Impaired Nitric Oxide Mediated Vasodilation In The Peripheral Circulation In The R6/2 Mouse Model Of Huntington’s Disease

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Recent evidence shows that the Huntington’s disease (HD) extends beyond the nervous system to other sites, including the cardiovascular system. Further, the cardiovascular pathology pre-dates neurological decline, however the mechanisms involved remain unclear. We investigated in the R6/2 mouse model of HD nitric oxide (NO) dependent and independent endothelial mechanisms. Femoral artery reactivity was determined by wire myography in wild type (WT) and R6/2 mice at 12 and 16 weeks of adulthood. WT mice showed increased endothelial relaxation between 12 and 16 weeks (Rmax: 72 ± 7% vs 97 ± 13%, P < 0.05). In contrast, R6/2 mice showed enhanced endothelial relaxation already by 12 weeks (Rmax at 12w: 72 ± 7% vs. 94 ± 5%, WT vs. R6/2, P < 0.05) that declined by 16 weeks compared with WT mice (Rmax at 16w: 97 ± 13% vs. 68 ± 7%, WT vs. R6/2, P < 0.05). In WT mice, the increase in femoral relaxation between 12 and 16 weeks was due to enhanced NO dependent mechanisms. By 16 weeks of adult age, the R6/2 mouse developed overt endothelial dysfunction due to an inability to increase NO dependent vasodilation. The data add to the growing literature of non-neural manifestations of HD and implicate NO depletion as a key mechanism underlying the HD pathophysiology in the peripheral vasculature.
endothelial dysfunction in peripheral resistance circulations, which pre-dates the neurological manifestations of HD. Given that HD patients and R6/2 mice display evidence of sympathetic autonomic dominance\textsuperscript{8–10}, we further investigated femoral artery $\alpha_1$-adrenergic constrictor function in addition to NO-dependent and independent dilator function in the R6/2 mouse at 12 and 16 weeks of age, corresponding to early symptomatic and established impaired neurological stages of the disease process, respectively\textsuperscript{16}.

**Methods**

**Animals.** All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were overseen by the United Kingdom Home Office. Further local ethics committee approval was obtained from the University of Cambridge. Only male mice were used to control for sex differences. Male mice were taken from a colony of R6/2 transgenic mice established in the Department of Pharmacology, University of Cambridge, and maintained by backcrossing onto CBA × C57BL6N F1 female mice\textsuperscript{17}. Genotyping and CAG repeat length measurement were carried out by Laronen (Los Angeles, CA, USA) as described previously\textsuperscript{18}. The transgenic mice used in this study had a mean CAG repeat length of 242 ± 1 (range 237–251). Mice were kept in home cages comprising single sex, single genotype groups of ten. All of the mice lived in an enhanced environment with increased amounts of bedding and nesting materials. Clean cages were provided twice weekly with grade 8/10-corn cob bedding, and finely shredded paper for nesting. The mice were maintained on a 12:12 hour light/dark (LD) cycle, at a temperature of 21–23°C and a humidity of 55 ± 10%. The mice had ad libitum access to water and dry laboratory food (RM3(E) rodent pellets, Special Diet Services, Witham, UK). In addition, once a day, a mash was prepared by soaking 100 g dry food in 230 ml water until the pellets were soft and fully expanded. The mash was placed on the cage floor, improving access to food and water for the R6/2 transgenic mice. This feeding regime has been shown previously to be beneficial\textsuperscript{19}.

