

Regulation of Inducible Nitric Oxide Synthase (iNOS) and its Potential Role in Insulin Resistance, Diabetes and Heart Failure

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Abstract: Nitric oxide synthases (NOS) are the enzymes responsible for nitric oxide (NO) generation. NO is a reactive oxygen species as well as a reactive nitrogen species. It is a free radical which mediates several biological effects. It is clear that the generation and actions of NO under physiological and pathophysiological conditions are regulated and extend to almost every cell type and function within the circulation. In mammals 3 distinct isoforms of NOS have been identified: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The important isoform in the regulation of insulin resistance (IR) is iNOS. Understanding the molecular mechanisms regulating the iNOS pathway in normal and hyperglycemic conditions would help to explain some of vascular abnormalities observed in type 2 diabetes mellitus (T2DM). Previous studies have reported increased myocardial iNOS activity and expression in heart failure (HF). This review considers the recent animal studies which focus on the understanding of regulation of iNOS activity/expression and the role of iNOS agonists as potential therapeutic agents in treatment of IR, T2DM and HF.

Keywords: Inducible nitric oxide synthase, insulin resistance, diabetes, heart failure.

I. INTRODUCTION

Nitric oxide (NO), one of the smallest known bioactive products of mammalian cells, can be produced by almost all cells [1]. In mammals, 3 distinct isoforms of nitric oxide synthase (NOS) neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) have been identified [1]. These enzymes are products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity, and with 51-57% homology between the human isoforms [2]. NOS can produce NO, superoxide anion (O₂⁻) or peroxynitrite [3]. The enzyme transforms L-arginine (L-Arg) to L-citrulline and NO, which mediates relaxation of the blood vessels through the activation of cyclic guanosine monophosphate (cGMP)-dependent pathways in vascular smooth muscle cells (VSMC) [4-7]. A second major pathway of L-Arg metabolism is *via* arginase, a hydrolytic enzyme responsible for converting L-Arg to urea and L-ornithine, the synthesis precursor of polyamines [8]. Vascular arginase activity is suspected to modulate intracellular levels of

L-Arg, which is a limiting factor in NO production by NOS [4, 8]. iNOS is a high output Ca²⁺-independent NOS whose expression can be induced in a wide range of cells and tissues by cytokines and other agents. After induction, iNOS continuously produces NO until the enzyme is degraded [1, 9]. Chronic inflammation has been postulated to play an important role in the pathogenesis of insulin resistance (IR) [10].

It has been shown that iNOS has been implicated in many human diseases associated with inflammation [10, 11]. iNOS deficiency was shown to prevent high-fat diet-induced IR in skeletal muscle of mice but not in the liver [10]. A role for iNOS in fasting hyperglycemia and hepatic IR, however, remains to be investigated in obesity-related diabetes [10]. IR associated with type 2 diabetes mellitus (T2DM) contributes to impaired vasorelaxation in diabetic rats [12]. Impaired cardiovascular function in T2DM is partially attributed to pathological overexpression of iNOS in cardiovascular tissues of diabetic rats [13]. Increasing evidence now implicates the abnormal activation of protein kinase C beta 2 (PKCβ₂), secondary to increased formation of diacylglycerol (DAG) by hyperglycemia, in a number of cardiovascular T2DM complications [13-15]. Several studies have found preferential increases in expression and/or activation of the PKCβ₂ isoform in cardiac and vascular tissues of diabetic

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rats [13, 15-17]. Excessive NO formation is thought to contribute to contractile dysfunction; in macrophages, increased iNOS expression is associated with increased arginase expression, which competes with iNOS for arginine in rabbits [18]. With substrate limitation, iNOS may become uncoupled and produce reactive oxygen species (ROS) [18]. Concomitant increases in iNOS and arginase expression result in unchanged NO species and protein S-nitrosylation; with substrate limitation, uncoupled iNOS produces superoxide anions and contributes to contractile dysfunction heart failure (HF) [18]. The role of iNOS on cardiac function during the development of left ventricular hypertrophy in mice has been investigated [19]. Recent data demonstrate that NO production *via* iNOS plays an important role in modulating cardiac function after moderate aortic banding (AoB) that mimics long-term hypertension in humans [19]. This review focuses

on recent advances from animal studies in the understanding of regulation of iNOS activity/expression and role of iNOS agonists as potential therapeutic agents in treatment of complex diseases such are IR, T2DM and HF (Table 1).

