

■ H Y P O T H E S I S

Exercise and possible molecular mechanisms of protection from vascular disease and diabetes: the central role of ROS and nitric oxide

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A B S T R A C T

It is now widely accepted that hypertension and endothelial dysfunction are associated with an insulin-resistant state and thus with the development of T2DM (Type 2 diabetes mellitus). Insulin signalling is impaired in target cells and tissues, indicating that common molecular signals are involved. The free radical NO[•] regulates cell metabolism, insulin signalling and secretion, vascular tone, neurotransmission and immune system function. NO[•] synthesis is essential for vasodilation, the maintenance of blood pressure and glucose uptake and, thus, if levels of NO[•] are decreased, insulin resistance and hypertension will result. Decreased blood levels of insulin, increased AngII (angiotensin II), hyperhomocysteinaemia, increased ADMA (asymmetric ω-N^G,N^G-dimethylarginine) and low plasma L-arginine are all conditions likely to decrease NO[•] production and which are associated with diabetes and cardiovascular disease. We suggest in the present article that the widely reported beneficial effects of exercise in the improvement of metabolic and cardiovascular health are mediated by enhancing the flux of muscle- and kidney-derived amino acids to pancreatic and vascular endothelial cells aiding the intracellular production of NO[•], therefore resulting in normalization of insulin secretion, vascular tone and insulin sensitivity. Exercise may also have an impact on AngII and ADMA signalling and the production of pro- and anti-inflammatory cytokines in muscle, so reducing the progression and development of vascular disease and diabetes. NO[•] synthesis will be increased during exercise in the vascular endothelial cells so promoting blood flow. We suggest that exercise may promote improvements in health due to positive metabolic and cytokine-mediated effects.

Key words: diabetes, endothelial vascular cell, exercise, nitric oxide, pancreatic β-cell, reactive oxygen species (ROS).

Abbreviations: ADMA, asymmetric ω-N^G,N^G-dimethylarginine; AMPK, AMP-activated protein kinase; AngII, angiotensin II; AT1R, AngII type 1 receptor; CAT, catalase; CVD, cardiovascular disease; DDAH, dimethylarginine dimethylhydrolase; GPX, glutathione peroxidase; GSIS, glucose-stimulated insulin secretion; IGF-1, insulin-like growth factor-1; IL, interleukin; L-NMMA, N^G-monomethyl-L-arginine; MLC, myosin light chain; MRP, multidrug-resistance protein; NF-κB, nuclear factor κB; NOS, NO[•] synthase; eNOS, endothelial NOS; iNOS, inducible NOS; O₂^{•-}, superoxide radical; ONOO[•], peroxynitrite; nNOS, neuronal NOS; PI3K, phosphoinositide 3-kinase; RAS, renin-angiotensin system; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, Type 2 diabetes mellitus.

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INTRODUCTION

The beneficial effects of aerobic exercise of moderate intensity in T2DM (Type 2 diabetes mellitus) are well documented with regard to glycaemic control and multiple CVD (cardiovascular disease) risk factors. Improvements in glycaemic control included improvements in Hb_{1Ac} (glycated haemoglobin), insulin sensitivity and AUC (area under the curve) during an OGTT (oral glucose tolerance test); improvements in vascular function included changes in endothelial function (e.g. flow-mediated dilation), carotid artery intima-media thickness and arterial distensibility; and improvements in metabolic control included glycaemic control, weight loss and improvement in lipid profile [1].

Although the exercise-induced improvements in the parameters described above are widely accepted, the molecular basis underlying the improvement is still open to debate. We wish to put forward a hypothesis linking amino acid availability, ROS (reactive oxygen species) generation, RNS (reactive nitrogen species) generation and muscle–islet–vascular endothelial cell communication.

Diminished insulin sensitivity is a characteristic feature of various pathological conditions, such as the cardiometabolic syndrome, T2DM and hypertension. Persons with essential hypertension are more prone than normotensive subjects to develop diabetes, and this propensity may reflect the decreased ability of insulin to promote relaxation and glucose transport in vascular and skeletal muscle tissue respectively [2].

The free radical NO• is one of the most widespread signalling molecules in mammalian biology and participates in virtually every cellular and organ function in the body, for example physiological levels of NO• produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leucocyte adhesion, platelet aggregation, angiogenesis and thrombosis. In addition, NO• produced by neurons serves as a neurotransmitter, and NO• produced by activated macrophages is an important mediator of the immune response [3]. However, as an oxidant and as an inhibitor of proteins containing an iron-sulfur centre, excess production of NO• may exert detrimental effects in sensitive cells and tissues and thus may have an impact on cardiovascular function [4].

