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# Forum Review

# Oxidant Stress, Immune Dysregulation, and Vascular Function in Type I Diabetes

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## ABSTRACT

**Although high glucose is an important contributor to diabetic vasculopathies, complications still occur in spite of tight glycemic control, suggesting that some critical event prior to or concurrent with hyperglycemia may contribute to early vascular changes. Utilizing previously published and new experimental evidence, this review will discuss how prior to the hyperglycemic state, an imbalance between oxidants and antioxidants may contribute to early vascular dysfunction and set in motion proinflammatory insults that are further amplified as the diabetes develops. This imbalance results from the resetting of the equilibrium between vessel superoxide/$H_2O_2$ production and/or decreased antioxidant defenses. Such an imbalance may cause endothelial dysfunction, characterized by abnormal endothelium-dependent vasoreactivity, as the first sign of blood vessel damage, followed by morphological changes of the vessel wall and inflammation. As such, increased oxidant stress in preglycemic states may be a critically central initiating event that underlies the pathogenesis of life-threatening vascular diseases in autoimmune diabetes. This review focuses on the relationship between oxidative stress, immune dysregulation, and vascular injury in type 1 diabetes, and how the discovery of novel pathways of vascular disease in nonobese diabetic mice may direct future studies in patients with type 1 diabetes.** *Antioxid. Redox Signal.* 9, 879–889.

## INTRODUCTION

The vascular endothelium, although not traditionally considered an organ, is intimately involved in maintaining organ and tissue homeostasis, and is a dynamic structure that responds to local changes in the environment. It exhibits a spectrum of behaviors that ranges from the protective to the pathogenic, contributing to end-organ damage. Under extreme circumstances, and once a certain threshold has been reached, the endothelium can become pro-thrombotic and pro-inflammatory (2). Indeed, the endothelium is a particularly sensitive target of inflammatory end-organ disease. Autoimmune diseases, including type 1 diabetes (T1D), rheumatoid arthritis, and systemic lupus erythematosus, show the presence of antiendothelial cell antibodies that appear not to be mere markers but may play a pathogenic role and contribute to endothelial dysfunction (19, 82, 113).

Although the pathogenesis of vascular complications in type I diabetes (T1D) is currently under intense investigation, the exciting results from the long-term Diabetes Control and Clinical Trials Group/Epidemiology of Diabetes Interventions and Complications Research Group (DCCT/EDIC) have helped to narrow the gap about our understanding of the role that uncontrolled glucose levels play in the pathogenesis of complications (76, 77, 92). These studies illustrate the importance of tight glycemic control and its positive influence in attenuating the progression of carotid intimal thickening and the urinary albumin excretion associated with renal glomerular changes. However, a level of pathology remained in some patients (92), suggesting that a critical event prior to or concurrent with hyperglycemia may contribute to early vascular changes. This review will show the possible basis for endothelial pathobiology in T1D, a disease with clearly unique but sometimes overlapping features compared to the vasculopathy of type 2 diabetes (44).

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In diabetes, complications such as microangiopathies, arteriosclerosis, sclerosis of small and large vessels, remodeling of extracellular matrix (ECM), and reprogramming of the gene expression pattern of vascular cells are commonly found (4, 13, 36, 37, 61). Endothelial cells (EC) regulate vascular tone by releasing endothelium-derived constricting factors, such as endothelin-1, and endothelium-derived relaxing factors, such as nitric oxide ($NO^{\bullet}$) (23). In diabetes, endothelial dysfunction occurs, and this leads to decreased responses to $NO^{\bullet}$, increased expression of proinflammatory and procoagulant factors (ICAM-1, VCAM, E- and P-selectin, and plasminogen activator inhibitor-1) and secretion of chemokines and cytokines (15, 24, 27, 91, 94, 97, 107, 120). Regardless of whether they are spontaneous or induced, a universal finding in a variety of diabetes models is impairment of endothelium-dependent relaxation (27, 57, 62, 78, 81). Endothelial dysfunction has been described in older diabetics (27, 29, 115) and in younger patients with T1D (18, 86). Vessels in asymptomatic young type I diabetics appear particularly susceptible to damage from LDL cholesterol, and very young pediatric patients show microvascular abnormalities as well (18, 21). Studies emanating from our laboratory (described below) and others suggest that endothelial dysfunction in a subset of patients who are autoimmune-prone may antedate the hyperglycemia and injure the blood vessels and accelerate complications later on.