II. REGULATION OF iNOS ACTIVITY AND EXPRESSION

II.1. iNOS Activity

iNOS was originally identified in macrophages and recognized as part of the cytostatic and cytotoxic mechanisms that operate in these cells [20, 21]. Unlike eNOS, iNOS is mostly transcriptionally regulated and is not normally produced in most cells [22, 23]. Although the rank order of intrinsic activity of the isomers of NOS per unit time is

Table 1. List of Some Provided Animal Studies According to Pathophysiological Condition

Author	Reference	Animal	Pathophysiological condition
Fujimoto M <i>et al.</i> , 2005	[10]	mice	IR
Bitar MS <i>et al.</i> , 2010	[58]	rats	IR
Shimabukuro M <i>et al.</i> , 1998	[66]	rats	IR
Ceriello A <i>et al.</i> , 2002	[67]	rats	IR
Sugita H <i>et al.</i> , 2002	[69]	rats	IR
Zhou YT <i>et al.</i> , 2000	[73]	rats	IR
Dobashi K <i>et al.</i> , 2000	[77]	rats	IR
Kim JK <i>et al.</i> , 2001	[83]	rats	IR
Kido Y <i>et al.</i> , 2000	[84]	mice	IR
Valverde AM <i>et al.</i> , 2003	[86]	mice	IR
Suzuki R <i>et al.</i> , 2004	[87]	mice	IR
Shimomura I, 2000	[88]	mice	IR
Kerouz NJ <i>et al.</i> , 1997	[89]	mice	IR
Anai M <i>et al.</i> , 1998	[90]	rats	IR
Kim F <i>et al.</i> , 2008	[91]	mice	IR
Rizzo NO <i>et al.</i> , 2010	[92]	mice	IR
Charbonneau A <i>et al.</i> , 2010	[97]	mice	IR
Lee JH <i>et al.</i> , 2009	[12]	rats	T2DM
Nagareddy PR <i>et al.</i> , 2009	[13]	rats	T1DM
Inoguchi T <i>et al.</i> , 1992	[15]	rats	T1DM
Xia Z <i>et al.</i> , 2007	[16]	rats	T1DM
Lin G <i>et al.</i> , 2009	[17]	rats	T1DM
Aliev G <i>et al.</i> , 1998	[51]	rabbits	T2DM
Kubota N <i>et al.</i> , 2000	[85]	mice	T2DM
Yang P <i>et al.</i> , 2010	[108]	mice	T2DM
Gealekman <i>et al.</i> , 2002	[145]	rats	T2DM
Zhang P <i>et al.</i> , 2007	[3]	mice	HF
Dias FA <i>et al.</i> , 2010	[19]	mice	HF
Funakoshi <i>et al.</i> , 2002	[146]	mice	HF
Shiomi <i>et al.</i> , 2004	[172]	mice	HF

Abbreviations: IR-insulin resistance; T2DM = Type 2 diabetes mellitus; T1DM = Type 1 diabetes mellitus; HF = heart failure.

nNOS>iNOS>eNOS [24], iNOS generates 100 to 1000-fold more NO than eNOS [22, 25] since, once it is expressed in response to immunological stimuli, its activity persists for many hours [20]. The literature data together with our previously reported data [4, 7, 26, 27] suggested that protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) p42/44 or extracellular signal-regulated kinase (ERK1/2) are involved in regulation of iNOS activity or expression in cardiovascular tissues.