Muscle contraction and exercise increase glucose uptake into skeletal and cardiac muscle, via a pathway independent to that stimulated by insulin (Figure 1A). Indeed, skeletal muscle glucose uptake is similar during exercise in insulin-resistant individuals or patients with diabetes compared with healthy individuals [5]. By the use of NOS (NO• synthase) inhibition, it was

demonstrated that NO• was required for glucose uptake during exercise in individuals with T2DM compared with healthy controls [6]. Moreover, NO• donors, such as SNP (sodium nitroprusside), increased glucose uptake in primary human skeletal muscle cells derived from both healthy individuals and patients with T2DM [7]. Thus, in skeletal muscle and other insulin-sensitive tissues, inhibition of NO• production will culminate in blunted glucose transport and, subsequently, in insulin resistance (Figure 1B).

The role of the NO• in the cardiovascular system and regulation of blood pressure has been studied extensively [8]. This gas is continuously synthesized from the amino acid L-arginine by endothelial cells, utilizing the constitutive Ca²⁺/calmodulin-dependent enzyme NOS. The principal physiological stimulus for NO• synthesis and release from the endothelium is probably the shear stress of blood flowing over the surface of the vessel by a non-receptor-dependent mechanism. NO•, released from the endothelium as a gas or attached to other molecules, stimulates soluble guanylate cyclase in vascular smooth muscle underlying the endothelium, producing increased concentrations of cGMP, thus activating cGMP-dependent kinases that promote relaxation [9] (Figure 1A). Endothelial dysfunction, which is defined by decreased endothelium-dependent vasodilation, is associated with an increased number of cardiovascular events. NO• bioavailability is reduced by altered endothelial signal transduction or increased formation of O₂^{•-} (superoxide radical) reacting with NO• to produce ONOO• (peroxynitrite). Endothelial dysfunction is therapeutically reversible and physical exercise, calcium-channel blockers, ACEIs (angiotensin-converting enzyme inhibitors) and angiotensin receptor antagonists improve flow-evoked endothelium-dependent vasodilation in patients with hypertension and diabetes [10].

Physiological concentrations of NO• are essential for normal skeletal muscle and endothelial function, and are also required for optimal insulin secretion from pancreatic β -cells [11]. It has long been recognized that L-arginine, the immediate precursor of NO•, is one of the most potent nutrient secretagogues with respect to β -cell insulin release [12], while L-arginine deficiency is associated with insulinopenia and a failure to secrete insulin in response to glucose [13]. Hence, although NO• may be cytotoxic for β -cells at pathological high concentrations (for review, see [11]), at physiological conditions L-arginine-derived NO• is likely to contribute to the process of stimulating insulin release [14].

In summary, decreased production of NO• within the cells of the body will result in dysfunction resulting in decreased blood flow, glucose transport, insulin action, insulin secretion and, thus, give rise to hypertension and diabetes.

HYPERTENSION, INSULIN RESISTANCE, DIABETES AND EXERCISE: A UNIFYING HYPOTHESIS BASED ON NO• AVAILABILITY

Hypertension in most patients is associated with sustained increases in systemic arteriolar tone compared with normotensive subjects [9]. It was reported by Panza et al. [15] that L-NMMA (*N*^G-monomethyl-L-arginine; a non-selective NOS inhibitor) infused into the brachial artery of hypertensive patients was less effective on basal forearm flow compared with normotensive subjects, suggesting that the basal release of NO• is deficient in hypertension. The RAS (renin–angiotensin system) is activated in diseased vascular beds, with the up-regulation of the two known AngII (angiotensin II) receptors: AT1R (AngII type 1 receptor) and AT2R (AngII type 2 receptor). Increased AT1R-mediated activity in the vasculature is central to the development of increased arterial stiffness and is enhanced in insulin-resistant states [16].

There is increasing evidence that AngII inhibits insulin and IGF-1 (insulin-like growth factor-1) signalling through the PI3K (phosphoinositide 3-kinase)/Akt pathway (downstream serine/threonine kinases activated by insulin and IGF-1 receptor signalling), resulting in inhibition of mechanisms involved in the vasodilator and glucose transport properties of insulin and IGF-1 [17]. The main mechanism involved in these effects of AngII appears to be by the generation of ROS such as O₂^{•-} [18]. ROS are able to inhibit actions mediated through PI3K/Akt, including activation of eNOS (endothelial NOS) activity, Na⁺ pump activation and Ca²⁺-MLC (myosin light chain) desensitization [2].