## CONCEPT OF OXIDANT/ANTIOXIDANT BALANCE IN DIABETES

As scientists, we have been discussing and pondering the role of reactive oxygen species in disease. The question often is whether a particular effect is just a response to injury or some pathophysiological condition or whether the biochemical changes are part of the normal physiological processes. Oxidative stress is characterized by increased production of cellular oxidants and/or decreased concentrations of antioxidants and antioxidant enzymes (including glutathione, vitamin E, ascorbate, glutathione peroxidase, superoxide dismutases, and catalase). Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hypochlorous acid, and the most reactive of all, the hydroxyl radical. We know that an imbalance between reactive nitrogen/oxygen species and antioxidants results in tissue injury and dysfunction. However, we do not really know what the magic "balance" is. *In vivo*, one source of free radical production often leads to additional sources, as ischemia leads to inflammation and vice versa (74). Furthermore, all of these species, in one way or another, initiate lipid peroxidation processes and inflammation. Reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$), also play an important role in inflammation. $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ to produce the powerful oxidant peroxynitrite, $ONOO^-$ (10). $ONOO^-$ reacts with proteins, nitrating cysteine or tyrosyl residues, and resulting in an accumulation of nitrotyrosyl groups (NTY) (51, 105). Stimulation of endothelial cell $NO^{\bullet}$ synthase (eNOS) by various agonists produces $NO^{\bullet}$, which then causes vasodilation through its actions on the smooth muscle cells. When this endothelial/smooth muscle axis is impaired, vessel dysfunction occurs, and in fact, $NO^{\bullet}$ inactivation by $O_2^{\bullet-}$ has been associated with the pathogenesis of hypertension (6). Nevertheless, complete inhibition of ROS production can also lead to pathologies, as mutants of the superoxide-generating enzyme NADPH oxidase as well as mutants that lack p47 phox are more susceptible to arthritis (49), suggesting that the system behaves more like a rheostat and it is not necessarily just "on" or "off." In diabetes, there is a severe deficiency in the capacity of pancreatic β-cells to detoxify reactive oxygen species (54, 68), and diabetic patients have decreased glutathione levels (26). What is not clear is whether this defect also translates into increased susceptibility to vascular complications. The redox imbalance, as it pertains to the susceptibility to diabetic vascular complications, has been proposed to be a function of the endogenous antioxidant status (37). In this context, we recognize the role that high glucose plays in the pathogenesis of diabetic complications, particularly as high glucose conditions lead to an oxidant-mediated EC dysfunction (75, 101). Glucose plays a central role in increasing oxidant burden via accumulation of advanced glycation end (AGE) products and AGE-receptor-mediated activation of vascular NADPH oxidase (35, 94, 114). In addition, high glucose leads to mitochondrial uncoupling and activation of PARP (13, 30, 75, 79). In either case, it is clear that the increased oxidant burden leads to endothelial dysfunction.

Because oxidative stress is likely to play a central role in the development of endothelium-dependent vascular complications in T1D, the identification of similar abnormalities in the nonobese diabetic (NOD) mouse allows the dissection of the biochemical mechanisms responsible for this dysfunction. In almost every reported case of endothelial dysfunction in a clinical setting or in animal models, antioxidant treatment has restored normal vascular responses, suggesting a common underlying mechanism (41, 58, 60, 63, 83, 107, 115, 118, 119). A study that examined parameters of oxidative stress in children with T1D and their nondiabetic relatives showed that oxidant burden was high in both groups, although these differences did not achieve statistical significance (108). Given that we find vascular dysfunction in prediabetic healthy mice (70), it is plausible to suggest that endothelial dysfunction may also be found in these healthy relatives of T1D patients. The question becomes then, why is oxidant burden high prior to hyperglycemia and why is it that in some patients, tight glycemic control does not completely restore normal endothelial function? A logical assumption is that the immune dysregulation and its associated endothelial dysfunction may be the common denominator linking these conditions in susceptible families. Indeed, autoimmune diseases are now recognized as having a vascular component, and these diseases usually cluster in families. Thus, it is not uncommon to find T1D patients who have relatives with other autoimmune diseases other than T1D.

As it is difficult to predict with certainty who will develop T1D, no studies of endothelial dysfunction have been performed in prediabetic patients at risk or their healthy first-order relatives. In published studies performed on prediabetic children, the authors demonstrated elevated C-reactive protein (CRP) levels in subjects who had one or more islet autoantigens; CRP levels were more predictive of subsequent disease development (17), lending support to the idea that vascular dysfunction and inflammation can be dissociated from the hyperglycemia in some T1D patients.

In addition, once oxidant burden becomes high, this results in further amplification of the inflammatory process, upregulating adhesion molecules, inflammatory cytokines, and

chemokines, many of which are regulated by NF-κB. Thus, it is difficult to distinguish events temporally or spatially. Nevertheless, we propose a novel mechanism that may partly account for lack of correlation between tight glycemic control and endothelial dysfunction in diabetes that implicates a specific autoreactive T cell subset. In this new paradigm, the mechanisms that allow for a diabetogenic T cell clone to expand also allow for activation of NADPH oxidase-mediated oxidant stress and subsequent vascular dysfunction.

## NADPH OXIDASES IN VASCULAR FUNCTION

The NADPH oxidase of phagocytes is a high output superoxide-generating enzyme whose purpose is bactericidal. The phagocyte enzyme consists of five subunits: a gp91 and p22 phox that assemble at the membrane to form cytochrome b558, the cytosolic p47 and p67 phox, and a regulatory small G-protein rac. It has become clear, however, that a variety of nonphagocyte cells have oxidase homologues. These have now been termed NOX to distinguish them from the phagocyte phox family. The vascular oxidase, however, produces little superoxide and appears to be constitutively active. Cytokines, vasoactive hormones, pressor and inflammatory mediators result in increased levels above baseline. Oxidase activation has been associated, in one form or another, with diseases such as atherosclerosis, hypertension, cardiomyopathy, coronary artery disease, and even cell transformation in cancer (38, 66, 99). The high glucose-mediated angiotensin II-dependent cardiac contractile dysfunction could be abrogated by NADPH oxidase inhibitors, highlighting the central role of this enzyme in the pathogenesis of diabetic hypertension (87). NOX isoforms in a wide variety of tissues and even plants (32) have been associated with growth, the angiogenic switch (5), differentiation, and cancer (99). For reviews see Refs. 34, 64, and 104.