**In vitro wire myography.** Segments of femoral arteries were mounted on a wire myograph, as previously described in detail\textsuperscript{20,21}. In brief, under a bifocal dissecting microscope (Brunel Microscopes Ltd., Wiltshire, UK), the first branch from the femoral artery of the left hind limb was excised and placed in ice cold saline solution. The vessel was carefully cleaned of excess connective tissue and cut to a 2 mm long ring. Two 40 μm diameter stainless steel wires were carefully threaded through the lumen of the femoral sections, maintaining the endothelium intact. The wires were then placed between the mounting support jaws of a 4-chamber small-vessel wire myograph (Multi Wire Myograph System 610M; DMT, Aarhus, Denmark) containing warmed oxygenated Krebs buffer (NaCl 118.5 mM, Fisher Scientific; KCl 4.75 mM, Sigma; MgSO$_4$·7H$_2$O 1.2 mM, Sigma; KH$_2$PO$_4$ 1.2 mM, Sigma; NaHCO$_3$ 25.0 mM, Sigma; CaCl$_2$ 2.5 mM, Sigma; glucose 11.1 mM, Sigma, UK; bubbled continuously with 95% O$_2$/5% CO$_2$ mix, 37°C).

Force data from the myograph were recorded at 4 Hz (Labchart 6.0, Powerlab 8/30; AD Instruments, Chalgrove, UK), and each vessel was standardized to an optimal working tension of 100 mM H$_2$O\textsuperscript{22}. Both vasoconstrictor and vasodilator function was assessed in the vessels. All vessels were contracted with increasing concentrations of potassium chloride (KCl, 4.75–100 mM). Vasoconstrictor function to phenylephrine (PE: 10$^{-4}$ to 10$^{-1}$ M; Sigma Aldrich, Poole, UK) and vasodilator responses to sodium nitroprusside (SNP: 10$^{-5}$ to 10$^{-1}$ M; Sigma Aldrich) and endothelium-dependent vasodilatation with the acetylcholine analogue methacholine (10$^{-9}$ to 10$^{-4}$ M; Sigma Aldrich) were assessed. Vasoconstrictor responses to phenylephrine were normalised to the response of KCl at 40 mM for each vessel to standardise for any differences in muscle mass. Vasodilator responses with methacholine were assessed with no additive in the Krebs and separately with N(G)-nitro-L-arginine methyl ester (L-NAME, an endothelial nitric oxide synthase inhibitor; Sigma Aldrich, Poole, UK). All vasodilator responses were assessed with the vessels pre-contracted with PE (10$^{-5}$ M) at a stable plateau. Responses were recorded for 2 minutes after each dose, whereupon the next cumulative dose was given. Vessels were repeatedly washed with Kreb's solution and allowed to equilibrate for at least 20 minutes between different concentration-response curves.

**Analysis and statistics.** Individual vessel responses were fitted to a Boltzmann sigmoidal (K’) or non-linear log (agonist) vs. response (PE, SNP, MetCh; GrpahPad Prism), as previously described in detail\textsuperscript{23}. Maximal responses for each vessel and drug were taken from the curve fit value. Sensitivity was defined as the concentration of agonist required to elicit fifty percent of the maximal response expressed as either the EC$_{50}$ (Excitatory concentration to achieve 50% maximal response) or pD$_{50}$ (where pD$_{50} = \log_{10} EC_{50}$)\textsuperscript{24}. An index of total endothelial relaxation (NO dependent + NO independent) was calculated in each vessel by calculating the area above the methacholine relaxation curve between 10$^{-4}$ and 10$^{-3}$ M as previously described\textsuperscript{21,23}. An index of NO independent relaxation was calculated by the area above the methacholine relaxation curve in the presence of L-NAME and NO dependent relaxation was calculated as the difference between total and NO independent relaxation\textsuperscript{21,23}. All data were compared statistically by Two-Way ANOVA followed by post hoc Bonferroni test where significant effects of WT vs R6/2, 12 vs 16 weeks of age or an interaction were found. Significance was accepted when P < 0.05 (SigmaStat 2.0; SPSS Inc., Chicago, USA).