AngII, acting through AT1R, increases the generation of O₂^{•-} in the vasculature, primarily through the activation of membrane-bound NADPH oxidase [18]. O₂^{•-} reacts with bioavailable NO• to form ONOO• in endothelial cells, a very reactive and destructive molecule. Moreover, O₂^{•-} is a known inhibitor of a key regulatory enzyme, DDAH (dimethylarginine dimethylhydrolase), which controls the metabolism of ADMA (asymmetric ω-*N*^G,*N*^G-dimethylarginine).

ADMA is an endogenous methylated amino acid that inhibits the constitutive eNOS and nNOS (neuronal NOS) isoforms, but is a less potent inhibitor of the iNOS (inducible NOS) isoform [19]. ADMA is released by protein hydrolysis and exported from the cell and taken up by other cells via system y⁺ carriers of the cationic amino acid family, and is eliminated both by renal excretion and metabolic degradation [20]. As DDAH utilizes ADMA as a substrate and regulates plasma levels of ADMA, it can determine the bioavailability of NO• and thus blood pressure.

Increased levels of AngII and the activation of its receptor (AT1R) could lead to the production of O₂^{•-}, followed by inhibition of DDAH and thus increased levels of ADMA in the kidneys, as suggested previously [21].

The high concentration of ADMA could result in a reduction in NO• synthesis, culminating in increased intra-kidney blood pressure and, chronically, to nephropathy.

Curiously, many cardiovascular risk factors, such as hyperhomocysteinaemia, are found to be elevated in diabetic patients with renal complications, indicating that the kidneys have a key function in homocysteine metabolism [22]. Homocysteine (an indirect substrate for the synthesis of ADMA) is also an inhibitor of the enzyme DDAH (by oxidation of a sulfhydryl group) [23]. Thus homocysteine is capable of inducing a further increment in ADMA level thus decreasing the availability of NO•, resulting in impaired cardiovascular and renal function [24].

The production of NO•, by any of the NOS enzymes, is dependent on the extracellular levels of the amino acid L-arginine. The kidneys have a key role in the endogenous synthesis of this amino acid, being the main organ for L-citrulline uptake and metabolism. L-Citrulline is released from the metabolism of L-glutamine in the gut. The kidney is able to convert L-citrulline into L-arginine and release the latter into the blood. Other tissues express the key enzymes for arginine synthesis from citrulline (argininosuccinate synthetase and lyase), but do not promote net delivery to the circulation [11]. In fasted humans, the contribution of glutamine via L-citrulline to the *de novo* synthesis of L-arginine is approx. 65% [25]. Considering that the kidney is the main supplier of L-arginine for physiological NO• synthesis, renal complications could induce a reduction in L-arginine concentrations, leading to negative consequences in the vasculature and pancreatic islets. In agreement with our hypothesis is the fact that the plasma and tissue concentrations of L-arginine are decreased in patients with T2DM [26], and that L-arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP levels. Moreover, low levels of L-arginine can result in ‘uncoupling of NOS enzymes’, when in the absence of sufficient L-arginine the enzyme donates electrons to oxygen-forming O₂^{•-} [27].

Of additional relevance, the levels of ET-1 (endothelin-1; a locally produced vasoactive peptide released from vascular endothelial cells) are often elevated in patients with hypertension, obesity, T2DM and peripheral vascular resistance, as well as other disease states associated with CVD. ET-1 release may block the action of insulin with respect to haemodynamic regulation and thus may contribute to localized insulin resistance [28].

To summarize, the actions of AngII on the AT1R could stimulate O₂^{•-} formation, inhibition of DDAH, raised levels of ADMA and inhibition of NOS, so blunting NO• production in endothelial cells, which then gives rise to renal and cardiovascular complications. Chronic kidney disease could result in increased homocysteine levels (exerting a positive feedback on the production of ADMA and oxidative stress), resulting

