Expression of Nitric Oxide Synthases in the Pathophysiology of Cardiovascular Diseases

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Nitric oxide (NO) is not only a potent vasodilator, it also inhibits platelet adherence and aggregation, reduces adherence of leukocytes to the endothelium, and suppresses proliferation of vascular smooth muscle cells. The knowledge of nitric oxide synthases (NOSs) is of extreme scientific importance, not only for understanding new pathophysiological mechanisms but, also as a target for therapeutic intervention [1]. Therefore, the intention of this overview is to review the role of nitric oxide synthases in the physiological and pathological mechanisms of the cardiovascular system.

Basic concepts

NOSs, from the biochemical point of view, are a family of complex enzymes that catalyze the oxidation of L-arginine to form NO and L-citrulline. The three human NOS isoforms have been identified to date, ecNOS(endothelial constitutive NOS), nNOS (neuronal NOS), and iNOS (inducible NOS), their genes are found on human chromosomes 7, 12, and 17, respectively, and so were named for the tissue in which they were first cloned and characterized [1,2] .

Endothelial constitutive nitric oxide synthase (ecNOS) - The role of nitric oxide in regulating vascular tone and mediating platelet function is attributable to the ongoing activity of the endothelial constitutive form of NOS. Inactivation of the ecNOS pathway limits the contribution of NO to vessel homeostasis and results in increased vascular tone and platelet adhesion and aggregation. The complete signal transduction pathway of ecNOS activation is represented in figure 1, where the activities of ecNOS are regulated by the intracellular free calcium concentration and calcium-calmodulin complexes. ecNOS is a constitutively expressed protein predominantly associated with the particulate subcellular fraction, suggesting that the native enzyme is a membrane-bound protein. A recent detailed analysis of the membrane association of ecNOS showed that this enzyme is localized to the Golgi apparatus as well as to specific structures in the plasmalemmal membrane called caveolae. The association of ecNOS with a region of the plasma membrane in which several key signal transducing comple–xes are concentrated (such as G-proteins) is likely to have profound repercussions on enzyme activity as well as on its accessibility to intracellular mechanisms of the pathway release, including mechanisms independents of intracellular calcium release [3-5].

Neuronal nitric oxide synthase (nNOS) - This isoform is present in central and peripheral neuronal cells and certain epithelial cells. Its activity is also regulated by Ca^{2+} and calmodulin. Its functions include long-term regulation of synaptic transmission in the central nervous system, central regulation of blood pressure, smooth muscle relaxation, and vasodilation via peripheral nitrergic nerves. It has also been implicated in neuronal death in cerebrovascular stroke [6].

Inducible nitric oxide synthase (iNOS) - The expression in this enzyme is induced in a multitude of different cells, including macrophages, endothelial cells, vascular smooth muscle cells and cardiac myocytes after stimulation with lipopolysaccharide (LPS), cytokines (such as IL-1β, TFN-α, IFN-γ, IL-6), and others; thus it has an important role in antimicrobial, antiparasitic and antineoplastic activity [7]. This isoform is not regulated by Ca^{2+}. It produces large amounts of NO that have cytostatic effects on parasitic target cells by inhibiting iron-containing enzymes and causing DNA fragmentation. The induction of iNOS is involved in the pathophysiology of autoimmune diseases and septic shock [6].

Based on the difficulties the authors of this review had in relation to the inconsistent NOS isoform nomenclature we decided to create a table of the various names used for each isoform to help futures research and reading on the subject (table I).

Methods for studying NOS expression

Laboratory methodology is not the main motivation of this review, but brief comments about methodology will be helpful. This involvement may be documented by: a) direct detection using spectrophotometric or electrochemical methods or more often by indirect methods and; b) indirect methods for detection of nitric oxide effects include localization of nitric oxide synthase enzyme by immunoche-

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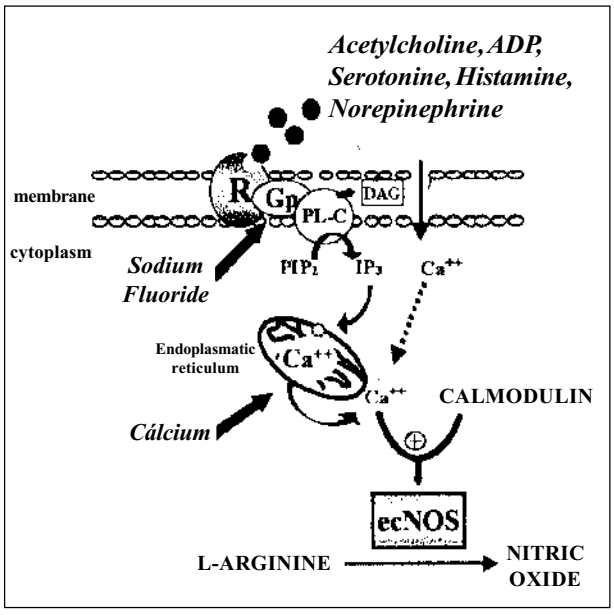

Fig. 1 - Pathway of release of nitric oxide (NO); PIP2= phosphatidylinisitol 4,5-biphosphate; IP3= inositol triphosphate; ecNOS=endothelial constitutive nitric oxide synthase.

Table I - Nomenclature of oxide nitric synthases (NOSs)

Enzyme	Nomenclature
Endothelial NOS	eNOS, ecNOS, cNOS, NOS III ou NOS3
Neuronal NOS	bNOS(brain),NOS I ou NOS1
Inducible NOS	iNOS, NOS II ou NOS2

mistry or messenger ribonucleic acid (mRNA) by in situ hybridization, bioassays, inhibition of nitric oxide synthase activity, iron responsive element-binding protein activity, and production of nitrate/nitrite, L-citrulline, or cyclic guanosine monophosphate (cGMP). Careful evaluation of potential pitfalls associated with these indirect methods of detecting nitric oxide effects prior to their use will prevent misinterpretation of results [8].

Unspecific inhibitors - Inhibitors of both ecNOS and iNOS are metilates and nitrics forms of L-arginine that act by competition. Among them the most popular are L-NMMA, o L-NAME, e and o L-NOARG. Another example is ADMA (asymmetric dimethylarginine), a circulating endogenous NOS inhibitor. Studies have shown that plasma ADMA level is positively correlated with risk factors for atherosclerosis thus suggesting that this endogenous antagonist of NO synthase may be a marker of atherosclerosis [9].

Specific inhibitors - Specifically inhibit the isoform INOS. They include the glucocorticoids (such as dexamethasone), aminoguanidine, L-canavanine, N6- (1-imioetyl) lisine (L-NIL) and 2,4-diamino 6-hydroxy-pyrimidine [10].

Knowledge about the specific NOSs inhibitors is of pivotal importance for experimental and therapeutical pharmacologic assays (figure 2).

Some comparisons of the differents NOS isoforms expressions would be helpful in a review of basic concepts.

As already mentioned ecNOS is an enzyme that is membrane associated whereas iNOS and nNOS are largely cytosolic [1]. Another difference between the NOS isoforms is amount and duration of NO produced. Molecule NO is synthesized for short periods of time (seconds to minutes) following enzyme activation of ecNOS or nNOS. In contrast, the iNOS is expressed after cell activation only and then produces NO for comparatively long periods of time (hours to days).

Anatomic distribution of NOS in the normal heart

Most of the constitutive nitric oxide synthase activity in the normal heart is present in endothelium along the extensive network of arteries, veins and capillaries within the myocardium. This endothelial isoform of nitric oxide synthase also exists in the endocardium lining the cardiac cavities. Neuronal nitric oxide synthase appears much less prominent, although the exact amount of this isoform in the heart is uncertain. Although no inducible nitric oxide synthase occurs in the normal heart, macrophages associated with repair of various types of cardiac damage contain this isoform. For all nitric oxide synthases, however, species variation and variability among models underscore the importance of correlative studies of structure and function [11].