**Results**

**Femoral artery vasodilator function.** Both SNP and methacholine led to dose dependent vasodilatation in femoral arteries from WT and R6/2 mice at 12 and 16 weeks of age in vessels pre-contracted with phenylephrine (Fig. 1, P < 0.05). There were no significant differences in the maximal response to SNP in any group, suggesting no differences in vascular smooth muscle potential to vasodilate (Fig. 1). WT mice showed an increase in endothelial dependent relaxation in response to methacholine between 12 and 16 weeks of adulthood (Figs 1 and 2). In contrast, R6/2 mice showed an increase in endothelial dependent relaxation already at 12 weeks and a significant decline by 16 weeks compared to WT mice at corresponding ages (Fig. 1). When NO dependent
and independent mechanisms underlying the endothelium-mediated relaxation were investigated, the increase in vasodilator capacity in the WT mice at 16 weeks was due to an increase in NO-dependent relaxation (Fig. 2). In contrast, this increase in NO-dependent endothelial mediated relaxation in the femoral resistance vessel with increasing age was not present in the R6/2 mouse (Fig. 2).
Femoral artery vasoconstrictor function. At both 12 and 16 weeks of age, application of KCl and PE led to dose dependent increases in femoral arterial tension in both WT and R6/2 mice (Fig. 3). WT mice showed a decrease in the maximal vasoconstrictor response to PE with advancing age from 12 to 16 weeks. In contrast, the R6/2 mouse showed impaired maximal constrictor responses to PE already at 12 weeks compared to WT, which was not worsened further by 16 weeks (Fig. 3). There were no differences in the maximal response or sensitivity to KCl.

Discussion
The striking neurological manifestations of HD have led investigators to focus on the nervous system in search of the underlying pathology and management of this disorder. However huntingtin, the mutated protein, is expressed extensively at many extra-neuronal sites including the heart and circulatory system. Here, we show that endothelial function is significantly altered in peripheral resistance arteries of the R6/2 mouse from 12 weeks of age and grossly impaired by 16 weeks. In contrast, the R6/2 mouse showed impaired maximal constrictor responses to PE already at 12 weeks compared to WT, which was not worsened further by 16 weeks (Fig. 3). There were no differences in the maximal response or sensitivity to KCl.

Several mechanisms may be involved in triggering dysfunction in peripheral resistance circulations. In the present study, our data suggest that in the R6/2 mouse model of HD alterations in endothelial function and in NO biology may contribute to the pathophysiological decline. It is now understood that the cellular oxidant milieu is an important modulator of peripheral vascular resistance. In resistance vessels, the balance between superoxide (O$_2^-$) and NO has important effects on endothelial function, both under physiological regulation and in disease pathology. Under physiological conditions, the ratio of vascular NO:O$_2^-$ is an important determinant of vascular tone. Therefore, during conditions in which NO production outweighs O$_2^-$ production, the increase in concentrations of superoxide is likely to compromise NO-dependent mechanisms, leading to impaired endothelial function and an increase in vascular tone.
in the NO:O$_2^-$ ratio promotes vasodilation$^{25,26}$. When vascular O$_2^-$ production outweighs NO, this leads to vascular constriction and an increase in peripheral vascular resistance$^{23,24}$. Under pathological conditions, excess generation of reactive oxygen species and oxidative stress may therefore decrease NO bioavailability and promote endothelial dysfunction. Interestingly, it is already known that the huntingtin protein promotes oxidative stress$^{27}$. Indeed, reactive oxygen species mediated mitochondrial DNA damage$^{28}$, general protein nitrosylation$^{27}$ and lipid peroxidation$^{27,28}$ are all found in HD. Further, mitochondrial dysfunction has been strongly associated with a rise in oxidative stress in HD$^{27,30}$. Specifically the R6/2 mouse displays dysfunction of voltage-dependent anion channel 1$^{31}$, which is a key component of the mitochondrial permeability transition pore and a regulator of superoxide anion leakage from mitochondria$^{30,32}$. Collectively, past and present data therefore suggest impaired NO signaling as one mechanism underlying the inability of the R6/2 mouse to increase femoral artery vasodilation from 12 to 16 weeks of adult age. The enhanced femoral artery dilatation at 12 weeks of age in the R6/2 mouse may represent a compensatory response to impaired NO bioavailability, an adaptive response which is later decompenesated as NO-dependent vasodilation fails at 16 weeks.