ERK1/2 signaling pathway is a distinct Ser-Thr kinase cascade consisting of 3 enzymes: MAPK kinase kinase, MAPK kinase (MAPKK, MEK, MKK), and MAPK [4]. Upstream activators of the MAPK pathways include small GTP-ases of the Ras family, and downstream effectors include transcription factors and other kinases [4, 28, 29]. Akt is another Ser/Thr protein kinase an important downstream target of phosphatidylinositol 3-kinase (PI3K) [29]. Activation of receptor Tyr kinases leads to phosphorylation and binding to PI3K lipid products to the Akt pleckstrin homology domain resulting in recruitment of Akt to the plasma membrane where it is phosphorylated at the Thr³⁰⁸ and Ser⁴⁷³ residues and thus becomes activated [30, 31]. However Hausel *et al.* described regulation of iNOS activity and protein stability by src-mediated tyrosine phosphorylation [1, 32]. As iNOS activity depends on arginine availability, regulation of arginine transport [33, 34] or consumption of arginine by other biochemical pathways [4] (e.g. arginase) [8, 35-37] has been shown to regulate iNOS enzyme activity. The active iNOS enzyme is a homodimer. Homodimerization of iNOS depends on the availability of its essential cofactor tetrahydrobiopterin (BH4) [38, 39]. Therefore, mechanisms regulating BH4 synthesis and consumption regulate iNOS activity [40, 41]. Additionally some proteins have been identified that interact with iNOS and regulate its activity. By yeast two hybrid screens using murine iNOS as bait the protein kalirin was shown to interact with the iNOS protein. This protein inhibits iNOS activity by preventing enzyme dimerization [42]. In murine macrophages, a 110-kDa protein (named NAP110) has been identified, that directly interacts with the amino terminus of iNOS, thereby preventing dimer formation and inhibiting NOS activity [43]. In rat (VSMC) iNOS protein has been found to interact with the calcium/calmodulin-dependent protein kinase II (CaMKII) [23]. Beside regulation of cellular iNOS protein localization all treatments that chronically affected CaMKII activity or expression significantly inhibited iNOS-specific activity following cytokine induction [1, 44].

II.2. iNOS Expression

iNOS is now known to be expressed in almost every cell type [4, 7, 20, 45, 46]. Atherosclerosis is associated with increases in iNOS expression, and this has been shown in humans to co-exist with a decrease in eNOS mRNA expression in the endothelial cells overlying advanced atherosclerotic plaques [47, 48]. This pattern of increased iNOS accompanied by reduced eNOS has been reported in response to ischaemia [49], hypercholesterolaemia [50] and ROS in endothelial cells of rabbits [20, 51]. The transcription factor nuclear factor-kappa beta (NF-κB) is persistently activated in advanced atherosclerotic lesions and its activation is linked to a wide variety of processes, including inflammation, pro-

liferation, differentiation, and apoptosis [52-54]. Cytokines such as interleukin-1β (IL-1β) activate NF-κB in many cell types, including VSMC, and activation of NF-κB is a requirement for iNOS expression [55, 56]. Activation of the redox-sensitive PI3K/Akt pathway in rats stimulates NF-κB by promoting the dissociation of phosphorylated inhibitor of NF-κB alpha (IκBα) [54, 57, 58].

Increasing evidence indicates that the members of the MAPK family of protein kinases (ERK1/2, c-Jun kinase, p38) are important modulators of proinflammatory cytokine-dependent expression of iNOS in multiple cell types [11, 54, 59, 60]. Of particular importance in VSMC is the role and mechanisms that couple ERK1/2 to iNOS expression in response to proinflammatory cytokines. A few studies reported that stimulation with IL-1β results in a coordinate activation of ERK1/2 and NF-κB leading to increased expression of iNOS in rat VSMC [11, 56]. Furthermore, the study reported by Jiang *et al.*, showed that IL-1β-dependent activation of NF-κB is dependent upon ERK1/2 through the ability of ERK1/2 to phosphorylate inhibitor of NF-κB beta (IκBβ) resulting in its degradation and subsequent translocation of the p65 and p50 subunits to the nucleus to initiate gene expression. Further studies showed that ERK1/2 selectively phosphorylates IκBβ rather than IκBα, resulting in sustained NF-κB activity, which is required for iNOS gene expression [56, 61, 62]. The intermediate between ERK1/2 and IκBβ is ribosomal S6 kinase (RSK) 1 [54, 62]. Our study indicated that both p38 MAPK and ERK1/2 are necessary for IL induction of iNOS and cyclooxygenase-2 (COX-2) and that they also seem to target COX-2 activity at post-translational level [11]. Thus IL activation of p38 and ERK1/2 results in multiple levels of regulation of the synthesis of the inflammatory mediators NO and prostaglandin E₂ (PGE₂) in rat cardiac myocytes [11]. In addition, our recent data from rat hearts show that iNOS activity/expression is altered by ghrelin [4] which is a peptide hormone and it is an endogenous ligand for growth hormone (GH) secretagogue receptor (GHSR) [63]. There is a suggestion that ghrelin treatment might activate insulin-like growth factor-1 (IGF-1) signaling pathway, which uses Akt/ERK1/2 signaling pathways and in that way participates in the regulation of iNOS activity/expression in the rat hearts [4, 64].