Although studies done on macrophages, antigen presenting cells (APC), and dendritic cells show a clear role for their oxidases in adapting to a changing immune microenvironment, little is known about the functional link between immune dysregulation and vascular oxidases. Most studies have been done *in vitro*, but several studies have managed to make the connection between immune dysregulation and oxidase activation in end-organ targets. For example, some patients with early HIV-1 infection develop a thrombocytopenia because of the presence of autoantibodies against an epitope of the beta3 (GPIIIa) integrin that, when engaged, induces platelet fragmentation via $H_2O_2$ derived from the interaction of platelet NADPH oxidase and 12-lipoxygenase (69). Pre-eclamptic patients develop agonistic antibodies that increase the chronotropic response of isolated murine cardiomyocytes and activate human smooth muscle cell and trophoblast NADPH oxidase by engaging the angiotensin receptor 2 (28, 112). Whereas the NAD(P)H oxidase of phagocytes is a bactericidal, high output $O_2^{\bullet-}$-generating enzyme, the vascular counterpart produces little $O_2^{\bullet-}$ and appears to be constitutively active. Tumor necrosis factor-α (TNF-α) has been shown to increase p22 phox levels, and as a consequence, ROS generation increases (25). It is possible that similarly to what has been observed in pre-eclamptic patients, this immune dysregulation leads to inappropriate activation of vascular oxidases and production of ROS. It is now clear that the output of ROS can be modulated in several tissue types and a variety of cells by antibody-mediated stimulation of cell surface receptors. Whether vasculopathogenic antibodies are present in autoimmune-prone T1D patients remains to be established.

## ROLE OF T CELLS IN VASCULAR AND AUTOIMMUNE DISEASES

The destruction of the pancreatic beta cells in type 1 diabetes occurs through an autoimmune process, and T cells are the primary mediators. These pathogenic T cells are reactive with as yet unknown islet antigens, and their expansion is a result of a failure of the homeostatic mechanisms that prevent destruction of self-antigens. Interestingly, cross-linking of the T-cell receptor (TCR) activates a T-cell oxidase through recruitment of Fas and Fas ligand (52) and mutations in the ncf gene (p47phox) have been associated with an increased susceptibility to arthritis (49).

Regulatory T cells play a central role in the maintenance of immune homeostasis. Breakdown of their regulatory role is thought to contribute to the autoimmune destruction of pancreatic β-cells (45). Apart from Th2 T cells, several types of regulatory T cells (Treg) have been described with distinct phenotypes and mechanisms of action (48, 65). Partly due to the difficulty of maintaining cell lines *in vitro*, the various subsets of regulatory T cells have been poorly defined and there is often overlap in phenotypic properties. More recent reports have provided somewhat clearer definition of these subsets, which in addition to Th2 T cells, include Th3 cells, Tr1 T cells and "natural" regulatory T (Tregs) cells (73, 100). Much recent emphasis has been directed at Tregs, which are CD25+, IL-2-dependent, and also highly specific for expression of Foxp3. Natural Tregs in addition express CTLA-4 and GITR (glucocorticoid-induced TNF receptor family) (80) and blockade of these molecules abrogates suppression, although their function in Tregs is not well understood (59). Therapeutic approaches aimed at modulating Treg activity are currently being attempted in the management of T1D.

## DIABETES AND INFLAMMATION

The inflammatory infiltrates, presence of anti-islet antibodies, and identification of diabetogenic T cells indicate that inflammation is a central participant in the pathogenesis of diabetes, and these processes can be initiated or amplified by oxidant stress. Just as the immune system has initiator and effector phases, so does inflammation have similar temporally segregated events. Oxidant stress can itself initiate an inflammatory process, but once it happens, oxidant stress will lead to activation of endothelial cells that will synthesize inflammatory cytokines and chemokines and recruit additional inflammatory cells to the site of injury. Leukocytes secrete oxidants and toxic cytokines at inflammatory sites, further injuring surrounding tissues. These interactions may be the result of high glucose, accumulation of advanced glycation end-products, increased ROS, or a combination of these (117),

Endothelial activation mediated by inflammatory cytokines, high levels of triglyceride-rich lipoproteins and/or ROS adversely affect vessel function and disrupt normal communication with smooth muscle cells. Adhesion molecules, elevated in plasma and blood vessels of diabetic patients (26), also contribute to inflammatory damage.

When NF-κB and activating protein-1 (AP-1) are activated by oxidants and/or cytokines (3, 22), expression of pro-inflammatory genes is increased (85, 90, 93). Chemokines and metalloproteases are regulated by ROS and NF-κB (98). As an inflammatory transcriptional factor, NF-κB is a central player. It is activated by many of the same signals that lead to diabetes. Peripheral blood monocytes from patients with diabetic nephropathy show increased activation of NF-κB. This activation was partially inhibited by the antioxidant alpha lipoic acid (47). CD40 also activates NF-κB (1, 46, 103), which then activates a host of κB-regulated genes. Thus, a vicious cycle of inflammation and increased oxidant burden can be initiated by CD40/CD40 ligand interactions.

In summary, in autoimmune-prone individuals, alterations in immune homeostasis may have an impact on blood vessel oxidant burden and when antioxidant defenses are inadequate to keep up with the increased generation, a positive feedback loop mediated by oxidants and amplified by cytokines results in further imbalances in cytokine production and adhesion molecule expression.