Autocrine and paracrine function of NOS in the normal heart

From the point of view of autocrine and paracrine function, the different cell types comprising cardiac muscle express one or more of the three isoforms (neuronal NOS, or nNOS; inducible NOS, or iNOS; and endothelial NOS, or ecNOS) of nitric oxide synthase (NOS). The nNOS is

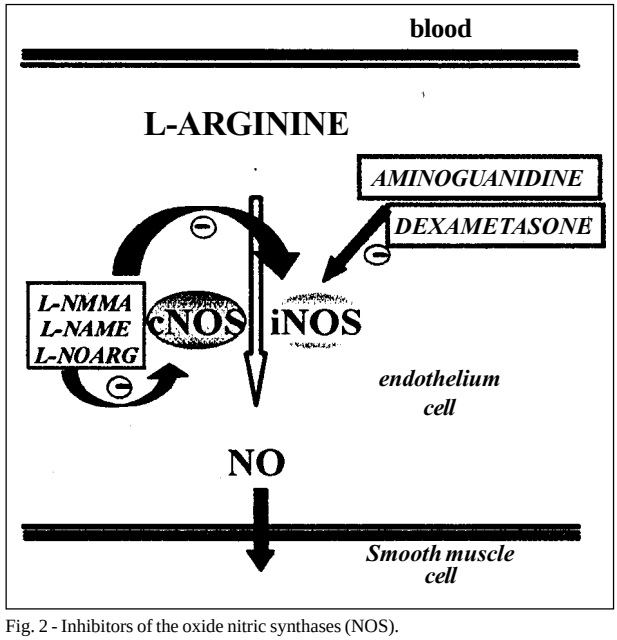

Fig. 2 - Inhibitors of the oxide nitric synthases (NOS).

expressed in sympathetic nerve terminals and regulates the release of catecholamines in the heart. The ecNOS constitutively expressed in endothelial cells inhibits contractile tone and the proliferation of underlying vascular smooth muscle cells, inhibits platelet aggregation and monocyte adhesion, promotes diastolic relaxation, and decreases O_2 consumption in cardiac muscle through paracrinally produced NO. The ecNOS is also constitutively expressed in cardiac myocytes from rodent and human species, where it autocrinally opposes the inotropic action of catecholamines after muscarinic cholinergic and beta-adrenergic receptor stimulation. The iNOS gene transcription and protein expression are induced in all cell types after exposure to a variety of inflammatory cytokines. Aside from participating in the immune defense against intracellular microorganisms and viruses, the large amounts of NO produced autocrinally or paracrinally mediate the vasoplegia and myocardial depression characteristic of systemic immune stimulation and promote cell death through apoptosis. In cardiac myocytes, NO may regulate L-type calcium current and contraction through activation of cGMP-dependent protein kinase and cGMP-modulated phosphodiesterases. Other mechanisms independent of cGMP elevations may operate through interaction of NO with heme proteins, nonheme iron, or free thiol residues on target signaling proteins, enzymes, or ion channels. Given the multiplicity of NOS isoforms expressed in cardiac muscle and of the potential molecular targets for the NO produced, tight molecular regulation of NOS expression and activity at the transcriptional and posttranscriptional level is necessary to coordinate the many roles of NO in heart function in health and disease [12].

NOS and cardiovascular diseases

Myocardial ischemia - This part of the text will include general aspects of myocardial ischemia: ischemia reperfusion injury, preconditioning and acute myocardial infarction (AMI).

Ischemia reperfusion injury - *In vivo* findings demonstrate a cardioprotective role for ecNOS-derived NO in ischemic-reperfused hearts. Myocardial ischemia-reperfusion injury is exacerbated in the absence of ecNOS [13]. Although endothelial dysfunction occurs following ischemia and has been attributed to altered NO formation, the biochemical basis for this dysfunction is unknown. Therefore, studies were performed to determine the effects of myocardial ischemia and reperfusion on ecNOS expression in isolated rat hearts subjected to periods of global ischemia or ischemia followed by reperfusion. While activity was preserved after 30 min of ischemia, it decreased by 77% after 60 min and became nearly undetectable after 120 min. Reperfusion resulted in only a partial restoration of activity. The decline in activity with ischemia was due, in part, to a loss of ecNOS protein. Hemodynamic studies have shown that the onset of impaired vascular reactivity paralleled the loss of functional ecNOS. Thus, the loss of endothelial function following ischemia parallels a loss of ecNOS activity. Itis due perhaps to a combination of pH-dependent denaturation and proteolysis [14].

Preconditioning (PC) - Brief periods of myocardial ischemia preceding a subsequent more prolonged ischemic period 24-72 h later confer protection against myocardial infarction ('delayed preconditioning' or the 'second window' of preconditioning). Three structurally different NOS inhibitors: 1) N-omega-nitro-L-arginine (L-NA - nonselective NOS inhibitor); 2) Aminoguanidine (AMG - selective iNOS inhibitors) e; 3) S-methylisothiourea sulfate (SMT - selective iNOS inhibitors) given 24 hours after the preconditioning (PC) ischemia, consistently abolish late PC against myocardial stunning in conscious rabbits, indicating that this cardioprotective effect is mediated by the activity of NOS. The results obtained with AMG and SMT specifically implicate iNOS isoform as the mediator of the protection on day 2. Previous studies have shown that NO triggers the development of late PC. The present results indicate that NO plays a dual role in late PC against stunning, acting initially as the trigger and subsequently as the mediator of the protection [15,16].

In the same line of research, one nice recent study examined the effects of specific iNOS blockers on delayed protection conferred by ischemic preconditioning 48 h later in an anaesthetized rabbit model of myocardial infarction. In rabbits receiving no pharmacological intervention, the percentage of myocardium infarcted within the risk zone was 43.9±5.0%. This percentage was significantly reduced to 18.5±5.6%, 48 h after ischemic preconditioning with four 5-min coronary occlusions. Administration of the iNOS expression inhibitor dexamethasone (4 $mg.Kg^{-1}$iv) 60 min before ischemic preconditioning completely blocked the infarct-limiting effect of ischemic preconditioning. Furthermore, administration of aminoguanidine (300 $mg.Kg^{-1}$ sc), a relatively selective inhibitor of iNOS activity, 60 min before sustained ischemia also abolished the delayed protection afforded by ischemic preconditioning. Neither aminoguanidine (AMG) nor dexamethasone *per se* had a significant effect on myocardial infarct size. These data provide pharmacological evidence that the induction of iNOS, following brief periods of coronary occlusion, is associated with increased myocardial tolerance to infarction 48 h later [17].

Pretreatment with monophosphoryl lipid A (MLA) can pharmacologically mimic the second window of ischemic preconditioning, significantly reduce infarct size and neutrophil infiltration. Inhibition of iNOS activity by AMG abolishes the infarct size reductive effect of MLA. Aminoguanidine also blocks the ability of MLA to significantly reduce neutrophil infiltration. These results suggest that MLA pretreatment may enhance iNOS enzyme activity during ischemia, which may be responsible for the observed cardioprotection [18].

Acute myocardial infarction (AMI) - The iNOS activity is significantly increased in infarcted rabbit myocardium as compared with to healthy myocardium 48 h after coronary occlusion. However, ecNOS did not increase significantly in infarcted regions, but it was immunohistochemically

localized in endothelial and endocardial cells in normal and infarcted tissues. The presence of iNOS activity in macrophages in infarcted myocardium was identified immunohistochemically, but cardiomyocytes and neutrophils did not label with the antibodies to ecNOS and iNOS. In summary: 1) infiltrating macrophages are the main site of increased iNOS activity in infarcted rabbit myocardium; 2) ecNOS activity is not significantly increased in infarcted tissues as compared with normal myocardium; 3) neutrophils and cardiomyocytes do not express NOS immunoreactivity in infarcted and normal rabbit myocardium [19].

Experimental data suggest that NO derived from the iNOS isoform contributes to some of the myocardial injury following AMI, possibly by causing myocardial cell death in lining areas of ischemic region of the heart. So far, induction of myocardial iNOS after 72 h of AMI contributes to the development of left ventricular dysfunction, and modulation of iNOS activity by SMT improves left ventricular performance and may be beneficial after AMI. These findings suggest that selective inhibition of iNOS activity may provide a therapeutic strategy in cardiac disorders such as AMI, by improving the left ventricular dysfunction and reduction of myocardial infarct size [20-22].