III. ROLE OF INOS IN PATHOPHYSIOLOGICAL CONDITIONS-EVIDENCE FROM ANIMAL STUDIES

III.1. iNOS in Insulin Resistance State (IR)

Although iNOS was originally identified in macrophages, it is now known that it is widely expressed in many tissues, including insulin-sensitive organs such as skeletal muscle, adipose tissue, and liver in normal rodents and humans [10]. The expression of iNOS is upregulated by most, if not all, inducers of IR, including proinflammatory cytokines, obesity [65], free fatty acids (FFA) [66], hyperglycemia [67, 68], endotoxins [69, 70] and oxidative stress (OxS) [71]. In fact, elevated expression of iNOS was observed in skeletal muscle of high-fat diet-fed mice [72], in heart of Zucker diabetic fatty rats [73], and in skeletal muscle [74] and platelets of patients with T2DM [75]. Furthermore, iNOS induction resulted in attenuated insulin-stimulated glucose uptake in cultured skeletal muscle cells [76]. Thiazolidinediones

(synthetic peroxisome proliferator-activated receptors (PPAR) gamma agonists), a class of insulin sensitizer, suppress iNOS expression in cultured cells and *in vivo* in rodents [77, 78]. Thus, inhibition of iNOS expression has been recently proposed to be a new mechanism of actions of insulin sensitizers [79, 80]. Chronic low-grade inflammation has been proposed to be involved in the pathogenesis in obesity-related IR and T2DM [10]. The activation of NF- κ B inhibitor κ B kinase β (IKK β), a crucial signaling cascade for inflammatory response, has been highlighted as a mediator of IR [10, 58]. The pharmacological inhibition or gene disruption of IKK β reversed obesity-related IR and fasting hyperglycemia in rodents and humans [81-83]. It has been shown that iNOS deficiency prevents high-fat diet-induced IR in skeletal muscle of mice but not in the liver [10]. A role for iNOS in fasting hyperglycemia and hepatic IR, however, remains to be investigated in obesity-related T2DM [10]. The effects of a specific inhibitor for iNOS, dihydrochloride L-N6-(1-Iminoethyl)lysine (L-NIL), in obese diabetic (*ob/ob*) mice have been examined [10]. Results from those study are that iNOS was significantly elevated in the liver as well as skeletal muscle and adipose tissue in *ob/ob* mice compared with wild type (*wt*) mice and that the iNOS inhibitor reversed fasting hyperglycemia and ameliorated whole-body IR in *ob/ob* mice [10]. In the liver, insulin receptor substrate 2 (IRS-2) rather than insulin receptor substrate 1 (IRS-1) plays a prominent role in metabolic actions of insulin, including the inhibition of hepatic glucose output, whereas IRS-1 has a major role in mouse skeletal muscle [84-87]. Previous studies [88-90] showed a marked reduction in IRS-2 expression in the liver of *ob/ob* mice, whereas IRS-1 expression was unaltered or modestly decreased [10]. These findings suggest that defective IRS-2-mediated insulin signaling is a major component of obesity-related hepatic IR [10]. Inhibitor of iNOS improved the protein expression of both IRS-1 and IRS-2 in the liver of *ob/ob* mice [10]. The mentioned study highlighted therapeutic potential of iNOS inhibitors to improve glycemic control in obesity-related T2DM [10].

A recent study employed a mouse model of diet-induced obesity induced by high-fat feeding to assess the natural history of inflammation and impaired insulin signaling in 4 different insulin-sensitive tissues: vascular tissue (thoracic aorta), liver, adipose tissue and skeletal muscle [91]. Their findings are that vascular tissue is adversely impacted much earlier in the course of diet-induced obesity than are key insulin-sensitive tissues involved in glucose metabolism implies a heightened susceptibility of vascular elements to the deleterious effects of obesity [91].