## THE NOD MOUSE AS A MODEL OF VASCULAR COMPLICATIONS IN T1D

The NOD mouse is an accepted model of type 1 autoimmune diabetes. It is known to have a generalized autoimmunity that is ultimately responsible for β-cell destruction (102). Interestingly, autoantibodies against EC antigens have indeed been identified in NOD mice (89) and in patients with T1D as well (53). In addition, diabetes can be transferred by specific immune components, and NOD.*scid* mice, or mice that have mutations in costimulatory pathways also fail to develop diabetes (8, 121, 122). In fact, a failure to limit the expansion of these effector T cells has been associated with diabetes onset (11). It is clear from these studies that a competent immune system is required for T1D. In the context of vascular dysfunction in the NOD, it is possible that there is a convergence between some immune system component, oxidant stress, and endothelial dysfunction in diabetes. However, less is known about the metabolic abnormalities and vascular complications in NOD mice, particularly in the prediabetic state. Significant hypercholesterolemia and increased triglyceride and hyperlipidemia levels were reported in hyperglycemic NOD mice fed a western diet (56) and plasma lipid concentrations were positively correlated with the duration of hyperglycemia (7). Interestingly, their low density lipoprotein (LDL) was also shown to be more susceptible to oxidation by copper, suggesting the provocative possibility that mediators in the circulation of the NOD mouse caused conformational changes that changed LDL sensitivity to oxidants. In spite of these evident abnormalities, the NOD mouse does not develop atherosclerotic plaques (56) and therefore, these mice are not a model for the study of atherosclerotic plaque burden, although this could be due to the way vascular macrophages dispose of their oxidized lipids in NOD mice. Nevertheless, NOD mice do develop endothelial dysfunction, even in prediabetic stages. Endothelial dysfunction, characterized by abnormal endothelium-dependent vasoreactivity, is the first sign of blood vessel damage and may be a sign of early vessel disease in asymptomatic (21) or normoglycemic patients (29). We found impaired endothelium-dependent vasodilation and paradoxical vasoconstriction that could be dissociated from the high glucose in NOD mice (70). This dysfunction requires activation of NADPH-oxidase and a reactive oxygen species-dependent production of vasoconstrictive arachidonic acid metabolites.

Endothelium-dependent relaxation in aortae from diabetic and hypertensive patients and from hypertensive animal models has been shown to be impaired (57). The abnormal vasoconstrictor activity of acetylcholine in the aortae of spontaneously hypertensive rats (SHR) is probably mediated by oxygen-derived radicals, since allopurinol, an inhibitor of xanthine oxidase, abolishes this effect (118). Additional studies concluded that cyclooxygenase-1 (COX-1), and possibly one of the endoperoxide products of this reaction (prostaglandin $H_2$ is a possibility) were responsible for this response (107). Interestingly, SHR aorta show increased COX-1 expression and increased production of endoperoxides (33). Finally, development of diabetes after streptozotocin resulted in hyperglycemia, as expected, but also a selective loss of endothelium-dependent vasodilation in the thoracic aorta and an early diastolic dysfunction of the heart were observed (81).

Our laboratory is interested in whether a redox imbalance antedates the rise in blood glucose that is the hallmark of diabetes. Carbonyl protein content, an index of increased protein oxidation from lipid hydroperoxides, was measured in aortic lysates from prediabetic 10-week-old NOD mice via immunoblots with an αDNPH antibody. NOD mice have increased oxidation of several proteins when compared with the age-matched BALB/c controls (Fig. 1, left top panel).

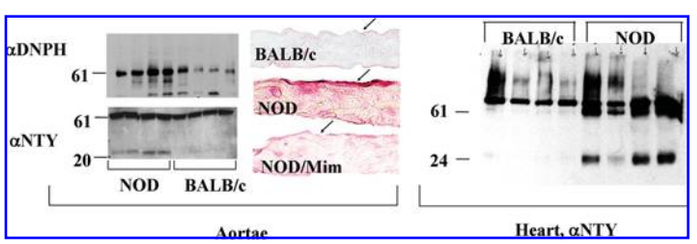


**FIG. 1. Increased markers of oxidant stress in 10-week-old prediabetic NOD and age-matched BALB/c aortae and hearts.** AORTAE: *left panel, top*, carbonyl protein content as determined by DNPH-immunoblot analysis of aortic lysates*; left panel, bottom,* nitrotyrosine (NTY); *right panel*, 4-hydroxynonenal (4-HNE) levels detected by IHC in BALB/c, NOD, and NOD + antioxidant mimetic treatment for 2 weeks. Each lane represents a different animal. HEARTS: Immunoblot analysis of nitrotyrosine levels in prediabetic 10-week-old NOD and age-matched BALB/c heart protein lysates. Numbers refer to molecular weight markers in kDa.


**FIG. 2.** *Left:* Immunoblot analysis of constitutive levels of NF-κB p65 protein in lysates from aortae of four separate 10-week old BALB/c and NOD mice. *Right*: NF-κB p65 levels in prediabetic aortae are decreased by antioxidant mimetic treatment (NOD/M) for 2 weeks *in vivo.*

Immunoblots for nitrotyrosine (NTY), an index of increased reactive nitrogen/oxygen species (Fig. 1, left bottom panel) confirmed the published immunohistochemical results (70). Levels of immunoreactive 4-hydroxynonenal (4-HNE, a lipid peroxidation product) were also higher in histological sections of prediabetic NOD aortae (middle panel). Heart tissue lysates also show more nitrated proteins (Fig. 1, right panel). These results demonstrate that steady-state levels of reactive nitrogen and/or oxygen species are higher in young (10-week-old) prediabetic aortae and hearts when compared to nonimmune BALB/c mice. A clinical correlate to these observations is found in both normal and diabetic subjects where high fat nutritional load and glucose alone produced a decrease of endothelial function, and an increase in nitrotyrosyl residues in proteins (16), suggesting that the endothelium in healthy and susceptible individuals is subject to constant postprandial oxidant damage.