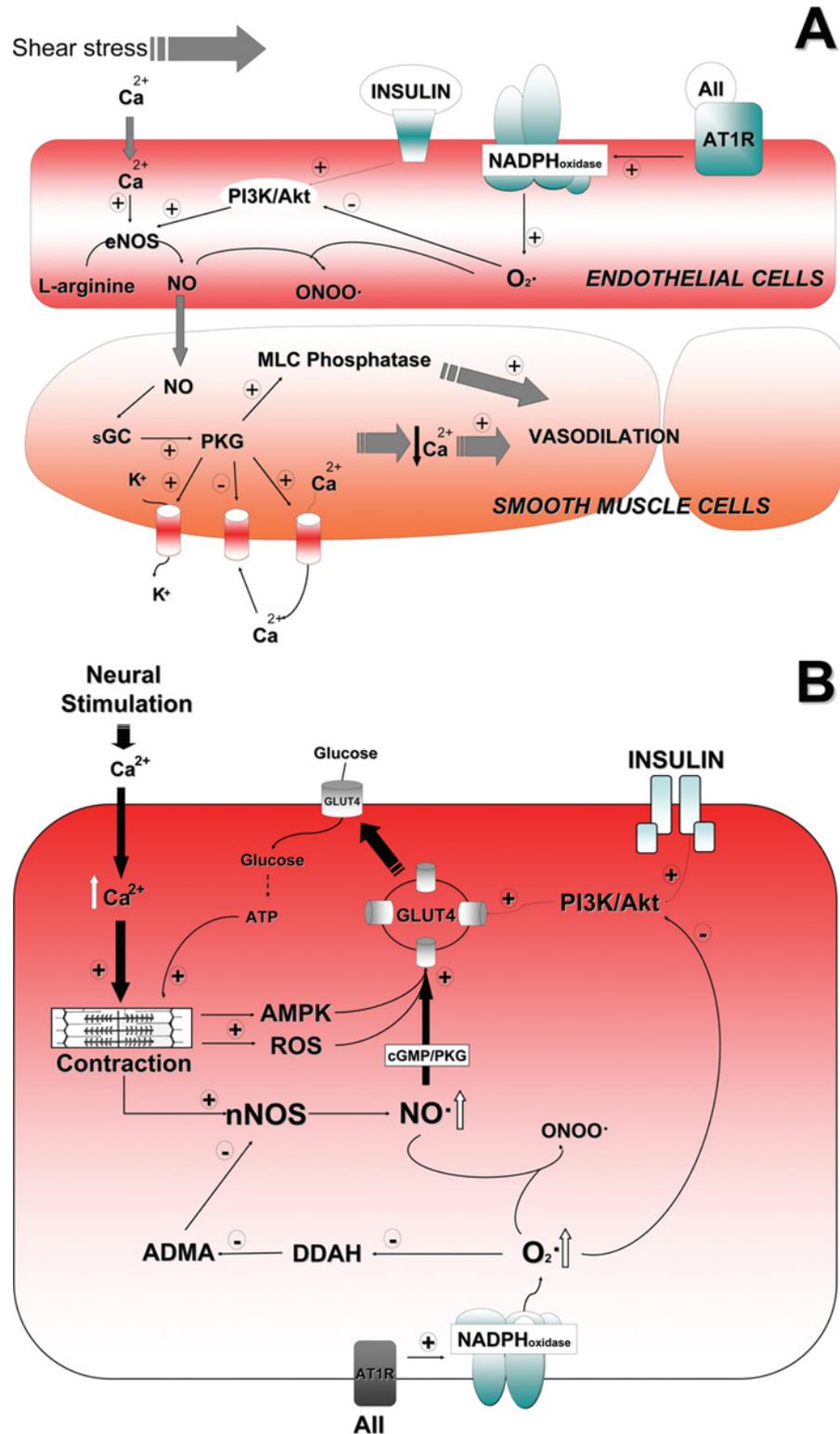


Figure 1 General mechanisms of NO \cdot actions on vessel relaxation (A) and glucose uptake by the skeletal muscle (B)

(A) Endothelial shear stress or other stimuli results in increased intracellular Ca^{2+} and, hence, eNOS activation. NO \cdot is released from the endothelial cells and diffuses to the smooth muscle cells located nearby. NO \cdot binds and activates soluble guanylate cyclase (sGC) in the vascular smooth muscle underlying the endothelium, producing increased concentrations of cGMP. The increase in cGMP thus activates cGMP-dependent kinases that promote relaxation by altering the activity of K^+ and Ca^{2+} channels, leading to cell hyperpolarization and decreased intracellular Ca^{2+} respectively. PKG (protein kinase G) can also activate MLC phosphatases that promote myosin dephosphorylation and then vessel relaxation. Insulin induces vasodilation via a PI3K/Akt-dependent mechanism. AngII (All) activates the NADPH oxidase

in impaired L-arginine production, leading to amino acid deficiency and further complications with respect to hypertension, insulin resistance and possibly β -cell function. This unifying hypothesis is summarized in Figure 2. Finally, low levels of L-arginine coupled with increased production of oxygen free radicals in pancreatic β -cells in patients with CVD and T2DM could result in an accelerated decline in function, as discussed below.