Hypertension - The available data on the role of the L-arginine/nitric oxide (NO) pathway in the genesis of hypertension in spontaneously hypertensive rats (SHR) are limited and contradictory. The expression of nNOS was revealed in the media of SHR arteries but not in healthy rats. The ecNOS expression was observed in the endothelium, but no detectable levels of iNOS were found in these tissues. These results demonstrate the expression of nNOS in rat vascular smooth muscle cells and its activation on stimulation by Ang II in spontaneously hypertensive, but not normotensive, animals [23]. Male SHR were studied during the early phase of evolution of hypertension (age 8 to 12 weeks) to distinguish the primary changes of NO metabolism from those caused by advanced hypertension (vasculopathy and aging) late in the course of the disease. The SHR exhibited a marked rise in arterial blood pressure and a significant increase in urinary excretion and plasma concentration of NO metabolites (nitrite/nitrate [NOx]). Likewise, the SHR showed a significant elevation of thoracic aorta NOS activity coupled with significant increases of kidney, aorta, iNOS, and ecNOS proteins. In an attempt to determine was whether the enhanced L-arginine/NO pathway was a consequence of hypertension, studies were repeated using 3-week-old animals before the onset of hypertension. The study revealed significant increases in urinary NOx excretion as well as vascular ecNOS and renal iNOS proteins. In conclusion, the L-arginine/NO pathway is up-regulated in young SHR both before and after the onset of hypertension. Thus, development of hypertension is not due to a primary impairment of NO production in SHR. On the contrary, NO production is increased in young SHR both before and after the onset of hypertension [24].

Considering the factors age and hypertension, the activity and protein expression of ecNOS and iNOS were investigated during the development of hypertension in spontaneously hypertensive rats (SHR). SHR and Wistar-Kyoto rats (WKY) were studied at three different ages: 4, 14 to 17, and 63 weeks of age. After treatment with saline or lipopolysaccharide (LPS, 10 mg/kg IV) for 3 hours, the aortas were removed for measurement of NOS activity and protein expression. Plasma levels of nitrite/nitrate (NO_2-/NO_3-) and tumor necrosis factor-alpha (TNF-alpha) were also determined. At 14 to 17 weeks and 63 weeks, the basal activity and protein expression of ecNOS in the aortas were significantly lower in SHR than in WKY. In addition, the aged WKY exhibited lower ecNOS activity than that of adult WKY, but this change was not seen in SHR. By comparison, the basal activity and protein expression of iNOS were only observed in SHR of the 14-to-17-week group and in the 63-week group. The SHR still exhibited higher activities of iNOS, and these differences were further exaggerated by treatment with LPS. These results demonstrated that alterations of activity and protein expression of ecNOS and iNOS occurred in SHR. In addition, aging may reduce the activity of ecNOS in WKY but not in SHR. The decline of either ecNOS activity, or expression or both may contribute to the development of hypertension whereas the increase of iNOS expression may be a consequence of the pathological state of vessels associated with hypertension in SHR. However, the augmented expression of iNOS in SHR was attenuated by antihypertensive therapy, suggesting that the abnormal expression of iNOS, which needs stimulation by citokines, is associated with hypertension, but this association is relatively rare and considered controversial [25].

Another study about the role of iNOS in hypertension using a different model of experimental hypertension deserves to be mentioned. Salt-sensitive hypertension in the Dahl/Rapp rat (S strain) is prevented by L-arginine. Based on the observations that dexamethasone prevented the antihypertensive effect of L-arginine in these animals and the suggestion that a locus in or near an iNOS gene on chromosome 10, one study explored the hypothesis that the vascular smooth muscle isoform of iNOS was abnormal in S rats. Primary cultures of aortic smooth muscle cells from S rats demonstrated impaired iNOS production, which improved with increased L-arginine in the medium. A possible mutation of iNOS and the role of this enzyme in the pathogenesis of salt-sensitive hypertension in the Dahl/Rapp rat require further investigation [26].

The medullary portion of the kidney plays a crucial role in the control of sodium and water excretion and arterial pressure, and this control is anomalous in hypertension and may be related to an impaired renal NO production. Calcium-dependent NOS activity (ecNOS or nNOS) was considerably higher in the renal medulla than in the other tissues studied, both in WKY and SHR. The medulla and heart of the SHR displayed a higher Calcium -dependent NOS activity compared to with that of WKY. No differences were found in the Calcium-independent NOS activity (iNOS), except for the renal cortex of the SHR, which was higher than in the rest of the tissues. These observations indicate that the renal medulla has a high relative

capacity to synthesize NO and suggest that the impaired renal medullary control of arterial pressure of genetic hypertension is not due to a reduced NO production by the kidney [27].

Current renal studies, in conjunction with studies in the heart and aorta, strongly suggest that in hypertension, increased ecNOS activity may provide a protective homeostatic role in all the end-organs that are targets of hypertensive injury [28]. The mRNA levels of NOS in rat kidneys during states of stimulated and reduced renin gene expression were determined, to find out whether renal mRNA levels of NOS were correlated with the activity of the renin system. Stimulation of the renin system was achieved by unilateral renal artery clipping (2-kidney/1-clip rats), treatment with the angiotensin II (ANG II) antagonist losartan (40 mg/kg), application of furosemide (12 mg/Kg x day) and a low-sodium diet (0.02% w/w Na^+). Inhibition of the renin system was achieved in the nonclipped (contralateral) kidneys of 2-kidney/1-clip rats and in the kidneys of rats that were fed a high-sodium diet. In both cases renin mRNA levels decreased to about 50% of the control values. The first screening of the gene expression of NOS (nNOS, ecNOS and iNOS) during all these alterations of the renin system indicated that only nNOS mRNA levels change concordantly with the levels of renin. These changes in nNOS mRNA levels were checked by further studies, which proved that the renal levels of nNOS mRNA were significantly increased by about 50% after a low-sodium diet and hypoperfusion of the kidney. Given a stimulatory role of NO on the renin system these findings may provide the first evidence that increases of in renal levels of nNOS mRNA and, as a consequence, of renal NO formation could be important mediators of the well-known effect of salt intake and hypoperfusion on the renin system [29].

The role of nNOS in arterial pressure, renal hemodynamics, and renal excretory changes that occur in Dahl salt-resistant (DR) and salt-sensitive (DS) rats during changes in Na intake is another interesting aspect of the expression of this NOS isoform. The expression of nNOS in these two types of animals was evaluated by its inhibition with 7-nitroindazole (7NI). After 7 days of 7NI, DS-high Na rats, which had a control arterial pressure 31 mmHg higher than the comparable DR rats, increased their arterial pressure to 114+/-3% control, which was not significantly different from the DS-high Na alone pressure. No significant changes occurred in the glomerular filtration rate, effective renal plasma flow, urinary Na excretion, or urine volume because of 7NI. However, plasma renin activity decreased significantly in DR and DS rats on low Na intake with 7NI infusion. The data demonstrate that the highly salt-resistant DR rat became salt-sensitive during nNOS inhibition with 7NI. However, the arterial pressure of the DS rat was not affected by 7NI. This suggests that nitric oxide produced by nNOS in the DR rat normally helps to prevent salt-sensitive hypertension and that low functional levels of nNOS in the DS rat may contribute to its salt-sensitivity [30].

The mechanism underlying the central hypertensinogenic effects of mineralocorticoids remains unclear. Given that NO is thought to act at autonomic sites in the brain to regulate arterial blood pressure, the effects of the potent mineralocorticoid aldosterone and 19-noraldosterone on the abundance of nNOS mRNA in the brain were investigated. Compared with controls, rats treated with aldosterone or 19-noraldosterone for 4 weeks showed significant decreases in the amount of nNOS mRNA in the hypothalamus and rostral and caudal ventrolateral medulla. These data suggest that reduced nNOS activity may contribute to the increase in blood pressure in rats with central mineralocorticoid-induced hypertension [31].

One criterious review must include data about special situations involving arterial hypertension: pre-eclampsia, aortic coarctation and cyclosporin A induced arterial hypertension. The syncytiotrophoblast (ST) cell layer of the human villous placenta expresses NOS. Because NO is a potent relaxant of vascular smooth muscle and inhibitor of platelet activity, it is possible to postulate that exaggerated intervillous aggregation of platelets and reduced fetoplacental blood flow in pre-eclampsia result from reduced expression of NOS (and production of NO) by the ST. But, contrary to any expectations, the NOS expression was not significantly different between villous placenta obtained from normal first pregnant and pre-eclamptic women.

The placental NOS were also comparable among multiparous normal and pre-eclamptic women, as well as women with gestational hypertension. When compared with the enzyme activity of the villous, that of the basal plate was reduced by approximately one-half in all placentae. The calcium-independent activity was consistently fortyfold less than the calcium-dependent activity, and it was similar between villous and basal plate, and between placentae from normal and hypertensive women. These data suggest that expression of NOS is not different in placentae obtained from normal and pre-eclamptic women [32]. The correlation nNOS activity with renal hypertension has been investigated in a coarctation rat model. Significant elevation of blood pressure occurred along with left ventricular hypertrophy 8 weeks after coarctation. The nNOS activity was also significantly reduced in coarctated animals. The results suggest that an inverse correlation exists between nNOS activity and blood pressure level in aorta coarctation [33].