The family of NF-κB transcriptional factors includes c-Rel, RelB, p52, p50, and p65. Constitutive p65 levels are a good reflection of whether a particular tissue will be hyperresponsive to an inflammatory stimulus. Thus, we measured constitutive p65 levels in aortic tissues from prediabetic NOD and BALB/c mice. The results in Fig. 2 demonstrate that levels of p65 were consistently higher in aortae from NOD mice; mimetic treatment prevented the increase. Areas of high shear stress that have a high probability of developing atherosclerotic plaques have been shown to express high levels of cytoplasmic p65, suggesting that NF-κB signal transduction pathways are already primed, increasing the susceptibility to atherosclerotic insults (42). Interestingly, basal levels of nuclear p65 and ICAM-1 protein expression were higher in cells from hypertensive rats (116). NF-κB is activated by extracellular signals such as those elicited at sites of inflammation: oxidants and cytokines. In addition, it is possible that increased p65 is not confined to the vascular tissues, as macrophages from the same mice have dysregulated cytokine production that could potentially be secondary to increased p65 and NF-κB. Furthermore, we (40) and others (12, 31, 50) have shown the reciprocal relationship between NADPH-oxidase-mediated reactive oxygen species generation and NF-κB activation in endothelial cells.

To further evaluate the level of vascular oxidative stress in T1D, we sought to determine whether aortae and hearts from prediabetic NOD have increased expression of NADPH oxidase subunits. Immunoblot analyses of aortic and heart lysates for several of the NADPH oxidase subunits revealed that levels of p67 as well as rac1 are higher in aortae from prediabetic NOD. Of note, the diabetes-resistant NOD.Lc7 showed levels of oxidase subunits that were similar to BALB/c (Fig. 3). Thus, NOD.Lc7 mice are phenotypically similar to BALB/c in diabetes resistance, low oxidant stress, no endothelial dysfunction, and normal oxidase expression. These results are significant because NOD.Lc7 is a NOD congenic strain that has a segment from C57L/J chromosome 7 that not only imparts diabetes resistance but restores normal endothelial-dependent vasodilation, suggesting that these may be linked, possibly by altered redox homeostasis. These results provide further evidence for disrupted responses to oxidative stress in diabetic vascular endothelium.

Although pharmacological NADPH oxidase inhibitors (apocynin, diphenyleneiodonium) prevent the paradoxical vasoconstriction in prediabetic NOD aortae, these inhibitors may have nonspecific effects. We used a targeted approach with a recombinant adenovirus expressing a dominant negative mutant of p67phox (Ad-dnp67) and an adeno-lacZ (Ad-LacZ) as a control. The dnp67phox adenovirus harbors a mutation in the p67 phox activation domain that prevents enzyme activation (39) and therefore acts as a transdominant negative inhibitor. Freshly isolated aortic rings from NOD mice were transduced with $3 \times 10^8$ plaque forming units/200 μl of either AdLacZ or Ad-dn p67 virus in sterile DMEM (containing antibiotic/antimycotic agents). After incubation, rings were treated with an inhibitor of $NO^{\bullet}$ synthase (NLA) to uncover the paradoxical vasoconstriction, and changes in developed tension in response to cumulative doses of acetylcholine were measured. Figure 4 shows that expression of the transdominant negative p67phox in the aortic rings of prediabetic mice abolishes the paradoxical vasoconstriction, while transduction of the adenoLacZ control had no effect, validating the specificity of the transgene.

We further established the role of ROS by inhibiting the paradoxical vasoconstriction with recombinant human Mn-superoxide dismutase or commercially available catalase (data not shown) administered *ex vivo* to aortic rings or with a metalloporphyrin superoxide dismutase mimetic administered *in vivo* (70). We concluded from these studies that antioxidants can inhibit the vasoconstriction whether used acutely or chronically.

A fundamental question that arises from the above findings is whether the endothelial dysfunction in prediabetic animals requires a competent immune system. The possibility that the increased reactive oxygen species seen in the prediabetic NOD mice was secondary to the autoimmunity present in these mice

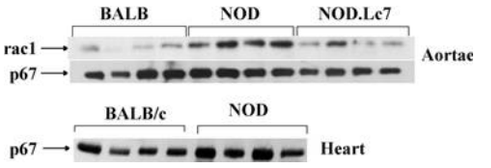


**FIG. 3. Immunoblot analyses of NADPH oxidase subunits in aortae and hearts of prediabetic 10-week-old NOD mice and age-matched BALB/c or NOD.Lc7.** All immunoblots were stripped and membranes stained with colloidal gold to ensure that equal amounts of protein were loaded in the wells.