ROS, NO[•] AND L-ARGININE METABOLISM IN PANCREATIC β -CELLS

Under conditions of elevated metabolism or enhanced mitochondrial activity, many tissue-specific cells are continuously subjected to insults from ROS. The damage inflicted by ROS has been implicated in conditions of inflammation, T2DM, age-related degeneration and tumour formation [29]. Intracellular O₂^{•-} may combine with NO[•] to generate ONOO[•], which may cause the inhibition of activity of a number of key signal transducing or metabolic enzymes [30]. Overproduction of ROS or a failure in intracellular defences against ROS will result in the pathogenesis of disease [30] including diabetes. Some of the important mechanisms related to the production of ROS and NO[•] and intracellular defence mechanisms are summarized in Figure 3. The molecular basis for excessive mitochondrial oxidative damage in diabetes has been expertly reviewed elsewhere [31].

Cells require antioxidant systems to neutralize ROS (Figure 3). For example, O₂^{•-} is enzymatically converted into H₂O₂ by a Mn-SOD [manganese SOD (superoxide dismutase)] within mitochondria. H₂O₂ can then be rapidly removed by the mitochondrial enzyme GPX (glutathione peroxidase). The inner mitochondrial membrane also contains vitamin E, which is a powerful antioxidant, as it can accept unpaired electrons to produce a stable product. A further antioxidant enzyme, CAT (catalase), is the major H₂O₂-detoxifying enzyme found exclusively in peroxisomes (Figure 3) [30]. In addition to the classic antioxidant enzymes, MRPs (multidrug-resistance proteins), such as the MRP1 pump (a transmembrane protein that acts by exporting intracellular glutathione disulfide, reducing accumulation and redox imbalance), are also important [32].

Interestingly, compared with many other cell types, the β -cell may be at high risk of oxidative damage with an increased sensitivity for apoptosis. This high risk may be due to (i) excessive levels of mitochondrial ROS generation; (ii) additional ROS generation through elevated β -cell NADPH oxidase activity (see below); and (iii) failure of antioxidant defence. With respect to T2DM, β -cell dysfunction and associated depressed insulin secretion must be evident before hyperglycaemia develops [33].

Specific isoforms of the O₂^{•-}-generating NADPH oxidase family (i.e. NOX 1–4, which differ in their requirement for, and translocation to the membrane of, cytosolic protein subunits) are an important source of ROS in non-phagocytic cells including pancreatic islets [34]. However, low levels of ROS have recently been shown to be essential for optimal GSIS (glucose-stimulated insulin secretion) [35].

The involvement of 'non-damaging' levels of ROS in signal transduction is now firmly accepted, and examples include roles in cell growth or programmed cell death (apoptosis) [36], kinase activation [37], immune responses [38], cell calcium signalling [39] and gene expression [40]. For example, increased iNOS expression in response to redox-dependent transcription factor NF- κ B (nuclear factor κ B) activation is a specific example of ROS-regulated gene expression. Vascular tone and inhibition of platelet adhesion is regulated by an NO[•]- and H₂O₂-dependent activation of guanylate cyclase. AngII, thrombin, PDGF (platelet-derived growth factor) and TNF- α (tumour necrosis factor- α) are known to increase ROS production in vascular smooth muscle cells through the activation of an isoform of the NOX family of NADPH oxidase.

A decrease in L-arginine concentration can lead to β -cell dysfunction, such as reduced insulin secretion, as it is a substrate for NO[•] synthesis [13]. Hypertension can lead to a decrease in levels of L-arginine, as discussed above, but, additionally in conditions of inflammation, macrophages release arginase [41] so reducing the availability of this critical amino acid. Indeed, L-arginine administration *in vivo* resulted in protection against the effects of many diabetic agents, such as alloxan and streptozotocin [42–46], by promoting β -cell neogenesis, increasing GPX [42], SOD and CAT activities, increasing GSH content [43], and reducing polyol and PKC (protein kinase C) pathway activation [45]. A single administration of watermelon, rich in L-citrulline (L-arginine precursor), reduced serum concentrations

in the endothelium cells, resulting in O₂^{•-} production, which, in turn, decreases NO[•] availability by reacting with NO[•], causing ONOO[•] formation. Skeletal muscle activation results in increased intracellular Ca²⁺, leading to contraction of muscle fibres and nNOS activation. (B) Skeletal muscle contraction also results in AMPK activation and ROS formation by different mechanisms, and both are likely to induce the translocation of GLUT4 (glucose transporter 4) to the plasma membrane. NO[•] also regulates GLUT4 translocation by a cGMP/PKG activation mechanism. Increased O₂^{•-} production from NADPH oxidase reduces the NO[•] availability due to reactivity with the gas and by the inhibition of a key enzyme, DDAH, leading to increased levels of ADMA, a known NOS inhibitor, causing reduced glucose uptake and insulin resistance.