Cyclosporine (CsA) for clinical use has greatly enhanced the outcome of organ transplantation.However, CsA can cause nephrotoxicity and hypertension (HTN). CsA administration resulted in a significant rise in arterial blood pressure (BP) coupled with a steady decline in urinary NOx excretion, suggesting depressed NO production. This was accompanied by a significant reduction in iNOS protein abundance in the kidney and thoracic aorta but no change in ecNOS protein. The fall in renal iNOS protein in CsA-treated rats was accompanied by a parallel decline in iNOS mRNA and enzymatic activity. In conclusion, administration of CsA for three weeks resulted in a significant rise in BP together with marked reductions in urinary NOX excretion and renal and vascular iNOS expression. These observations suggest that CsA-induced HTN may be, in part,

related to impaired NO production stimulated by the NOS isoform. If true, strategies designed to restore NO availability may mitigate HTN and other vascular complications of CsA therapy [34].

The adaptive changes that occur in the left ventricle (LV) and vessels in response to hypertension, namely, muscle hypertrophy/hyperplasia, endothelial dysfunction, and extracellular matrix increase, do not depend solely on blood pressure elevation. These changes are, in fact, maladaptive because they are forerunners of cardiac failure, stroke, and renal failure. Investigations were done about the relationships among LV and aortic ecNOS activity, with LV hypertrophy and aortic hypertrophy in spontaneously hypertensive rats (SHR) and Dahl salt-sensitive (DS) rats matched for blood pressure and age. Compared with their normotensive counterparts, aortic ecNOS activity was increased in SHR but reduced in DS rats. The correlation between blood pressure and aortic ecNOS activity was positive in SHR and negative in DS rats. LV ecNOS activity was increased in SHR compared with that in normotensive Wistar-Kyoto rats . On the other hand, LV ecNOS activity was not increased in hypertensive DS rats compared with normotensive DS rats. In SHR, aortic hypertrophy did not increase significantly and LV hypertrophy increased only 15% whereas in hypertensive DS rats the aorta and LV hypertrophied 36% and 88%, respectively. Moreover, in DS rats a negative correlation occurred between ecNOS activity and aortic hypertrophy. In DS rats, antihypertensive therapy consisting of an angiotensin-converting enzyme inhibitor, perindopril, and a diuretic, indapamide, normalized blood pressure, aortic ecNOS activity, and LV hypertrophy and reduced aortic hypertrophy. These studies imply that up-regulation of vascular ecNOS activity has a protective cardiovascular homeostatic role in hypertension. Clinically, the variable end-organ disease observed in individuals with similar severity of hypertension may be explained, at least in part, by genetically conditioned differences of vascular ecNOS activity in response to hypertension [35].

Diabetes mellitus - Investigations of platelet NOS activity in insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM), which are characterized by enhanced platelet activation and platelet membrane Na+/K+ ATPase activity were determined in 19 IDDM patients, 21 NIDDM patients and 31 healthy control subjects. Both NOS and Na+/K+ ATPase activity were significantly reduced in diabetic subjects compared with that in control subjects. NOS showed a significant positive relation with Na+/K+ ATPase activity in diabetic patients. It is hypothesized that the decreased NOS activity might play a role in the pathogenesis of diabetic vascular complications [36].

The radical NO is a possible mediator of pancreatic beta-cell damage in early insulin-dependent diabetes mellitus (IDDM). Different NOS isoforms exist, but in the context of immune mediated beta-cell damage the iNOS is the most relevant. The beta-cell iNOS is similar and encoded by the same gene on chromosome 17 as the iNOS expressed in macrophages and other nucleated cells. The iNOS activation depends on gene transcription and *de novo* enzyme synthesis, and NO seems to induce a negative feedback on iNOS expression. Although iNOS mRNA is induced by interleukin-1 beta (IL-1 beta) alone in rodent insulinproducing cells, a combination of two (IL-1 beta + interferon gamma) (IFN-gamma) or three (IL-1 beta + IFN gamma + tumour necrosis factor alpha), cytokines is required for iNOS activation in human pancreatic islets. Regulation of iNOS and other related genes in beta cells is complex and differs in several aspects from that observed in macrophages. Important differences also exist in iNOS regulation between rodent and human pancreatic islets. A detailed knowledge of the molecular regulation of these genes in beta cells may be instrumental in the development of new approaches to preventing beta-cell destruction in early IDDM [37].

Evidence indicates that insulin can down-regulate the iNOS pathway *in vivo*. The iNOS pathway is up-regulated in diabetes-prone rats and mice and is associated with an autoimmune process. However, some experiments indicate that macrophage NO production and iNOS mRNA expression are also elevated in rats or mice made diabetic by streptozotocin injection in which no primary autoimmune component exists. Insulin administration reduces NO production in autoimmune-prone and streptozotocin-induced diabetic rodents. Finally, insulin decreases macrophage NO production in normal hosts. These results indicate that the autoimmune paradigm is inadequate for explaining increased NO in diabetes. As a potential mechanism to for explaining insulin-mediated regulation of NO production, TGF beta 1 may be involved because 1) macrophages from diabetic mice produce less TGF-beta1 than macrophages from healthy hosts; 2) the circulating TGF-beta1 level is lower in diabetic mice; and 3) insulin administration increases circulating TGF-beta1 in normal mice. Together, these results provide evidence that increased NO in diabetes is not only a cause but also an effect of beta-cell destruction and results in part from a heretofore unrecognized immunomodulatory activity of insulin [38].

The overproduction of NO is reported in the diabetic kidney and is considered to be involved in glomerular hyperfiltration. The precise mechanism of NO production in the diabetic kidney is, however, not known. One recent report compares the localization ecNOS isoform expression in the kidney tissue of streptozotocin (STZ)-induced diabetic rats and 5/6 nephrectomized rats and clarifies the pivotal role of ecNOS for the glomerular hyperfiltration in the early stages of diabetic nephropathy. In diabetic rats, the diameters of afferent arterioles, the glomerular volume, creatinine clearance, and urinary NO^-_2/NO^-_3 were increased after the induction of diabetes. Efferent arterioles were, however, not altered. Insulin or L-NAME treatment returned the diameters of afferent arterioles, glomerular volume, creatinine clearance, and urinary NO^-_2/NO^-_3 to normal. The expression of ecNOS in afferent arterioles and glomeruli of diabetic rats increased during the early stages of the disease, but was not altered in efferent arterioles. Treatment with either insulin or L-NAME decreased ecNOS expression in afferent arterioles and in glomeruli. In contrast, the ecNOS expressi-

on was up-regulated in both afferent and efferent arterioles and in the glomeruli of 5/6 nephrectomized rats, where the dilatation of afferent and efferent arterioles and glomerular enlargement were observed. Treatment with L-NAME ameliorated the ecNOS expression and dilatation of arterioles. It was concluded that enhanced NO synthesis by ecNOS in afferent arterioles and glomerular endothelial cells in response to the hyperglycemic state could cause preferential dilatation of afferent arterioles, which ultimately induces glomerular enlargement and glomerular hyperfiltration [39]. Another study was aimed at investigating this role of NO in the pathogenesis of glomerular hyperfiltration and hyperperfusion in streptozotocin-induced diabetic rats. To evaluate the role of NO in diabetic hyperfiltration, plasma and urine concentrations of NO_2-/NO_3-, stable metabolic products of NO and protein expressions of three isoforms of NOS in streptozotocin-induced diabetic rats were measured. Also, renal hemodynamic changes, such as glomerular filtration rate (GFR) and renal plasma flow (RPF), in responses to acute and chronic administration of NO synthesis inhibitor, nitro-L-arginine methyl ester (L-NAME) were investigated in diabetic and control rats. Diabetic rats exhibited significantly elevated plasma and urinary NO^-_2/NO^-_3 levels at 28 days after streptozotocin injection, and total excretion of NO^-_2/NO^-_3 was approximately fivefold higher in diabetic rats than controls. The three isoforms of NOS (nNOS, iNOS, and ecNOS) were all increased in the renal cortex, whereas they remained unaltered in the renal medulla at day 28. GFR and RPF were significantly elevated in diabetic rats, and acute and chronic inhibition of NO synthesis by L-NAME attenuated the renal hemodynamic changes. These studies concluded that NO synthesis was increased due to enhanced NOS expression in diabetic rats, and chronic NO blockade attenuated renal hyperfiltration and hyperperfusion in diabetic rats. In addition, diabetic rats exhibited enhanced renal hemodynamic responses to acute NO inhibition and excreted increased urinary NO^-_2/NO^-_3. These results suggest that excessive NO production may contribute to renal hyperfiltration and hyperperfusion in early diabetes [40].