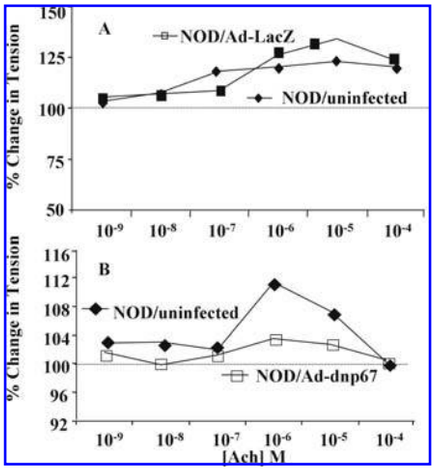


**FIG. 4. Percent change in tension as a response to Ach of aortic rings isolated from prediabetic NOD mice.** Rings were incubated *ex vivo* with the indicated recombinant adenovirus overnight, followed by tension measurements. Controls received media alone. Rings were placed in a 24-well tissue culture dish and incubated in atmospheric $O_2$/6.8% $CO_2$ for 3 h at 37°C, with mild agitation every 30 min. Rings were then removed from the DMEM mixture, washed, and immersed in sterile Earle's basic salt solution and incubated for another 24 h. Aortic rings retain their properties after 24 h incubated under these conditions (not shown).

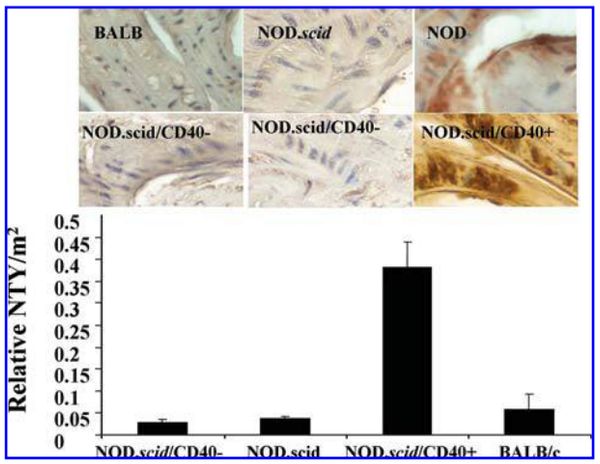


**FIG. 5. NTY IHC in aortae of mice.** $CD4^+CD40^+$ or $CD4^+CD40^-$ T cells were adoptively transferred into 4-week-old NOD.*scid* recipients. After 3 weeks, aortae were removed, used in functional studies and then fixed in formalin for NTY IHC. The *top panel* shows a representative image, and the *bottom panel* shows the quantification of image intensities. Multiple random images were captured with a digital camera and the relative staining per micron of tissue was analyzed with ImagePro software. Each of the groups was analyzed for differences with a one-way ANOVA test using the software package StatMost for Windows. The NOD.*scid* CD40+ animals were found to have statistically significantly higher staining than the NOD.*scid*/CD40-animals ($p < 0.001$, $n = 9$), the NOD.*scid* ($p < 0.001$, $n = 16$) and the BALB/c control animals ($p = 0.0076$, $n = 3$).

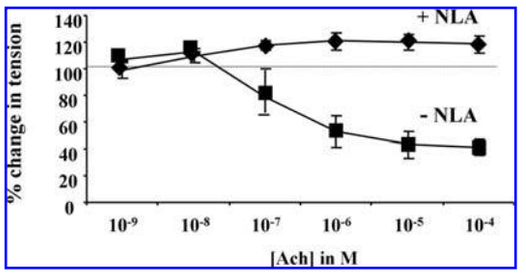


**FIG. 6. Tension measurements in NOD.*scid* mouse aortae +/–NLA. (*ANOVA*, $p < 0.005$, $n = 9$).**

can not be excluded. We have undertaken a careful examination of NTY levels in aortae of NOD.*scid* mice. These mice, which have no functional T or B cells, are also resistant to spontaneous diabetes, unless diabetogenic T cells are adoptively transferred. Indeed, we have used this model of accelerated diabetes (7–10 days from transfer to onset) to demonstrate how a metalloporphyrin SOD mimetic prevents or delays onset of diabetes after adoptive transfer (84). After a quantitative evaluation of NTY in aortic tissues from these mice, we conclude that levels of NTY in NOD.*scid* aortae are not different from levels in BALB/c aortae (Fig. 5). We then asked whether aortic rings from these mice show the paradoxical vasoconstriction present in the prediabetic NOD. The results in Fig. 6 demonstrate that there is no paradoxical vasoconstriction in NOD.*scid* mice, suggesting that a competent immune system is essential for the full spectrum of observed endothelial dysfunction.

## THE LINK BETWEEN THE IMMUNE-MEDIATED OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION IN PREDIABETIC NOD MICE

The above-described results with NOD. *scid* aortic rings indicated that a competent immune system is essential for the paradoxical vasoconstriction. We questioned what specific cellular component leads to this defect. CD4+ T cells play a major role in the pathogenesis of T1D, and adoptive transfer of CD4+ diabetogenic T cell clones into NOD.*scid* mice causes pancreatic infiltration and loss of insulin production (14, 43). Although CD8+ T cells are able to transfer diabetes when adoptively transferred, it is clear that for optimal diabetogenesis, CD4+ T cells are required when primary CD8+ T cells are used (67). A unique subset of CD4+ T cells expressing CD40 on their surface are autoaggressive and diabetogenic (110, 111). This population of CD40+ T cells constitutes a larger percentage of the CD4+ cells in NOD mice when compared to control BALB/c mice, indicating that this population is unique to autoimmunity. CD40 is a member of the TNF receptor superfamily and is expressed on B cells, T cells, APC, dendritic cells, and in nonimmune cells such as platelets, fibroblasts, smooth muscle, and endothelial cells (88, 106). In T cells, CD40 is thought to play a costimulatory role in antigen presentation and processing. Its ligand, CD40 ligand (CD154), is required for