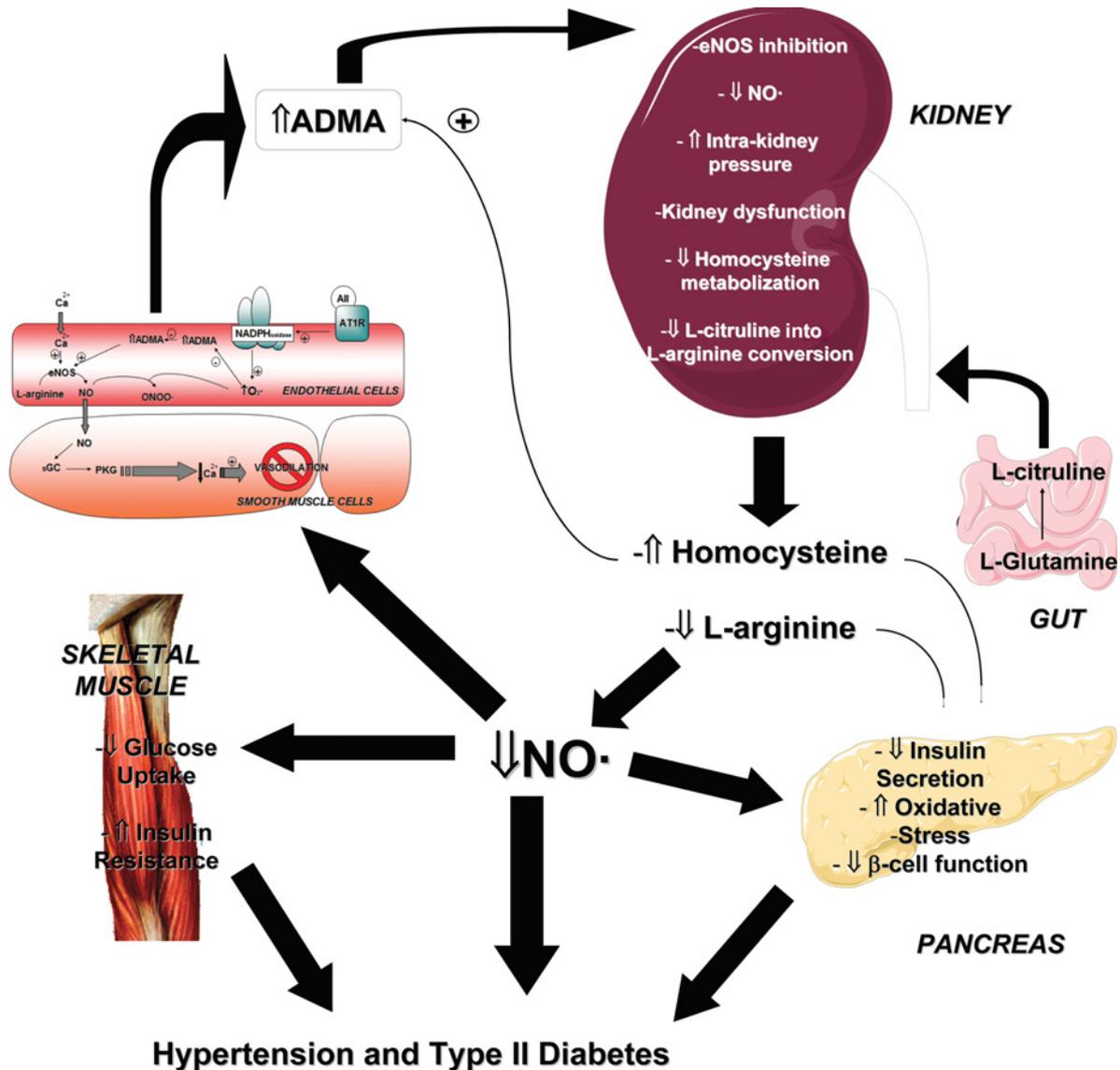


Figure 2 Unifying hypothesis of NO• bioavailability in hypertension, insulin resistance and diabetes