To determine the possible role of nNOS expression in the pathogenesis of diabetic neuropathy, nociception and nNOS expression in dorsal root ganglion (DRG) of rats with streptozocin-induced diabetes were evaluated. Paw withdrawal threshold to noxious mechanical stimuli was decreased in both L-NAME-treated and diabetic rats. The number of positive neurons to nNOS (by histochemistry) was significantly decreased in untreated diabetic rats compared with controls. Decreased expression of nNOS protein was confirmed by molecular biology techniques. Insulin treatment completely prevented decreases in withdrawal threshold and nNOS expression. Cyclic GMP content paralleled nNOS expression in experimental animals. These results suggest that decreased nNOS-cGMP system in DRG may play a role in the pathogenesis of diabetic sensory neuropathy [41].

With regard to diabetic retinopathy: 1. NOS activity was studied in the retinas from normal rats and in the retinas from two groups of streptozotocin-induced (8 days and 4 months) diabetic rats. In each animal group, the NOS activity correlated with the concentration of amino acids related to L-arginine metabolism and to L-arginine uptake. 2. Retinas from both groups of streptozotocin-induced diabetes (8 days and 4 months) showed an increased NOS activity compared with the NOS activity in retinas from healthy rats. In retinas lysate from healthy rats, the NOS activity was most potently inhibited by NO-Arg (1 mM) whereas in both groups of streptozotocin-induced diabetes, the NOS activity was most potently inhibited by the iNOS inhibitor aminoguanidine (0.5 mM). 3. The basal levels of the amino acids related to L-arginine metabolism-namely, L-arginine, L-citrulline, L-ornithine and L-glutamine in retinas from both groups of rats with streptozotocin-induced diabetes were decreased compared with the amino acid levels in retinas from normal rats. 4. The uptake of L-[3H]arginine in retinas from both groups of rats with streptozotocin-induced diabetes was increased compared with the uptake of of L-[3H]arginine in retinas from healthy rats. A close association of neuronal nitric oxide synthase-immunoreactive (nNOS-IR) neurons with the retinal vasculature has been reported, and it is proposed that activation of these neurons could be the mechanism by which retinal blood flow and metabolism are linked. These studies suggest that the action of Aminoguanidine in restoring the number of nNOS-containing retinal neurons is mediated by the inhibition of AGE formation. The depletion of nNOS-containing neurons may contribute to alterations in the autoregulation of blood flow that occurs in diabetes [42].

Nitric oxide is an important inhibitory neurotransmitter in the gut. Alterations in NO mediated responses have been described in diabetic animals. The presence of NOS reflects the potential for NO synthesis and is found in neurons in the myenteric plexus. A study was designed to determine changes in NOS expression in the myenteric plexus of the gastrointestinal tract of diabetic rats at three months of streptozotocin-induced diabetes. Diabetic animals showed a decrease in NOS expression in the antrum, compared with that in controls. The NOS expression in the duodenum, ileum, and colon of diabetic animals was not statistically different from that in controls. Decreased expression of NOS in the antrum may contribute to altered gastric emptying observed in diabetes [43].

Finally, trying to establish links among NOS, insulin and arterial hypertension is important. It has been reported that insulin treatment improves hypertension in patients with diabetes mellitus. The mechanisms of the antihypertensive effect of insulin, however, remain to be fully elucidated. One good study investigated a possible involvement of NO in insulin-induced reduction of blood pressure using the Zucker diabetic fatty (ZDF) rat, an animal model of non-insulin-dependent diabetes mellitus. The animals were divided into three groups and treated for 4 weeks with daily subcutaneous injections of insulin (25U/kg body weight) with or without oral administration of L-nitro-arginine methyl ester (L-NAME, 50mg/kg/day body weight as drinking

water), an unspecific inhibitor of NOS. Saline solution was injected subcutaneously in the control groups. During the experimental period, body weight gain was greater in the insulin-treated groups than in the control groups whereas water intake was considerably decreased in the insulin-treated groups. Insulin treatment resulted in a decrease in plasma glucose and blood pressure and an increase in both NO metabolites (NOx) in the plasma and NOS activity in the aorta tissue. L-NAME treatment blunted not only the antihypertensive effect of insulin but also the changes in NOx and NOS activity. These findings suggest that insulin reduces blood pressure in the ZDF rat by stimulating NOS activation and NO production [44].

Hypercholesterolemia - Hypercholesterolemia is associated with impairments in endothelium-dependent vascular relaxation. Paradoxically, endothelial production of nitrogen oxide is increased in early stages of hypercholesterolemia. Oxidized low-density lipoproteins (ox-LDL) inhibit vascular relaxation by decreasing the synthesis or rapid degradation of NO. Human neutrophils, which are also stimulated to generate NO, by lipoproteins, were incubated with native-LDL, ox-LDL, HDL or HDL+ox-LDL, and NO synthase activity was measured as the conversion of [3H]L-arginine to [3H]L-citrulline. Ox-LDL, but not native-LDL or HDL, significantly decreased NOS expression. This effect of ox-LDL was incubation time and concentration dependent. The incubation of cells with HDL or L-arginine diminished the effects of ox-LDL on NOS. Thus, ox-LDL decreases the activity of NOS, and this effect of ox-LDL can be modified by HDL and L-arginine [45]. Oxidized low-density lipoprotein has both stimulatory and inhibitory effects on ecNOS expression and has focused on lysophosphatidyl choline (LPC) as a component of oxidized LDL that may modulate this effect. Another biologically active component of oxidized LDL is 13-hydroperoxyoctadecadienoic acid (13-HPODE), an oxidized form of linoleic acid. Twenty-four-hour treatment of bovine aortic endothelial cells with HPODE caused a dose-dependent increase in ecNOS mRNA levels. The time response studies show that HPODE treatment significantly increased ecNOS mRNA levels at 12 and 24 h, inducing an up-regulation of ecNOS expression. These observations suggest that endothelial cells may attempt to compensate for oxidative injury by increasing expression of ecNOS in early stages of hypercholesterolemia [46].

Hypercholesterolemia is a central pathogenic factor of endothelial dysfunction caused in part by an impairment of endothelial NO production through mechanisms that remain poorly characterized. The activity of the ecNOS was recently shown to be modulated by its reciprocal interactions with the stimulatory Ca^{2+}-calmodulin complex and the inhibitory protein caveolin. Hypercholesterolemia may reduce NO production through alteration of this regulatory equilibrium. Bovine aortic endothelial cells were cultured in the presence of serum obtained from normocholesterolemic (NC) or hypercholesterolemic (HC) human volunteers. Exposure of endothelial cells to the HC serum up-regulated caveolin availability without any measurable effect on ecNOS protein levels. This effect of HC serum was associated with an impairment of basal NO release paralleled by an increase in inhibitory caveolin-ecNOS complex formation. Similar treatment with HC serum significantly attenuated the NO production stimulated by the calcium ionophore A23187. Accordingly, higher calmodulin levels were required to disrupt the enhanced caveolin-ecNOS heterocomplex from HC serum-treated cells. Finally, cell exposure to the low-density lipoprotein (LDL) fraction alone reproduced dose-dependent inhibition of basal and stimulated NO release, as well as the up-regulation of caveolin expression and its heterocomplex formation with ecNOS, which were unaffected by cotreatment with antioxidants. Together, these data establish a new mechanism for the cholesterol-induced impairment of NO production through the modulation of caveolin abundance in endothelial cells, a mechanism that may participate in the pathogenesis of endothelial dysfunction and the proatherogenic effects of hypercholesterolemia. The ecNOS function is rapidly regulated by agonists and blood flow and chronically by factors that regulate mRNA stability and gene transcription. Recently, localization of ecNOS to specialized plasma membrane invaginations called caveolae has been proposed to be required for maximal ecNOS activity. Caveolae are highly enriched in cholesterol, and hypercholesterolemia is associated with increased NO production. Reactive oxygen species (ROS) contribute to endothelial dysfunction in hypercholesterolemia. Experimental cholesterol treatment increases ecNOS expression whereas ROS treatment decreases ecNOS expression, suggesting that oxidative stress modulates endothelial function by regulating caveolae formation, ecNOS expression and ecNOS-caveolin interactions [47,48].

