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Short-term hyperglycemia increases arterial superoxide production and iron dysregulation in atherosclerotic monkeys

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ABSTRACT

The incidence and severity of atherosclerotic vascular disease are increased in diabetic patients, in part because of increased production of reactive oxygen species (ROS). Previously, we found both increased atherosclerosis and arterial protein oxidation 6 months after streptozotocin-induced diabetes in monkeys fed an atherogenic diet, the pattern of which was indicative of redox-active transition metal involvement. The goal of this study was to determine if short-term (1 month) hyperglycemia increases oxidative stress and dysregulates iron metabolism before differences in atherosclerosis. Cynomolgus monkeys with preexisting atherosclerosis were stratified by dietary history and plasma lipids and received either streptozotocin (STZ-DM; n = 10) or vehicle (control; n = 10). One month after diabetes induction, blood and artery samples were collected. There were no differences in plasma lipoprotein cholesterol, arterial cholesterol, and atherosclerosis between control and STZ-DM. However, plasma lipid peroxides were elevated 137% ($P < .01$); arterial superoxide was increased 47% ($P < .05$); plasma ferritin, an indicator of whole-body iron stores, was 46% higher ($P < .05$); and iron deposition within aortic atherosclerotic lesions was more prevalent in STZ-DM compared with controls. Arterial levels of the antioxidant enzymes, superoxide dismutase, catalase, and heme oxygenase-1 were not higher in STZ-DM, although superoxide was higher, suggesting impaired antioxidant response. The increase in ROS before differences in atherosclerosis supports ROS as an initiating event in diabetic vascular disease. Further studies are needed to determine if increases in iron stores and arterial iron deposition promote hydroxyl radical formation from superoxide and accelerate diabetic vascular damage.

1. Introduction

Cardiovascular disease, the primary cause of death among diabetic patients, occurs at an earlier age and results in 2- to 8-fold greater mortality rates than in nondiabetic patients [1]. The primary cause of this increased incidence of vascular disease, which includes cerebrovascular disease, coronary heart disease, and peripheral vascular disease, is atherosclerosis [2,3]. Although the exact mechanisms responsible for the increase in atherosclerosis are unclear, epidemiologic

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evidence suggests that only 25% of the excess coronary heart disease in diabetic patients can be accounted for by changes in traditional risk factors such as hypercholesterolemia, hypertriglyceridemia, hypertension, and obesity [3,4], suggesting that the increased vascular disease may involve factors unique to the diabetic patient.

Chronic hyperglycemia, a common feature of all forms of diabetes, contributes significantly to both the microvascular and macrovascular complications of the disease and has been linked to the increased incidence of atherosclerosis in diabetic patients [5,6], suggesting that glucose itself may be toxic to the vasculature [4]. Indeed, the United Kingdom Prospective Diabetes Study demonstrated that strict glycemic control dramatically lowered the incidence of microvascular diseases (retinopathy, nephropathy, and neuropathy); and a 10-year follow-up showed an emergent risk reduction for myocardial infarction for subjects receiving intensive glucose-lowering therapy during the trial [4,7]. Furthermore, it has been suggested that vascular endothelial cells and mesangial cells are particularly sensitive to the damaging effects of hyperglycemia because they are relatively inefficient in their ability to prevent glucose transport in response to hyperglycemia, resulting in high intracellular glucose levels [8].

Oxidative stress has been postulated as a major contributor to both microvascular and macrovascular diabetic complications; and it has been suggested that many, if not all, of the mechanisms that relate hyperglycemia to vascular disease involve the overproduction of reactive oxygen species (ROS) [4,9,10]. A consistent differentiating feature common to all cell types that are damaged by hyperglycemia is an increased production of ROS, in particular, the overproduction of superoxide [4,8,11-13]. In fact, it has been proposed that the overproduction of superoxide that occurs in response to mitochondrial dysfunction in diabetes is the unifying mechanism by which hyperglycemia exerts its role in producing vascular damage [4,8,14].