In conditions of nutrient excess, such as obesity and T2DM, elevated FFA levels are implicated in the pathogenesis of both inflammation and IR in a variety of tissues, including mouse endothelial cells [92-96]. Lipid-induced IR in muscle and liver is linked to overactivation of inflammatory signaling pathways known to impede insulin signal transduction [97-100]. A few studies have shown that iNOS is overexpressed in metabolic tissues of both dietary and genetic models of obesity and plays a pivotal role in the pathogenesis of IR and glucose intolerance in mice [10, 72, 99, 101]. The recent study [97] showed using iNOS $^{-/-}$ mice that iNOS underlies lipid-induced insulin resistance in both liver and skeletal muscle. Disruption of iNOS restored insulin's

ability to suppress glucose production and prevented lipid-induced elevations in basal glucose production that account for the initial hyperglycemia seen in *wt* mice [97]. It has been reported that iNOS induction in metabolic tissues and insulin target cells interferes with the insulin receptor beta (IR β)/IRS/PI3K/Akt insulin signaling pathway [72, 80, 99, 102]. Beside that iNOS impairs insulin action on glucose production by altering insulin signaling to IR β , IRS-1/-2 and Akt [97]. They found that iNOS $^{-/-}$ mice were protected from lipid-induced inhibitory phosphorylation of IRS-1 Ser³⁰⁷ and IRS-2 Ser¹³³, two well-established target sites of Ser/Thr kinases known to be activated by lipids through activation of inflammatory pathways (e.g. I κ B kinase, c-Jun NH₂-terminal kinase (JNK), PKC) [103-105]. Lack of iNOS prevented the lipid-induced impairment in Ser/Tyr phosphorylation of Akt observed in *wt* mice, resulting in normalization of hepatic Akt kinase activity [97]. These results suggest that iNOS causes hepatic IR by impairing insulin signaling through the coordinated action of 3 independent mechanisms: by promoting 1) inhibitory serine phosphorylation of IRS proteins, 2) tyrosine nitration of IR β and IRS-1 and IRS-2, as well as by, 3) directly impairing Akt activity through its tyrosine nitration [97]. These data provide genetic evidence that iNOS in mice is a key factor in the regulation of insulin sensitivity and hepatic glucose metabolism by FFA *in vivo* [97].

III.2. iNOS in Type 2 Diabetes Mellitus (T2DM)

IR associated with T2DM contributes to impaired vasorelaxation in rats [12]. The correlations between IR, defective Akt activation, insulin-resistant iNOS expression, and impaired insulin-induced vasodilation have been explored [12]. For the first time, Lee *et al.* have demonstrated that the presence of Akt-independent iNOS expression in the Goto-Kakizaki (GK) nonobese insulin-resistant diabetic rat model and that the defective insulin-induced vasodilation observed in the diabetic vasculature can be restored by the overexpression of active Akt, which advocates a novel therapeutic strategy for treating T2DM [12].

Numerous studies have led to the identification of multiple hyperglycemia-induced alterations in metabolism and signaling that have been linked to activation of protein kinase C (PKC) and an eventual increase in oxidative/nitrosative stress in T2DM [13, 14, 106, 107]. It remains unclear whether the increase in nitrosative stress, which is implicated in the etiology of T2DM secondary complications [106], is an independent manifestation of hyperglycemic injury or is linked to the activation of PKC [13]. In a recent study, the hypothesis that high glucose-induced activation of PKC β_2 increases iNOS-mediated nitrosative stress leading to cardiovascular abnormalities has been tested on rats [13]. The results of that study demonstrate that PKC β_2 is an obligatory mediator of nitrosative stress and that LY333531 (selective PKC β inhibitor) significantly reduced the formation of iNOS and improved cardiovascular abnormalities in streptozotocin (STZ)-diabetic rats [13]. Moreover, hyperglycemia-induced activation of PKC β_2 is antecedent to increases in superoxide, ERK1/2, NF- κ B, and iNOS expression in cardiovascular tissues, whereas inhibition of this pathway suppresses key signaling events that lead to increased nitrosative stress [13]. This data suggest that inhibition of PKC β_2 may be a useful approach for correcting ab-

normal hemodynamics in T2DM by preventing iNOS-mediated nitrosative stress [13].