Atherosclerosis - Normal and atherosclerotic human vessels were studied by *in situ* hybridization and immunocytochemistry by using probe-specific ecNOS, iNOS, and nNOS isoforms. The ecNOS was detected in endothelial cells overlying normal human aortas, fatty streaks, and advanced atherosclerotic lesions. A comparison of the relative expression of ecNOS to von Willebrand factor on serial sections of normal and atherosclerotic vessels indicated that a decrease occurs in the number of endothelial cells expressing ecNOS in advanced lesions. The iNOS and nNOS were not detected in normal vessels, but widespread production of these isoforms was found in early and advanced lesions associated with macrophages, endothelial cells, and mesenchymal-like intimal cells. These data suggest that (1) a loss of ecNOS expression by endothelial cells over advanced atherosclerotic lesions and (2) a significant increase in overall NO synthesis by other cell types in advanced lesions composed of the ecNOS, nNOS, and iNOS isoforms occur. The increased expression of NOS and presumably NO in atherosclerotic plaques may be related to cell death and necrosis in these tissues [49].

An excellent recent review reports the role of NOS in the context of atherosclerosis and hyperlipemia: 1) NO has important roles in physiological vasodilatation, cytotoxicity and vascular disease. NO and prostacyclin (PGI2), both

released from the endothelium, act synergistically to inhibit platelet aggregation and adhesion. These autacoids also inhibit the adhesion and migration of leukocytes and, in some arteries, they synergize in terms of vasodilation. 2) The development of atherosclerosis and hyperlipemia per se is accompanied by impairment of endothelium-dependent vasodilation. 3) Atherosclerosis is associated with marked changes in the activity of isoforms of NOS in the artery wall, including increased expression of iNOS isoform in complex human lesions as well as in the neointima of experimental animal models. 4) Failure of NO release from the endothelium with normal physiological stimuli, which has been attributed to a defect in the operation of the ecNOS, provides conditions propitious for leukocyte adhesion, vasospasm, thrombosis and, in addition, may promote increased proliferation of intimal cells. 5) NO and superoxide anions generated by inflammatory cells in atherosclerosis react to form cytodestructive peroxynitrite radicals, potentially causing injury to the endothelium and myocytes, and this may be a factor in apoptosis of cells leading to plaque rupture [50].

Regarding ecNOS expression in atherosclerosis, studies in normal human mammary arteries and atherosclerotic carotid arteries showed reduced NO release in atherosclerotic segments accompanied by marked reduction of immunoreactive ecNOS in luminal endothelial cells. Endothelial cells of vasa vasorum of atherosclerotic segments, however, remained positive for ecNOS, as was the endothelium of normal arteries. These studies show that in clinically relevant human atherosclerosis ecNOS protein expression and NO release are markedly reduced. This may be involved in the progression of atherosclerosis [51].

Concerning the effects of lipoproteins on NOS expression, molecular biology analyses indicate that both endothelial NO-synthase mRNA and protein are down-regulated by atherogenic concentrations of nLDL (180 and 240 mg cholesterol/dl) after 48 h of incubation, perhaps at a transcriptional level. Additionally, treatment of the cells with high-density lipoproteins, at human physiological concentrations (45 mg cholesterol/dl), does not appear to alter the expression of endothelial NO synthase, which seems to indicate that nLDL affect the gene transcription rate by a specific and concentration-dependent mechanism. These findings may have important implications because they provide a novel mechanism by which hypercholesterolemia induces early changes on endothelial cells that could have pathophysiological significance in the atherosclerotic process [52].

The expression of iNOS as well as its functional activity has recently been reported in atherosclerotic lesions. The iNOS expression was not revealed in arteries from control rabbits and in fatty streaks found in carotid and femoral arteries from hypercholesterolemic rabbits. In transitional lesions from the thoracic and abdominal aortas, the coronary and pulmonary arteries, a punctiform iNOS staining was detected in the intima. When lesions were more advanced, iNOS expression was found more intense and diffuse and localized in the subendothelial layer as well as in the media. Smooth muscle cell accumulation in intimal layers of the arteries is a marker of the degree of evolution of the atherosclerotic lesion. Based on these observations, it is possible to assume a correlation between the smooth muscle cell infiltration in the intima and the iNOS expression in the intima and the subendothelial layer, suggesting a link between the severity of the lesion and the iNOS expression [53]. Inflammatory cytokines associated with atherosclerosis may be capable of stimulating the synthesis and activity of iNOS, which could further influence the pathologic features associated with the disease. Studies assessing the localization of iNOS within healthy human and atherosclerotic vessels confirmed the presence of iNOS in atherosclerotic vessels, in which it was specifically localized to macrophages, foam cells, and the vascular smooth muscle. The distribution of immunostaining for nitrotyrosine, which means peroxynitrite formation, was virtually identical to that seen for iNOS and was present in macrophages, foam cells, and the vascular smooth muscle. In conclusion, these studies have demonstrated that stimulated expression of iNOS is associated with atherosclerosis and that the activity of this enzyme under such conditions preferentially promotes the formation and activity of peroxynitrite. This may be important in the pathology of atherosclerosis, which contributes to lipid peroxidation and to vascular damage [54].

Recently, a circulating endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), has been detected in human plasma. One study was planned to examine the relationship between plasma ADMA and atherosclerosis in humans who underwent a complete history and physical examination, determination of serum chemistries and ADMA levels, and duplex scanning of the carotid arteries. These individuals had no symptoms of coronary or peripheral artery disease and were taking no medications. Univariate and multivariate analyses revealed that plasma levels of ADMA were positively correlated with age, mean arterial pressure, and Sigma glucose (an index of glucose tolerance). Most intriguingly, stepwise regression analysis revealed that plasma ADMA levels were significantly correlated to intima-media thickness of the carotid artery (as measured by high-resolution ultrasonography). This study reveals that plasma ADMA levels are positively correlated with risk factors for atherosclerosis. Furthermore, plasma ADMA level is significantly correlated with carotid intima-media thickness. These results suggest that this endogenous antagonist of NOS may be a marker of atherosclerosis [55].

Heart failure - Evidence is increasing that alterations in NO synthesis are of pathophysiological importance in heart failure. In failing human hearts, ecNOS (NOS III) mRNA levels were increased to 180% in dilated cardiomyopathy, 200% in ischemic heart disease, and to 210% in post-myocarditis cardiomyopathy as compared to with that in nonfailing hearts. Molecular biology studies showed that NOS III protein expression was increased about twofold in failing compared with nonfailing hearts. Immunohistochemical studies with a selective antibody to NOS III showed no

obvious differences in the staining of the endothelium of cardiac blood vessels from nonfailing and failing human hearts. However, NOS III-immunoreactivity in cardiomyocytes was significantly more intense in failing compared with to nonfailing hearts. Low expression of iNOS (NOS II) mRNA was detected in only 2 of 30 failing human hearts and was not found in nonfailing hearts. These studies concluded that the increased NOS III expression in the ventricular myocardium of failing human hearts may contribute to the contractile dysfunction observed in heart failure or it may play a role in morphologic alterations such as hypertrophy and apoptosis of cardiomyocytes, or it may do both[56].

A number of studies have shown altered NO production by the ecNOS isoform, but very little information is available on the role of the inducible isoform. Inducible nitric oxide synthase (iNOS or NOS II) generates a prolonged release of large amounts of NO that may be cytotoxic or inhibit myocyte contractility, or both. It has been suggested that this mechanism specifically contributes to heart failure caused by dilated cardiomyopathy. To test this hypothesis an interesting study compared the myocardial amount and localization of iNOS in myocardial biopsies from patients with dilated cardiomyopathy or ischemic heart disease, collected during heart transplantation. Twenty-two patients included in this study were in NYHA class III-IV and iNOS was detected in all biopsies. Intriguingly, the amount of iNOS mRNA did not differ significantly between the two groups. Similarly, no intergroup differences in the amount of iNOS protein were observed. iNOS was invariably located in vascular endothelial and smooth muscle cells. In conclusion, on the basis of these studies it appears that iNOS is expressed in the myocardium of all patients with heart failure caused by either dilated or ischemic cardiomyopathy. iNOS is located prima–rily and invariably in the endothelium and vascular smooth muscle cells of the myocardial vasculature, and its expression appears to be associated with the condition of heart failure *per se* rather than related to the heart failure etiology. Also, this is true for valvular heart disease [57,58].

