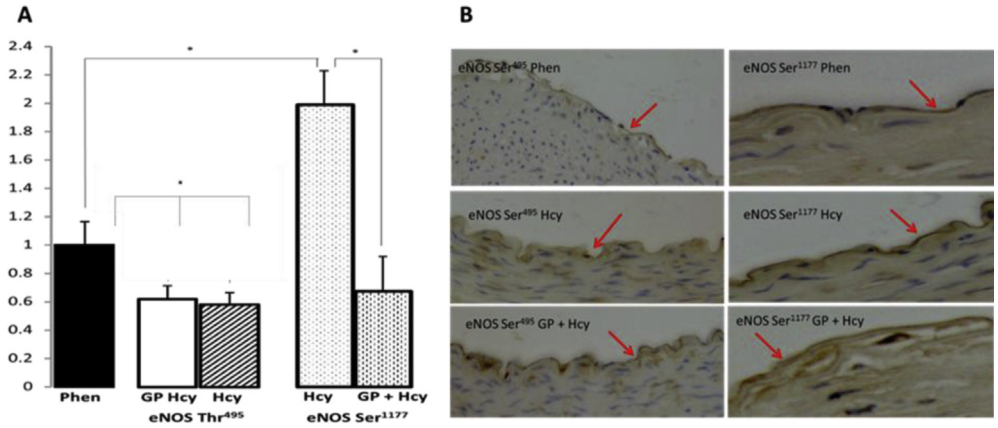

Figure 3 A) Quantification of eNOS phosphorylation at Thr^{495} and Ser^{1177} in response to Phen and with pre-treatment of Hcy or GP + Hcy in chow rings; * = $p < 0.05$). B) Comparison of phosphorylation of eNOS to Phen at Thr^{495} and $Ser^{1177}$with pre-treatment of Hcy or GP + Hcy. Arrows indicate the brown stain showing phosphorylation and presence of the eNOS protein. (Hcy = homocysteine, GP = gp91ds-tat, Phen = phenylephrine, eNOS = endothelial nitric oxide synthase, Ser = serine, Thr = threonine). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

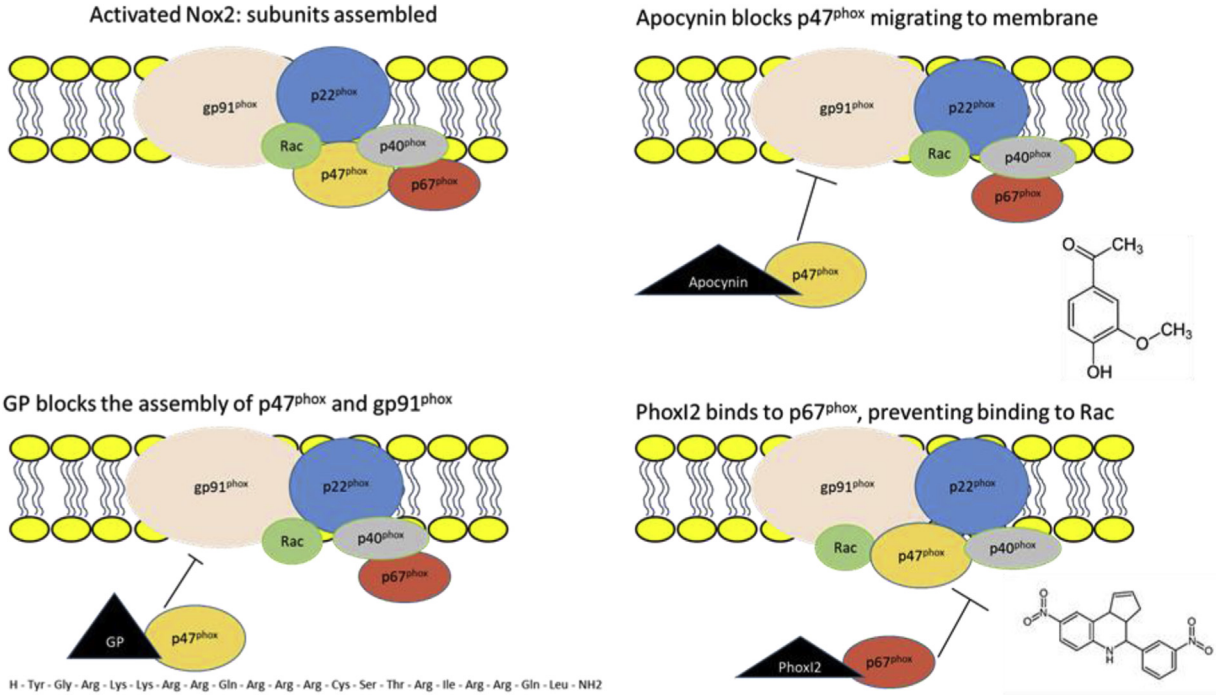

Figure 4 Nox2 assembly, inactivation by apocynin, GP or PhoxI2 and their structure. Nox2 inactivation results in reduced O_2^- production [37].

Our results indicate that apocynin, PhoxI2 and GP might not be suitable to normalize impaired acetylcholine-mediated vasorelaxation caused by Hcy-induced stress as they decrease the level of ROS required for NO release [40]. Pyrogallol is a benzenetriol [19], and has been studied (in doses > 100 μM) as an inducer of impaired acetylcholine-mediated vasorelaxation [14,30] and cardiomyocyte impairment [17]. Pyrogallol generates O_2^-, H_2O_2 and the hydroxyl radical [30]. Surprisingly, we found that putative Nox2 inhibitors worsened acetylcholine mediated relaxation, and exacerbated HHcy induced reduction in acetylcholine mediated relaxation and significantly lowers the potency (EC_{50}) of ACh; this was somewhat mitigated by incubation with pyrogallol (Fig. 1D). This implies that a reduction in O_2^-, and not drug structure, that impaired acetylcholine mediated vasorelaxation.