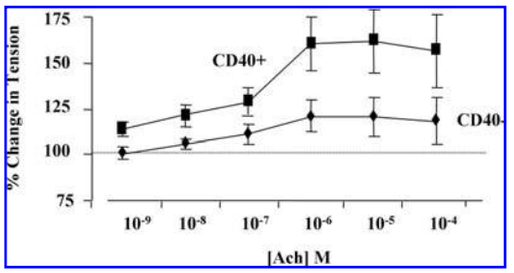


**FIG. 7. Ach dose response curves in NOD.*scid* mice aortae after adoptive transfer of CD4$^+$CD40$^+$ or CD4$^+$CD40$^-$ T cells ($n$ = 5 animals).**

these activities. CD40–CD40L interactions have been associated with atherosclerosis (72, 95, 96) and diabetes (8, 11). Exposure of fibroblasts and human intestinal microvascular endothelial cells to CD40L or CD40L-positive T cells results in increased adhesion molecule and RANTES expression in an antigen-independent manner (109). Type 2 and type 1 diabetic patients have significantly higher soluble CD40L (sCD40L) levels than controls, and improved metabolic control reduces its plasma levels (20). Cultured human umbilical vein endothelial cells (HUVEC) constitutively express a low level of CD40 antigen, but this expression is increased by incubation with inflammatory cytokines such as TNF and IL-1. Exposure of EC to CD40L induces adhesion molecule expression within hours of exposure (55). Many of these CD40L-dependent effects are mediated through NF-κB (9, 71).

When adoptively transferred into NOD.*scid* mice, this population of CD40+ T cells expands and rapidly induces diabetes (111). We were intrigued by the possibility that this population of T cells could also transfer endothelial dysfunction. To investigate this idea, CD40+ and CD40– T cells were isolated from the spleens of NOD mice by immunomagnetic absorption. Both CD4+/CD40+ and CD4+/CD40– T cell populations were transferred into individual NOD.scid mice (3–4/group). After 3 weeks, NOD.*scid*/CD40+ and NOD.*scid*/CD40– adoptively transferred mice were sacrificed, and aortic ring functional studies were performed. At the time of sacrifice, only 1 NOD.*scid* CD40+ mouse had a blood glucose level above 13.9 mmol/L, indicative of diabetes (data not shown). Despite this fact, all NOD.*scid*/CD40+ mice showed paradoxical vasoconstriction in response to acetylcholine (Fig. 7), suggesting that the paradoxical vasoconstriction depended on CD40+ T cells. NOD.*scid*/CD40– mice showed a much attenuated effect when compared to the effect caused by the CD40+ cells. In fact, all acetylcholine gradients performed on NOD.*scid*/CD40+ aortic rings showed significantly greater contraction than NOD.*scid*/CD40– aortic rings: $10^{-9}$ to $10^{-6}$ $M$ acetylcholine ($p < 0.006$), $10^{-5}$ to $10^{-4}$ M acetylcholine ($p < 0.05$). When examined individually, the differences in contraction between each acetylcholine concentration were clearly evident. For example, at an acetylcholine concentration of $10^{-5}$ $M$, NOD.*scid*/CD40+ aortic rings showed an average of 160.39% change in tension, as compared with only 119.88% in NOD.*scid*/CD40– mice. In summary, with a view to further characterizing the immune component potentially responsible for conferring vascular anomalies in T1D, we have found CD4+/CD40+, but not CD4+/CD40– T cells transfer endothelial dysfunction in NOD mice.

Given the impact of CD4+CD40+ cells on vascular pathology, we sought to determine whether adoptive transfer of these cells affected oxidative stress through activation of NADPH oxidase in vascular tissues. We first examined expression of several of the oxidase subunits in heart tissue lysates because aortic tissues had been extensively manipulated in the functional studies and were unavailable for immunoblot analyses. Both p47 and p67phox expression were higher in the NOD and NOD.*scid*/CD40+ but not in the NOD.*scid* and NOD.*scid*/CD40– mice examined in each group (Fig. 8). These results suggest two things: NOD.*scid* mice have lower levels of oxidase subunit expression than NOD mice and transfer of CD40+ cells results in upregulation of oxidase subunits in the heart. In summary, adoptive transfer of CD40+ cells into NOD.*scid* mice results in upregulation of NADPH oxidase subunit expression. Importantly, this finding provides a mechanistic link between the autoimmune response and the host oxidative stress response.

Finally, we also examined NTY levels in some of the aortic rings after adoptive transfer. Please note that these rings were stained after the functional studies were performed, so they have been extensively manipulated and not fixed *in situ*. In addition, there is always the possibility that the NTY turns over *ex vivo* and we may not see differences in NTY after the *ex vivo* manipulations. Nevertheless, we did find differences in levels of NTY between the NOD.*scid*, NOD.*scid*/CD40$^+$, and NOD.*scid*/CD40$^-$ (Fig. 5). These surprising results suggest that a dysregulated immune system leads to activation of vascular NADPH oxidase, which in turn leads to endothelial dysfunction. The basic concept remains the same: the redox imbalance and vascular dysfunction can be dissociated from the hyperglycemia.