Heart failure is associated with activation of cytokines and expression of iNOS (NOS II), which generates NO from L-arginine. Nitric oxide has been shown to modulate myocardial performance, raising the possibility that cardiac generation of NO by NOS II modulates cardiac contraction in the failing human heart. Cardiac production of NO by NOS II attenuates the positive inotropic effects of beta-adrenergic stimulation and hastens relaxation in failing human hearts. A significant activity of iNOS has been reported in biopsies from failing hearts due to idiopathic dilated cardiomyopathy. Thus, a potential pathophysiological role of iNOS in this type of heart disease has been stated. By measuring iNOS protein expression and cGMP content in left ventricular myocardium from nonfailing and failing human hearts it is possible to conclude that the induction of iNOS may play a role in contractile dysfunction observed in septic shock but is unlikely to be of major pathophysiological importance in end-stage heart failure of any cause [57] iNOS is capable of producing large amounts of NO once induced by mediators such as interleukin (IL-1, IL-2, IL-6), tumor necrosis factor (TNF)-alpha, and interferon-gamma. Endothelial NO synthase is present in the endocardium, cardiac myocytes, and cardiac conduction tissue. Inducible NO synthase is present in cardiac myocytes, endocardium, vascular smooth muscle cells, and infiltrating inflammatory cells. Evidence from both animal models and patients suggests that NO exerts a negative inotropic effect. Increased iNOs, TNF-alpha, and IL-6 have been found in patients with heart failure in several studies. In other studies, decreased ecNOS was found in patients with heart failure. TNF-alpha and IL-6 may be produced in heart failure and may induce iNOS, resulting in NO production, which acts as a negative inotrope. eNOS may be decreased as a result of down-regulation by TNF-alpha or inducible NO synthase. The possible role of these mediators in heart failure deserves further evaluation because these findings could have novel therapeutic implications [59].

Patients with heart failure exhibit high plasma levels of nitrite/nitrate (NOx), a stable metabolite of NO, and of cytokines. The increased iNOS activity in cardiac tissue from patients with dilated cardiomyopathy, raises the possibility of local or systemic overproduction of NO induced by cytokines. This exerts a chronic negative inotropic effect on the myocardium and may have detrimental effects on systemic hemodynamics in patients with heart failure. Plasma levels of NG-dimethylarginine (asymmetric dimethylarginine; ADMA), a circulating endogenous NO synthase inhibitor, were measured in control subjects and patients with valvular, hypertensive, ischemic heart diseases or idiopathic cardiomyopathy. The plasma levels of NOx and ADMA, assessed by high performance liquid chromatography, were significantly elevated in patients with heart failure. Both NOx and ADMA were positively correlated with New York Heart Association functional class. A significant inverse correlation occurred between plasma NOx and ejection fraction, as estimated by echocardiography. A significant relationship between plasma NOx and ADMA was found only in patients with moderate to severe heart failure. These findings suggest a compensatory role of a circulating endogenous NOS inhibitor against induced NOS activity in patients with heart failure [60].

In addition to left ventricular pump failure and low cardiac output, structural and metabolic alterations of skeletal muscle are thought to contribute to exercise intolerance seen in patients with CHF. Studies using cardiac myocytes have implicated NO elaborated by iNOS as a potential agent associated with the genesis of dilated cardiomyopathy. Studies designed to locate iNOS in the working skeletal muscle of patients with congestive heart failure showed that the expression of the enzyme was restricted to skeletal muscle myocytes and was increased five- to ninefold in patients with chronic heart failure. No statistically significant difference occurred in iNOS expression between patients with dilated cardiomyopathy and those with ischemic cardiomyopathy. The finding of a locally increased expression of iNOS and the experimental evidence that NO attenuates the con-

tractile performance of the skeletal muscle suggest that the expression of iNOS may be responsible for the exercise intolerance seen in patients with chronic heart failure [61].

Evidence exists indicating that NO availability is reduced in the peripheral vasculature of patients with congestive heart failure (CHF). In CHF animals, the ecNOS location in the aorta is altered: the endothelial protein expression is substantially reduced, whereas the expression of ecNOS in the smooth muscle is increased. The total aortic ecNOS is diminished in CHF compared with that in control animals. On the contrary, no difference in ecNOS protein expression was observed in the extensor digitorum longus and soleus muscles. Furthermore, iNOS was not detected in any of the tissues considered. In conclusion, experimental CHF causes a resetting of the ecNOS protein expression in the descending aorta but not in skeletal muscles. The marked reduction of ecNOS in the aortic endothelium is consistent with the impairment of the vasodilating function reported in patients with CHF [62].

The molecular mechanisms underlying exercise intolerance in CHF are still unclear. Expression of inducible nitric oxide synthase (iNOS) and reduced phosphocreatine resynthesis have been described in the skeletal muscle of patients with CHF. Increased expression of iNOS in skeletal muscle of patients with CHF was inversely correlated with mi-CK expression and exercise capacity. These findings extend our knowledge of the pathophysiology of exercise intolerance in CHF [63].

Flow-mediated dilatation (FMD) of the peripheral arteries may be impaired in chronic heart failure (CHF), and this may contribute to the increased peripheral resistance and exercise intolerance that occur with this disease. Physical exercise improves the FMD of large conduit arteries in CHF, but whether a similar impairment also occurs in smaller arteries is unknown. CHF abolishes the FMD of small arteries by impairing the nitric oxide pathway, increasing oxidant stress, and releasing a prostanoid-contracting factor. Exercise partially restores FMD by increasing expression of endothelial nitric oxide synthase and preventing the production of vasoconstrictor prostanoids and free radicals. Such restoration of FMD might contribute to the increase in exercise capacity after physical exercise in CHF [64].

Investigations of the cellular expression and activity of ecNOS and iNOS in failing human hearts with special reference to the underlying lesion and drug therapy have been tried. The expression of ecNOS but not iNOS in the myocytes was intimately associated with beta-adrenergic therapy, being more abundant in patients on beta-blockers compared with a diminished presence in patients on beta-agonists [65].

Some reports have identified iNOS within the myocyte component of the failing human heart, and NO is known to decrease the contraction amplitude of isolated ventricular myocytes. But treatment of myocytes from a failing human ventricle with a NOS inhibitor, NG-monomethyl-L-arginine (L-NMMA), in an attempt to restore contractile function, did not increase the isoprenaline/Ca^{2+} ratio in myocytes from failing hearts, which suggests no functional role for tonic NO production in this model of heart failure. Also, the beta-adrenoceptor desensitisation in myocytes from the failing human ventricle is improbable [66].

Whether cytokine inhibitors, which decrease iNOS expression, will offer a new therapeutic insight into heart failure is a matter of speculation. As we learn more about the pathophysiological and pathogenetic role of cytokines in heart failure, it should be possible to design better and more targeted pharmacological agents. Furthermore, the investigation of inotropic agents that are effective against the production of cytokines may help in the classification of these agents [67].

Conclusion

In concluding this review, it is important to add some information about gene therapy and try to analyse particularities of NOS expression in cardiovascular disease.

Gene therapy involves the transfer of a functional gene into host cells to correct the malfunction of a specific gene or to alleviate the symptoms of a disease. For gene transfer to the cardiovascular system, adenoviral vectors are the most efficient means of transfer. Recently, transfer and functional expression of recombinant NOS genes to cerebral and cardiovascular beds have been demonstrated both *ex vivo* and *in vivo*. Studies have demonstrated successful transfer of ecNOS into porcine coronary arteries as verified by histochemical localization of recombinant protein with an increase of NO release as demonstrated by enhanced nitrite production and an alteration in vasomotor function. Although the feasibility of the NOS gene transfer approach has been demonstrated in animal models, currently available vectors have a number of technical and safety limitations that have to be solved before human NOS gene therapy for cardiovascular disease can be attempted, and represent a potential therapeutic strategy in the treatment of vascular proliferative disorders. More than 100 protocols have been proposed for human gene therapy in the United States, but no effective results have been reported in the gene therapy field. This failure in gene therapy mainly results from the lack of effective gene transfer vectors. As far as somatic gene therapy is concerned, it will be very hard to control systemic disorders even after a much more powerful vector system is developed. However, local disorders will be better regulated by gene therapy. In this regard, cardiovascular diseases will be suitable and promising targets for future gene therapy [68,69].