Increased levels of AngII (AII) induces, by its interaction with the endothelial cell AT1R, the activation of NADPH oxidase and increased O₂^{•-} production. O₂^{•-} decreases the availability of NO• by three main mechanisms: (i) interaction with NO• that results in ONOO• synthesis; (ii) inhibition of DDAH, a key regulatory enzyme for control of ADMA levels, which is a potent NOS inhibitor; and (iii) through inhibition of the PI3K/Akt pathway. The high levels of ADMA in the bloodstream can induce changes in kidney blood flow through the inhibition of eNOS activity and NO• production. The high intra-kidney pressure culminates in kidney dysfunction. The kidney is a key site for homocysteine and arginine metabolism, thus kidney dysfunction could lead to increased levels of homocysteine and low levels of L-arginine. As L-arginine is the precursor of NO• synthesis, then NO• synthesis in vital cells may be decreased. In addition, homocysteine is able to inhibit DDAH activity and thus ADMA accumulation, leading to increased ADMA levels and hypertension. The low availability of L-arginine (and NO• formation) could also lead to pancreatic β-cell dysfunction and decreased insulin secretion, thus compounding the problematic metabolic status.

of cardiovascular risk factors such as homocysteine, improved glycaemic control and ameliorated vascular dysfunction in obese insulin-resistant animals [47].

Recently, we found that increased provision of extracellular L-arginine (5 mmol/l) to a clonal β-cell line (BRIN-BD11) resulted in enhanced synthesis of

GSH and thus antioxidant defence (even in the presence of an oxidative-stress-inducing pro-inflammatory cytokine cocktail) via a mechanism that appeared to be dependent on L-arginine metabolism through the formation of ornithine and glutamate (M.S. Krause and P. Newsholme, unpublished work).