Additional data in the present study show no alteration in peripheral vasoconstrictor function in response to potassium between the groups but a markedly impaired femoral artery $\alpha_1$-mediated constrictor response already at 12 weeks of life in the R6/2 mouse, akin to that measured in the WT mouse much later at 16 weeks of age. It is known that HD patients show a sympathetically dominated autonomic nervous system phenotype$^{8–10}$. As well at 12 weeks of life in the R6/2 mouse, akin to that measured in the WT mouse much later at 16 weeks of age. It is known that HD patients show a sympathetically dominated autonomic nervous system phenotype$^{8–10}$. As well as driving sympathetically dominant patterns of heart rate variability and structural and functional abnormalities in the heart, it is possible that the autonomic sympathetic outflow to the vasculature is also enhanced in the R6/2 mouse. In the R6/2 mouse, it has been suggested that the cardiomyopathy is caused by altered central autonomic pathways, since neither mutant huntingtin aggregates nor a HD-specific transcriptional dysregulation was identified in cardiac tissue, even at the end stage of disease$^{13}$. While aggregates of mutant huntingtin are a hallmark of HD brain, their presence (or lack thereof) does not always correlate with pathological cellular pathology. Therefore, it is possible that cell autonomous pathology in cardiac tissue is present. This remains to be investigated directly. Nevertheless, alterations in the metabolic and endocrine milieu which impact on vascular function are established in HD. For example, plasma cortisol levels are raised in both the R6/1 and R6/2 mouse model of HD, an effect which would also contribute to enhance $\alpha_1$-adrenergic receptor mediated peripheral vasoconstriction$^{15,33,34}$. In the R6/1 model of HD, plasma noradrenaline levels are reported to be 4–5x higher than WT animals at 7 months of age, when the R6/1 animal shows advanced HD-like phenotype$^{35}$. Sustained increases in sympathetic nervous system activity and in circulating catecholamine and glucocorticoids are strongly associated with endothelial dysfunction and cardiovascular disease in general$^{36}$. Therefore, the accelerated loss of the $\alpha_1$-adrenergic receptor mediated reactivity in peripheral resistance vessels of the R6/1 mouse may represent an adaptive response, down-regulating $\alpha_1$-adrenoceptor responsiveness to an increased sympathetic drive, which is exacerbated by increased circulating plasma glucocorticoid concentrations. Interestingly, in human patients with Huntington’s disease there appear to be no substantial differences in hypothalamic-pituitary-adrenal axis control$^{37}$.

Finally, changes in sympathetic stimulation combined with expression of the huntingtin protein may lead to structural alterations in resistance vessels. Defects have been reported in neurovascular tissue from humans with HD and in mouse models at the level of the blood brain barrier$^{26}$, although in the R6/1 mouse there is no apparent peripheral vascular remodelling in mesenteric or caudal arteries$^{15}$. In support of this, in the present study, responses to potassium chloride, which reflect a global measure of vascular smooth muscle structure, mass and function, were not altered in the R6/2 compared to WT mouse.

Translational perspective. Clinical studies show clearly that cardiovascular dysfunction is present in HD. Furthermore, the recent body of evidence in animal models of HD confirms that the systemic over-expression of huntingtin leads to premature cardiac and vascular dysfunction. Despite this, in clinical practice, HD is still perceived almost exclusively as a neurological disorder due to the overt neurological manifestations, with little or no clinical appreciation for extra-neuronal sites of expression of the disease. Using an established mouse model of HD, here we show that endothelial NO-dependent function is markedly impaired in the peripheral vasculature. Collectively, past and present data therefore support increasing attention to the monitoring of the cardiovascular system in patients with HD. In particular, investigation of whether treatment with drugs that enhance NO bioavailability already in common clinical use, such as statins, may protect peripheral vascular function in HD patients is warranted.

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Author Contributions
A.J.M. and D.A.G. conceived the study. A.D.K., Y.N. and E.A.H. performed the experiments. A.D.K., A.J.M. and D.A.G. performed the data analysis, prepared figures and wrote the manuscript. All authors read and approved the manuscript for submission.

Additional Information

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