Hyperglycemia resulting from diabetes has an adverse impact on embryonic development through induction of apoptosis in mice embryonic tissues [108]. The mechanisms underlying hyperglycemia-induced apoptosis are not completely understood [108]. Recently, it has been found that the proapoptotic JNK1/2 is activated in embryonic tissues exposed to maternal diabetes *in vivo* and hyperglycemic embryo cultures of both Sprague Dawley rats and C57BL/6J mice *in vitro* [108-111]. JNK1/2 agonist mimics the teratogenic effect of hyperglycemia to induce embryonic malformations, whereas targeted deletion of the *jnk2* gene significantly ameliorates diabetes-induced malformations [111]. Thus, JNK1/2 plays a causative role in the induction of diabetic embryopathy. Increased levels of NO are associated with the adverse impact of maternal diabetes on murine embryonic development [112]. NO has been shown to be involved in cell differentiation, proliferation, and apoptosis [113-115]. Although NO is of physiological importance, it can also be cytotoxic. iNOS and eNOS are expressed during murine early embryonic development [112]. Hyperglycemia increases NO production in embryonic tissues [112] inducing the production of reactive nitrogen species that leads to nitrosative stress. Yang *et al.*, 2010 hypothesized that hyperglycemia induced JNK1/2 activation mediates iNOS induction [108]. To test this hypothesis, they investigated the relationship between JNK1/2 activation and iNOS gene expression in diabetic embryopathy [108]. By using pharmacological inhibitors of JNK1/2 activation (SP600125) *in vitro* and target deletion of *jnk2* in mice, they have demonstrated that JNK1/2 activation is responsible for hyperglycemia-induced iNOS gene expression and consequent nitrosative stress [108].

Cardiovascular mortality is increased in patients with Type 1 diabetes mellitus (T1DM) [116, 117], and the incidence of HF, especially, following myocardial infarction, is much greater among diabetic than nondiabetic patients [117]. In experimental diabetes, the mechanical properties of the myocardium and cardiomyocytes *in vitro* are significantly altered, characterized by prolongation of contraction and relaxation as well as considerable slowing down of relaxation velocity in mice [118]. In rats with STZ-induced diabetes, abnormal cardiac function is seen as early as 7 days after induction of diabetes [116, 118, 119]. It has been shown that cardiomyocytes from diabetic rats [120], manifested reduced contractile responses to IGF-1 associated with altered NO metabolism [121]. Several studies suggest that NO production is reduced in diabetes such as in obese Zucker rats [122, 123]. Abnormal NOS expression and NO production in cardiovascular tissues may have various effects [116]. Therefore, investigation of NOS activity in cardiovascular tissues in the diabetic state may be of particular importance in understanding the etiology of cardiovascular dysfunction associated with chronic diabetes mellitus [116].

III.3. iNOS in Heart Failure (HF)

Mammalian cells synthesize NO through the 5-electron oxidation of 1 of the 2-guanidinonitrogens of L-Arg [54, 124]. All 3 isoforms are expressed in the vasculature. The

predominant isoform of NOS detectable in VSMC in response to inflammatory cytokines is iNOS [26, 125-127] and nNOS upregulation is induced in VSMC by shear stress, hypoxia, and growth factors [128-130]. In the healthy vessel, the endothelium serves as the main source of NO production through eNOS activity to maintain vascular tone and regulate platelet aggregation and leukocyte adhesion [124, 131-133]. Disruption of the endothelial layer and initial loss of eNOS is a hallmark of the development of atherosclerosis as well as restenosis [54]. Traditionally, the upregulation of iNOS is perceived to compensate for the loss of a functional endothelium and eNOS during injury and atherosclerosis [134], although the presence of excess NO and ROS coincidentally may lead to additional tissue damage and dysfunction [71, 126]. More recent studies support a dual role for iNOS in the development of the atherosclerotic plaque. In the ApoE^{-/-} mouse model, iNOS is expressed in both macrophages and smooth muscle cells of the developing plaque, although smooth muscle cells are not present in early lesions [135]. Upregulation of iNOS at the mRNA level is observed in the rat carotid artery by 24 h postinjury and it is sustained throughout 14 days [54, 134].