The ecNOS gene was effective in inhibiting of neointima formation in the rat carotid artery. These results indicate the possibility of gene therapy to prevent restenosis after angioplasty. We present here novel targets for future gene therapy. Restenosis remains the main limitation of interventional cardiology. Restenosis occurs when angioplasty-induced intimal hyperplasia as well as arterial remodeling result in flow-limiting renarrowing of the arterial lumen at the angioplasty site. Intimal hyperplasia is an important candidate for gene therapy because it is related to smooth muscle cell proliferation, which is an inviting target for molecular

antiproliferative strategies. To date, adenoviral vectors are, by far, the most efficient vectors to perform in vivo arterial gene transfer. These vectors, as well as others, have been recently used to demonstrate that therapeutic genes encoding cytotoxic (herpes virus thymidine kinase) or cytostatic (hypophosphorylatable Rb, Gax, endothelial nitric oxide synthase) products successfully inhibit smooth muscle cell proliferation and related intimal hyperplasia. Despite substantial progress, major technical issues, including the toxicity of first-generation adenoviral vectors, inefficient transduction of atherosclerotic arteries, and the risk of extra-arterial transfection remain to be addressed before gene therapy is applied to clinical restenosis [70,71].

Conventional antithrombotic treatments with antiplatelet, anticoagulant or fibrinolytic drugs are not uniformly successful and are associated with hemorrhagic side effects. Thus, new approaches to the prevention and treatment of arterial thrombosis are desirable. The gene transfer approach is particularly attractive because of its unique ability to express an antithrombotic gene at selected sites of the vessel wall (where thrombosis is threatened) while avoiding systemic anticoagulation. Clinical conditions potentially amenable to antithrombotic gene therapy include coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, peripheral artery angioplasty or thrombectomy, intravascular stenting, and vascular graft prostheses. Gene therapy may prove effective in preventing subacute thrombosis in these settings and, eventually, may play an adjuvant role to systemic thrombolysis in the treatment of acute arterial occlusion. The introduction of an antithrombotic gene into the arterial wall can be achieved either by direct in vivo gene transfer (e.g., by luminal administration of a viral vector) or by *in vitro* genetic manipulation of cells before their seeding onto vascular grafts, stents, or denuded arteries. The direct gene transfer approach has been used to deliver antithrombotic genes to animal arteries in vivo. Antithrombotic genes used to date include those encoding enzymes of the prostacyclin synthetic pathway, nitric oxide synthase, the thrombin inhibitor hirudin, and thrombomodulin. The in vitro gene transfer approach has been used to enhance the fibrinolytic activity of vascular grafts by overexpressing plasminogen activators. If the initial successes of gene therapy for thrombotic disease in animal models are confirmed by longer-term experiments, and if new vectors are developed that permit prolonged transgene expression without inflammation, human studies can be initiated [72].

Finally, to understand the NOS isoform's real role in cardiovascular diseases is a giant task. The simplest idea is consider the constitutive isoforms (ecNOS and iNOS) as "hero" and the inducible isoform (iNOS) as "villain". But this single idea many times is not true, because the isoform's NOS expressions have a dual effect. In other words, the NOS expression can act as either hero or villain, or both in certain situations related to the same cardiovascular disease.

Considering myocardial ischemia, in vivo findings demonstrate a cardioprotective role for ecNOS-derived NO in the ischemic-reperfused mouse heart. Myocardial ischemia-reperfusion injury is exacerbated in the absence of ecNOS ("hero"). Otherwise, studies indicate that NO plays a dual role in late preconditioning against stunning, acting initially as the trigger ("villain") and subsequently as the mediator of the protection ("hero"). As reviewed in this text administration of the iNOS expression inhibitor dexamethasone before ischemic preconditioning completely blocked the infarct-limiting effect of ischemic preconditioning ("hero"). Furthermore, administration of aminoguanidine, a relatively selective inhibitor of iNOS activity, 60 min before sustained ischemia also abolished the delayed protection afforded by ischemic preconditioning ("hero"). Neither aminoguanidine nor dexamethasone per se had a significant effect on myocardial infarct size (neither "hero" nor "villain"). These data provide pharmacological evidence that the induction of iNOS, following brief periods of coronary occlusion, is associated with increased myocardial tolerance to infarction 48 h later, showing iNOS's cardioprotective role ("hero"). Induction of myocardial iNOS after 72 hours of AMI contributes to the development of left ventricular dysfunction ("villain"), and modulation of iNOS activity by SMT improves left ventricular performance and may be beneficial after AMI. These findings suggest that selective inhibition of iNOS activity may provide a therapeutic strategy in cardiac disorders such as AMI by improving the left ventricular dysfunction ("hero") and reduction of myocardial infarct size (not clear, because the same authors did not prove that the effect of iNOS inhibition demonstrated a significant effect on myocardial infarct size).

In hypertension the role of ecNOS is clearly that of the "hero", because its activity declines and its expression may contribute to the development of hypertension. The increase of iNOS expression may be a consequence of the pathological state of vessels associated with hypertension in SHR, because the augmented expression of iNOS in SHR was attenuated by antihypertensive therapy, suggesting that the abnormal expression of iNOS is associated with hypertension ("villain"). But, by reviewing papers concerning hypertension and NOS, it is possible to observe that the great majority of the investigations in clinical and experimental hypertension show a pivotal "villain" nNOS role. The curious experimental data concerning the nNOS expression in hypertension and its apparent controversial effect in mice genetically lacking ecNOS, suggesting that NO released by non-ecNOS isoforms increases blood pressure. If this is true, nNOS can be elected as the "great villain". To release a kind of "vasoconstrictive NO" is too much, even for all great villains.

In diabetes, in the context of immune mediated beta-cell damage, the iNOS is the most relevant "villain" and evidences indicates that insulin can down-regulate the iNOS pathway in vivo.

In hypercholesterolemia observations suggest that endothelial cells may attempt to compensate for oxidative injury by increasing expression of ecNOS in early stages of hypercholesterolemia ("hero"). Experimental data suggest

that (1) a loss of ecNOS expression by endothelial cells occurs over advanced atherosclerotic lesions ("hero") and (2) a significant increase occurs in overall NO synthesis by other cell types in advanced lesions composed of the ecNOS, nNOS, and iNOS isoforms. The increased expression of NOS and presumably NO in atherosclerotic plaques may be related to cell death and necrosis in these tissues. Studies have demonstrated that stimulated expression of iNOS is associated with atherosclerosis and that the activity of this enzyme under such conditions preferentially promotes the formation and activity of peroxynitrite. According to these data, all three NOS isoforms could be "villains" considering the existence of atherosclerotic plaques.

Molecular biology studies have shown that ecNOS expression increases about twofold in failing compared to with nonfailing hearts. Studies concluded that the increased ecNOS expression in the ventricular myocardium of failing human hearts may either contribute to the contractile dysfunction observed in heart failure or may play a role in morphologic alterations such as hypertrophy and apoptosis of cardiomyocytes (here ecNOS as a "villain"), or may do both. The reduction of ecNOS in the aortic endothelium is consistent with the impairment of the vasodilating function reported in patients with CHF ("hero" again). The iNOS is expressed in the myocardium of all patients with heart failure caused by either dilated or ischemic cardiomyopathy ("villain"). The increased iNOS activity in cardiac tissue from patients with dilated cardiomyopathy, raises the possibility that local or systemic overproduction of NO induced by cytokines exerts a chronic negative inotropic effect on the myocardium and may have detrimental effects on systemic hemodynamics in patients with heart failure ("villain").

The above considerations may be consequences of our difficulties to in understanding some of concepts concerning the NOS expressions. The main idea of this review, is to call attention to these difficulties and to express feelings that the "end of the question" is very far from here. Finally, the idea remains of thinking about NOS expression and NO released (set free for the expression of each one of them) always as "heroes", coming back to the classic representation of the two plates balanced in equilibrium with relaxing factors on one side and contracting factors on the other side. Thus, the NOS isoform expressions would be interpreted as "heroes" who have lost the war with "villains". Unhappily, this war many times is, definitively, lost as in real life. For this idea, experiments would have to be delineated in parallel studies of genetic expressions of relaxing, antithrombotic and antiproliferative factors and genetic expressions of factors with opposing effects to these.

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