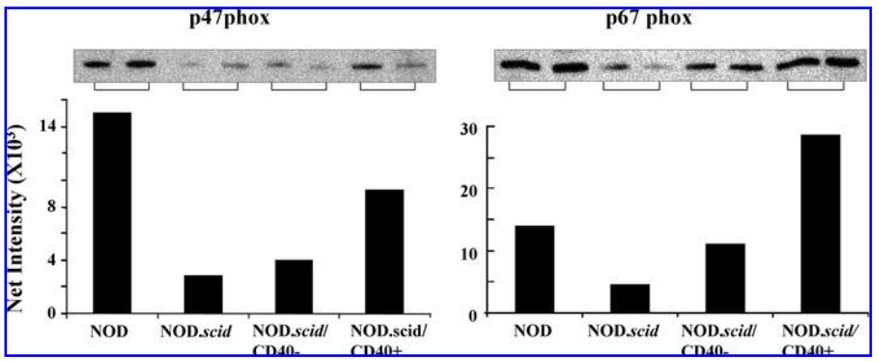


**FIG. 8. NADPH oxidase expression in hearts of NOD.*scid* mice after adoptive transfer of the indicated T cell population.** Three weeks after transfer, p67 and p47phox expression were examined via immunoblots analyses. Heart lysates from wild-type NOD mice are included for comparison purposes.

In summary, we have demonstrated that (a) the aortae and other tissues from prediabetic NOD mice have increased biochemical indices of oxidant stress associated with vascular dysfunction; (b) the vascular dysfunction can be attenuated with antioxidants *ex vivo* and *in vivo*; (c) metabolites of arachidonic acid are involved; and (d) these effects are mediated by a vascular NADPH oxidase. Our recent preliminary data implicate the immune system, whereby $CD4^+CD40^+$, but not $CD4^+CD40^-$ cells can adoptively transfer the dysfunction. Finally, adoptive transfer of these pathogenic T cells appears to cause dysfunction by NADPH oxidase activation. These exciting results suggest a mechanistic link between vascular dysfunction in autoimmune diseases and reactive oxygen species.

## CONCLUSION

Although tight glucose control delays the increase in carotid-intimal thickness that is the hallmark of late diabetic vascular complications, some diabetic patients still develop complications. The assumption that endothelial dysfunction in diabetes is solely due to abnormal glucose and perhaps exacerbated by dyslipidemia is inadequate to explain some clinical and experimental observations. In the NOD mouse, the preferred and accepted animal model of spontaneous autoimmune T1D, there is an endothelial dysfunction characterized by a paradoxical vasoconstriction that precedes the hyperglycemia. This could explain the clinical observation that certain people are very prone to complications (despite good glycemic control), while others are not, regardless of glycemic status. In susceptible individuals, immune dysregulation and alterations in redox homeostasis may be the initiating mechanism, and this process continues even in a background of good glycemic control. We have reviewed what is known about autoimmunity, oxidative stress, and vascular injury in T1D, as well as presented experimental data from our group in the NOD mouse. The case that is being built is that autoimmune injury may affect more than the destruction of pancreatic β-cells and include injury to the vascular endothelium; an injury that is likely facilitated by an enhanced oxidative state and perhaps diminished defenses against oxidative injury. Given the relative novelty of some of these concepts, it is hoped that this improved understanding between the phenomena that link autoimmunity, oxidative stress, and vascular injury will culminate in new avenues of therapy for patients with T1D.

## ABBREVIATIONS

Ach, acetylcholine; Ad-dnp67, a dominant negative mutant of p67phox; Ad-LacZ, adeno-LacZ; AP-1, activating protein-1; APC, antigen presenting cell; AGE, advanced glycation endproduct; CD4, CD4 co-receptor-positive T lymphocytes; CD8, CD8 co-receptor-positive T lymphocyteslCD25, CD25 antigen-positive T lymphocytes ;CD40, CD40 antigen-positive; CD40L, CD40 ligand antigen-positive; COX-1, cyclooxygenase-1; CRP, C-reactive protein; CTLA-4, cytotoxic T-lymphocyte antigen 4 ; DCCT/EDIC, Diabetes Control and Clinical Trials Group/Epidemiology of Diabetes Interventions and Complications Research Group; DMEM, Dulbecco's Modified Eagle Minimal Essential Medium; DNPH, dinitrophenylhydrazine); EC, endothelial cells; ECM, extracellular matrix; eNOS, endothelial cell nitric oxide synthase; Foxp3, forkhead/winged-helix family of transcriptional regulators transcriptional regulators; GITR, glucocorticoid-induced TNF receptor family; $H_2O_2$, hydrogen peroxide; 4-HNE, 4-hydroxynonenal; HUVEC, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; IL-2, interleukin-2; LDL, low density lipoprotein; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NF-κB, nuclear factor kappaB; $NO^{\bullet}$, nitric oxide; NOD, nonobese diabetic; NOD.Lc7, NOD congenic strain with a segment from C57L/J chromosome 7; NOD.scid, nonobese diabetic severe combined immunodeficiency; $O2^{-}$, superoxide; $O2^{\bullet-}$, superoxide radical; $ONOO^{-}$, oxidant peroxynitrite; p67phox, adenovirus expressing a dominant negative mutant of p67phox; PARP, poly(ADP-ribose) polymerase; RANTES, regulated upon activation, normal T-cell-expressed and secreted; RNS, reactive nitrogen species; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; TCR, T-cell receptor; Th1 cells, Th1 subset of T lymphocytes; Th2 cells, Th2 subset of T lymphocytes; Th3 cells, Th3 subset of T lymphocytes; Treg cells, regulatory T lymphocytes; Tr1 cells, Tr type 1 Treg cells; TNF-α, tumor necrosis factor-alpha; T1D, type I diabetes; VCAM, vascular cell adhesion molecule-1.

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