We performed immunohistochemical analysis on rabbits incubated with acute HHcy. Pharmacological targeting of eNOS to improve phosphorylation has been effective in reducing atherosclerotic lesions in $apoE^{-/-}$ mice [56] and improving acetylcholine mediated vasorelaxation in obese C57BL/6 mice [24]. To assess eNOS phosphorylation, we saved tissue as soon as the ACh curve was complete, so as to preserve phosphorylation. Importantly, there was clear presence of endothelial eNOS, indicating that there was no loss of tissue to either apoptosis or necrosis. Reduced

phosphorylation of eNOS at Thr^{495} and increased phosphorylation at Ser^{1177} is required for proper eNOS activation [29]. At Thr^{495}, Hcy alone or in combination with GP significantly reduced phosphorylation compared to control (Fig. 3A, B). This is in line with the literature however does not correlate with our isometric tension studies, where we saw a reduction in relaxation from these treatments. This implies that, while important, dephosphorylation of eNOS at Thr^{495} may play a smaller role in vascular relaxation than phosphorylation at Ser^{1177}. There was increased phosphorylation of eNOS at Ser^{1177} in HHcy, suggesting eNOS activation as a compensatory mechanism to normalise vasodilation, perhaps by activation of the Akt pathway, which stimulates eNOS phosphorylation at Ser^{1177} to increase NO availability [27,55]. Paradoxically, we saw a reduction in ACh-dependent relaxation in HHcy, therefore we expected to see reduced eNOS phosphorylation at Ser^{1177}, suggesting that O_2^- is essential for downstream eNOS activation. This is supported by the improvement in relaxation when pyrogallol and SC79 were added. GP + Hcy virtually abolished acetylcholine mediated relaxation, suggesting that other Nox isoforms cannot compensate for this effect. However, GP + Hcy were not significantly reduced at Ser^{1177} compared to Phen, which supports the ACh constriction curve outcome. This was unexpected, as it has been well reported that a reduction in Nox2 activation is related to an increase in endothelial-dependent relaxation [25]. The increase in phosphorylation of Ser^{1177} on Hcy and concomitant reduction in GP + Hcy provides evidence that Nox2 is essential for eNOS activation. Reduced eNOS phosphorylation could be explained by an inactivation of Akt due to reduced activation of Nox2, which could imply that activation of this pathway in the development of arterial pathogenesis could be partially regulated by Nox2. Further analyses in the changes to phosphorylation at these sites could be expanded to include an eNOS overexpressing murine model. To our knowledge, this has not yet been investigated and would provide further insight into the mechanisms of Hcy-induced dysfunction.

Support for an essential vasodilatory role for O_2^- has been shown in a novel Nox2 over-expressed mouse, where the authors reported improved coronary vasodilation, eNOS activation and NO synthesis [46]. Additionally, exercise training in rats was shown to increase O_2^- and consequently eNOS phosphorylation, which is essential for proper ACh mediated vasodilation [6]. It is currently unknown whether the improvement in endothelial function in the presence to O_2^- was due to increased SOD activity or increased signalling directly activating eNOS; together both could have augmented downstream NO release [57]. In contrast, purported NOS uncoupling has been linked to excess oxidative products, possibly attributing to endothelial dysfunction in the mesenteric arteries of *Mthfr*$^{=/-}$ mice [54]. While these results provide an interesting insight in what can happen to the smaller, mesenteric vessels, they do not provide clear consensus on whether oxidative products help or hinder vasodilation in the aorta.

We used acetylcholine to measure endothelial-dependent relaxation, which is an established method of assessing NO release [20,36]. These isometric tension results are in line with previous studies from our lab, wherein a recent paper used the NO donor sodium nitroprusside (SNP) to show that normal relaxation occurs to the same extent in HHcy incubated vessels, and that a novel peptide of the renin angiotensin system can restore normal acetylcholine mediated vasorelaxation after exposure to Hcy [35].

Limitations

Pharmacological Nox inhibitors were used in this study based on previous findings [11,39,53]. Additionally, pharmacological Nox inhibitors are not 100% specific [8] and have not been fully ascertained [48], therefore may have off-target effects and limit the possible clinical implications. Additionally, there is evidence that in the smaller mesenteric vessels, NO bioavailability is impaired by oxidative stress caused by the uncoupling of NOS rather than NADPH [54]. As well, 3 mM Hcy is a supra-physiological dose, as levels up to 0.3 mM have been reported in some cases of chronic kidney disease. We have used 3 mM Hcy as previous studies in our laboratory using lower doses reduce, but do not significantly impair, blood vessel function after 1–2 h incubation. We suggest clinical monitoring of patient's blood pressure or FMD once these inhibitors are used clinically.

Conclusion

The results presented here provide evidence that the current putative Nox2 inhibitors, are unlikely to reduce HHcy induced vascular damage, and might even worsen damage. Further research into developing new Nox2 inhibitors should be sought for HHcy-induced disease.

Conflicts of interest

The authors report no conflict of interest.

Ethics approval and consent to participate

I hereby declare that ethics approval was sought and approved by the appropriate ethics committee (VUAEC#14/005). There were only animals used in this study.

Consent for publication

Consent for publication was sought from all authors.

Availability of data and materials

All authors have access to data and material.

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Author contributions

Ms. Smith wrote the manuscript with additional editing from Dr. Kruzliak, Prof. Hayes, and Dr. Zulli. The experimental data was compiled by Ms. Smith, Mr. Rai and Dr. Zulli. The analysis was performed by Ms. Smith with input from Dr. Zulli.

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