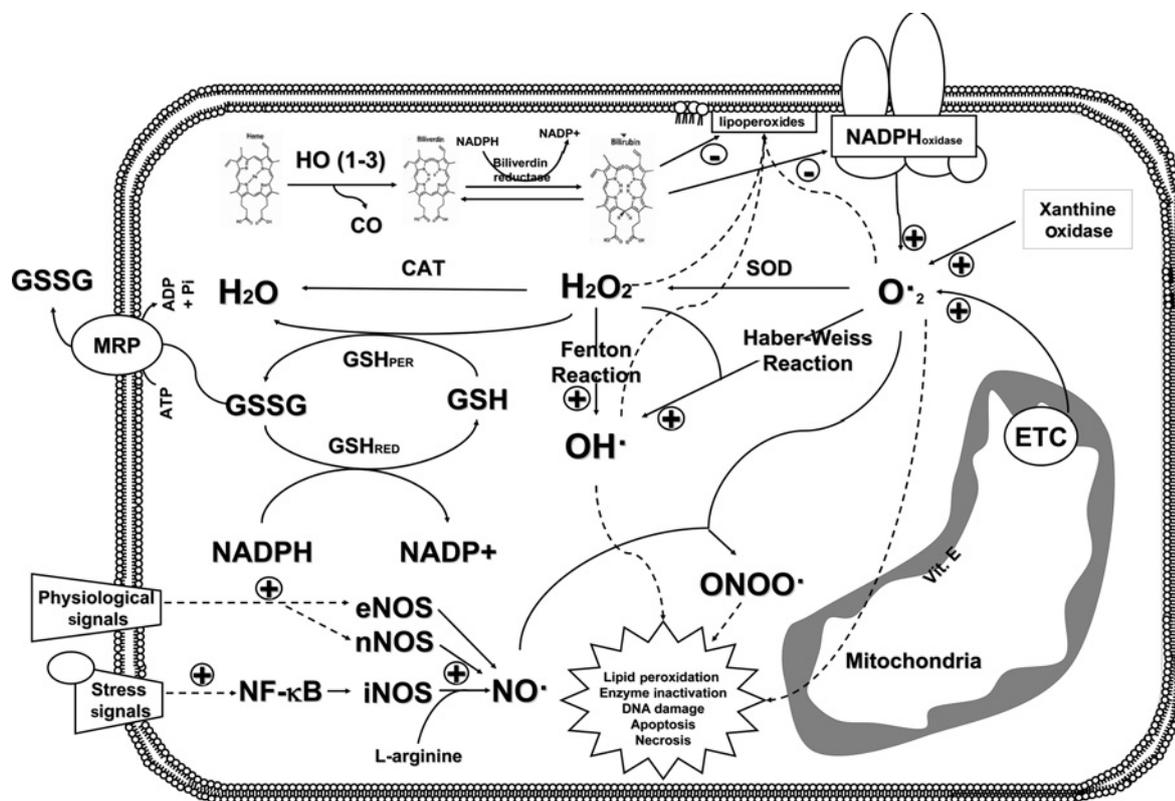


Figure 3 ROS/RNS species formation and antioxidant defences

ROS and NO may be generated by appropriate/inappropriate stimuli. These molecules may become toxic unless rapidly removed by the pathways described. GSSG, glutathione disulfide; GSHred, glutathione reductase; GSHper, glutathione peroxidase; ETC, electron transport chain; HO, haem oxygenase; Vit. E, vitamin E.

POSSIBLE THERAPEUTIC EFFECTS OF PHYSICAL EXERCISE ON PHYSIOLOGICAL AND METABOLIC PARAMETERS ASSOCIATED WITH T2DM AND VASCULAR DYSFUNCTION

Moderate-intensity aerobic exercise is known to reduce cardiovascular risk. Pellegrin et al. [48] recently demonstrated that 5 weeks of swimming training resulted in significantly reduced aortic AT1R levels and sympathetic tone. In addition, decreases in the expression of key NADPH oxidase components in the vascular wall occurred in response to exercise training, thus reducing vascular ROS production and enhancing NO• bioavailability. Importantly, it has now been demonstrated in human arteries that exercise training reduced vascular AT1R expression and AngII-induced vasoconstriction, while enhancing endothelium-dependent dilation. Changes in RAS components are also promoted by acute exercise [49].

Thus physical training is likely to decrease the expression of the AT1R, so diminishing the activation of NADPH oxidase and O₂•⁻ production in the vessel endothelial cells. The normalization of NO• production may decrease the risk of cardiovascular and kidney dysfunction. Indeed, exercise training decreased both

oxidative stress and the circulating concentrations of ADMA in patients with the metabolic syndrome [50].

Physical exercise, from a metabolic perspective, may promote increased plasma glutamine and arginine levels by increasing amino acid mobilization from skeletal muscle, thus normalizing cellular production of NO•, GSH synthesis and antioxidant defences [11]. Moreover, the release of hormones, such as catecholamines, cortisol, growth hormone and glucagon, during exercise is essential for provision of metabolic fuel to meet the requirements of skeletal and cardiac muscle for enhanced fatty acid and glucose oxidation. Mobilization of fuels, especially fatty acids, has long-term beneficial effects in patients with T2DM. Physical exercise can promote the activation of AMPK (AMP-activated protein kinase) in many cells. This enzyme is activated in conditions of low 'energy charge' indicated by a decreased ATP/AMP ratio, which occurs in conditions of high energy demand, such as exercise. AMPK may act as a metabolic 'switch' point, stimulating energy-generating processes, such as glucose and fatty acid oxidation, and decreasing energy-consuming processes, such as protein and lipid synthesis. Hence AMPK activation can contribute to the molecular mechanisms that result in improved

insulin secretion and action, reduced hypertension and improved metabolic function [51].

Finally, exercise, through its positive effects on the immune system, could also exert beneficial effects in diseases associated with a pro-inflammatory state, such as diabetes and CVD. The disease-associated pro-inflammatory state is mediated, at least in part, by the activation of the inducible transcription factor NF- κ B in many cells, which is a key regulator for the production of inflammatory proteins [52]. A large number of studies have reported increased plasma concentrations of anti-inflammatory cytokines, such as IL (interleukin)-6, IL-1ra, IL-4 and IL-10, after various forms of exercise [53]. We propose that IL-6, a muscle-derived cytokine, is essential for exercise-induced changes in immune and endocrine function. In fact, the level of circulating IL-6 has been shown to increase dramatically (up to 100-fold) in response to exercise [53]. A long-term anti-inflammatory response elicited by an acute bout of exercise was partly mediated by muscle-derived IL-6 [54]. In our hands, exercise-associated concentrations of IL-6 (50 pg/ml), when added to pancreatic β -cells in culture, induced increased GSIS via a mechanism that altered L-arginine metabolism and NO \cdot production (M.S. Krause and P. Newsholme, unpublished work).

CONCLUDING REMARKS

Exercise, by stimulating beneficial changes in metabolism, cytokine and hormone levels plus the activity of free-radical-generating enzymes, may enhance vascular endothelial cell, pancreatic β -cell and muscle function, thus leading to normalization of endocrine and cardiovascular function.

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