Whereas iNOS is not detectable in normal cardiomyocytes, iNOS expression is increased in HF in rabbits [18, 136] and patients [137-139]. Increased iNOS expression results from increased cardiomyocyte stretch secondary to protein kinase activation and through positive feedback, from increased NO concentration [140]. NO mediates its effects either through cGMP and protein kinase G [141, 142] or more directly through protein nitrosylation [143]. In the rat and mouse heart, NO reduces β -adrenergic responses [144-146], although that is controversial [147], and the L-type Ca²⁺ channel current [148], inhibits the mitochondrial respiratory chain [149-152] and increases the mitochondrial permeability transition pore opening probability [153], thereby increasing cardiomyocyte apoptosis [154]. However, the contribution of increased iNOS expression to HF development has been questioned in transgenic mice with chronic cardiac-specific upregulation of iNOS [155, 156] and only in the presence of a simultaneous knockout of myoglobin did iNOS overexpression result in HF [157]. In murine macrophages, increased iNOS expression is associated with a concomitant increase in arginase expression [158]. Recent data from wound healing studies in rats suggest that there might be a self-limiting negative feedback cycle, in that increased iNOS-derived NO increases arginase II activity, which subsequently reduces L-Arg concentration and thereby limits NO production [159]. Limitation of substrate availability also leads to uncoupling of iNOS with ultimate ROS formation [143, 160]. Increased ROS formation induces myofibrillar oxidation in rat hearts and subsequently contributes to the development of contractile dysfunction [161, 162]. An increase in iNOS expression does not necessarily imply increased NO-induced myocardial damage [18]. Nevertheless, uncoupled iNOS secondary to substrate limitation might contribute to contractile dysfunction through increased oxidative stress (OxS) [18]. Increased arginase expression might limit NOS substrate availability and contribute to the persistence of hypertension [163, 164]. Whether increased arginase expression in mice also contributes to the development/progression of HF by promoting iNOS uncoupling warrants

further investigation [18]. Certainly, blockade of enhanced arginase expression/activity might be a promising new therapeutic approach since iNOS, as long as it is functionally coupled, does not contribute to HF development [18, 155].

The role of NO in the regulation/modulation of cardiac performance has been extensively studied since the early 1990's [19, 165]. The expression of iNOS in cardiomyocytes only increases following induction by events such as hypertrophy or HF [19]. In such cases, iNOS appears to contribute to myocardial dysfunction and alters the myocardial response to β -adrenergic stimulation [19, 145, 166, 167]. However, since NO can be produced by 3 different isoforms of NOS within the myocardium, it has been difficult to separate their specific effects in physiological and pathological conditions without genetic manipulations [19]. Recently however, different mouse models have been generated in which one or more isoforms of NOS have been knocked out or over-expressed allowing for the specific, independent effects of the NOS isoforms to be studied on cardiac function [168]. Recent data demonstrate for the first time that the absence of iNOS during the development of hypertrophy induced by moderate aortic constriction, mimicking long-term hypertension in humans, delays both the increase in hypertrophic gene marker expression and contractile dysfunction associated with hypertrophy development [19]. Moreover, after long-term aortic banding, iNOS knock-out mice exhibited increased basal cardiac function as well as an improved response to β -adrenergic stimulation compared to *wt* mice [19]. These findings support a direct role for the expression of iNOS in the development of the cardiac dysfunction but not the hypertrophy that results from pressure overload [19].

Myocardial hypertrophy and HF are associated with increased O_2^- production [3, 169] and accumulation of oxidized lipid and protein products such as nitrotyrosine (a marker for peroxynitrite) and 4-hydroxy-2-nonenal (4-HNE, a marker of lipid peroxidation) [3, 170]. Oxygen free radicals are linked to fibrosis and matrix turnover involving the activation of matrix metalloproteinases (MMPs) [171]. Overexpressing glutathione peroxidase in mice [172], or administering BH4 to decrease myocardial O_2^- production [173] decrease myocardial MMP abundance [3]. A study examined the role of iNOS in the ventricular hypertrophy and congestive HF that develops in response to sustained pressure overload produced by transverse aortic constriction (TAC) in mice with or without the iNOS gene [3]. Consistent with these findings, TAC resulted in marked increases of myocardial atrial natriuretic peptide (ANP), 4-HNE and nitrotyrosine in *wt* mice but not in iNOS deficient mice [3]. In response to TAC, myocardial eNOS and iNOS was expressed as both monomer and dimer in *wt* mice, and this was associated with increased ROS production, suggesting that iNOS monomer was a source for the increased OxS [3]. In this study, the decreased myocardial OxS in the iNOS deficient mice was associated with decreased MMP-1 content, supporting the notion that OxS affects myocardial matrix turnover [3]. The conclusion was that iNOS deletion reduced the evidence of TAC-induced myocardial OxS, indicating that iNOS contributed to OxS in the *wt* mice, either directly through iNOS uncoupling or by iNOS-dependent eNOS uncoupling [3]. However, iNOS might also decrease intracellu-

lar BH4 and L-Arg availability to eNOS and thereby induce eNOS uncoupling [3]. The mentioned study on mice provided the first evidence that iNOS deficiency (iNOS^{-/-}) attenuates TAC-induced ventricular hypertrophy and congestive HF, and that iNOS expressed in response to systolic overload serves as a source for myocardial ROS that contribute to left ventricular dilatation and hypertrophy [3].

CONCLUSIONS

It has been shown that iNOS has been implicated in many human diseases associated with inflammation [10, 11]. To identify individuals with NO deficiency and increased cardiovascular risk, new diagnostic tools, apart from vasomotor testing to assess NO bioactivity, based on the recent advances in the understanding of NO metabolism have been developed. In parallel, new modes of NO delivery to patients have been studied, and new NO donating compounds have been developed to not only substitute for NO deficiency but also to release exogenously supplied NO at specific cellular targets and to overcome disadvantages of conventional NO donors such as organic nitrate and nitrite esters [174]. Nitrite delivery to humans *via* infusion or inhalation may counterbalance pathophysiologic processes occurring in disease states with a relative or absolute lack of NO, such as hypertension, atherosclerosis, diabetes, acute respiratory distress syndrome of the newborn, neonatal pulmonary hypertension, delayed-onset vasospasm due to subarachnoid hemorrhage and sickle cell disease [175]. Complete understandings of the mechanism of dysregulation of iNOS are needed in order to develop appropriate therapies for these conditions. The recent studies suggest that upregulation of iNOS may be a protective mechanism against excessive contraction, abnormal signaling resulting from OxS and due to enhanced inflammation in the diabetic vasculature [12]. There are strong reasons for further studies on new therapies involving the inhibition of iNOS activity to improve glycemic control in obesity-related T2DM and cardiac performance in HF and hypertrophy [19]. Knowledge gained from recent provided investigations mentioned in this review suggested that inhibitors of iNOS will have potentially important therapeutic implications.

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ABBREVIATIONS

iNOS	=	inducible nitric oxide synthase
NO	=	nitric oxide
nNOS	=	neuronal nitric oxide synthase
eNOS	=	endothelial nitric oxide synthase
O_2^-	=	superoxide anion
L-Arg	=	L-arginine
cGMP	=	cyclic guanosine monophosphate
VSMC	=	vascular smooth muscle cells
Ca^{2+}	=	calcium ions

IR	=	insulin resistance
T2DM	=	type 2 diabetes mellitus
PKC	=	protein kinase C
PKC β_2	=	protein kinase C beta 2
DAG	=	diacylglycerol
ROS	=	reactive oxygen species
HF	=	heart failure
AoB	=	aortic banding
Akt	=	protein kinase B
MAPK	=	mitogen-activated protein kinase
ERK1/2	=	MAPK p42/44 or extracellular signal-regulated kinase
PI3K	=	phosphatidylinositol 3 kinase
BH4	=	tetrahydrobiopterin
NAP110	=	iNOS inhibitor
CaMKII	=	calcium/calmodulin-dependent protein kinase II
NF- κ B	=	nuclear factor-kappa beta
IL-1 β	=	interleukin-1beta
I κ B α	=	inhibitor of NF- κ B alpha
I κ B β	=	inhibitor of NF- κ B beta
RSK	=	ribosomal S6 kinase
COX-2	=	cyclooxygenase-2
PGE ₂	=	prostaglandin E ₂
GH	=	growth hormone
GHSR	=	GH secretagogue receptor
IGF-1	=	insulin-like growth factor-1
FFA	=	free fatty acids
OxS	=	oxidative stress
PPAR	=	peroxisome proliferator-activated receptor
IKK β	=	NF- κ B inhibitor κ B kinase β
L-NIL	=	iNOS inhibitor
wt	=	wild type
IRS-2	=	insulin receptor substrate 2
IRS-1	=	insulin receptor substrate 1
IR β	=	insulin receptor beta
JNK	=	c-Jun NH ₂ -terminal kinase
LY333531	=	selective PKC β inhibitor
STZ	=	streptozotocin
SP600125	=	inhibitor of JNK1/2 activation
T1DM	=	type 1 diabetes mellitus

4-HNE	=	4-hydroxy-2-nonenal, a marker of lipid peroxidation
MMPs	=	matrix metalloproteinases
TAC	=	transverse aortic constriction
ANP	=	atrial natriuretic peptide

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