It is unlikely that superoxide per se is the ROS that is directly responsible for all of the vascular damage because it has a relatively limited reactivity with biomolecules [15]. Superoxide is, however, readily converted to more reactive species such as the hydroxyl radical, through the actions of superoxide dismutase (SOD) and transition metal-catalyzed Fenton reactions (Eqs. 1 and 2), and peroxynitrate, through its reaction with endothelial cell–derived nitric oxide [4,16,17].

$$\text{SOD reaction} \quad 2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

$$\text{Fenton reaction} \quad H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + {}^{-}OH + Fe^{3} \qquad (2)$$

Studies in streptozotocin-induced diabetic (STZ-DM) monkeys have suggested that hydroxyl radical is responsible for hyperglycemia-induced vascular damage [16]. Considered the most destructive ROS in nature [18-21], hydroxyl radicals rapidly react with most biomolecules [22,23]. However, because they are short-lived and diffuse only a finite distance before reacting, hydroxyl radicals must be produced within the vasculature to exert damage there.

The development of diabetes has been shown to be associated with an increase in whole-body iron stores, with reports of elevated serum ferritin not only in type 2 diabetes mellitus patients but also in insulin-resistant nondiabetic individuals [24-27]. In addition, there is considerable evidence that increases in whole-body iron stores, such as those in patients with familial hemochromatosis, also predispose to insulin resistance and diabetes [24] and that repetitive phlebotomy and/or iron chelation therapy using agents such as deferoxamine not only reduces ferritin levels in these patients but also improves insulin sensitivity and glycemic control [24].

However, as these studies are primarily associative in nature, it has been difficult to determine whether hyperglycemia per se increases whole-body iron stores and, if so, the time frame in which this may occur. Evidence argues against a generalized increase in oxidative stress in diabetes but rather supports localized generation of ROS species within microenvironments of the vasculature [4]; the role of iron in the ROS-mediated vascular damage induced by hyperglycemia in diabetes is appealing. Unfortunately, information on the role of oxidative damage and its relationship to vascular dysfunction and atherosclerosis in diabetic humans is limited. This is in part the result of both dietary variations within the human population and variability in dietary and supplemental intake of vitamins and minerals that may either have antioxidant properties or modify iron metabolism.

To study early oxidative events in the development of diabetes, we conducted studies examining ROS production, iron deposition, and atherosclerosis in vascular tissue of monkeys fed a standardized Western-type diet in which diabetes was induced with streptozotocin [16,28-30]. In previous studies using this model, we demonstrated that 6 months of hyperglycemia resulted in both an increase in atherosclerosis extent [29] and arterial oxidation [16]. Unfortunately, as oxidative stress and atherosclerosis increased concomitantly at this time point, we could not determine whether the oxidation preceded and possibly contributed to the hyperglycemia-induced atherosclerosis development or occurred as a consequence of the increased atherosclerosis.

In the present study, we assessed differences in systemic and arterial ROS production as well as iron deposition within the vasculature after a shorter, 1-month period of hyperglycemia in STZ-DM monkeys, a time at which treatment-induced differences in atherosclerosis development were not anticipated and therefore would not confound interpretation.

2. Methods

2.1. Animals

All monkeys were fed a moderately atherogenic diet containing 0.18 mg of cholesterol per kilocalorie with 35% of calories from fat for a minimum of 3 months before induction of diabetes and throughout the 1-month study period. Before this study, monkeys had been fed variable experimental diets, accounted for by calculating historical dietary cholesterol exposure (milligram cholesterol/calorie in diet × number of days fed diet). Monkeys were stratified into control (n = 10) and STZ-DM (n = 10) groups by their dietary cholesterol history and their pretreatment plasma total cholesterol to

high-density lipoprotein cholesterol (HDLC) ratio to balance atherosclerosis risk at baseline [29]. All animal procedures performed during this study were done in accordance with the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine.

2.2. Induction of diabetes mellitus

Diabetes was induced with streptozotocin (Zanosar; Upjohn, Kalamazoo, MI) as previously described [28-30]. Seven of the 10 monkeys became diabetic (fasting blood glucose >126 mg/dL) 3 days after streptozotocin treatment. The remaining 3 monkeys received 2 additional doses of streptozotocin to induce diabetes. The STZ-DM monkeys were maintained on Novolin 70/30 insulin (Novo Nordisk, Princeton, NJ) to mimic moderately controlled diabetes in humans (8%-10% glycated hemoglobin [HbA_{1c}]). Seven days after diabetes induction, intravenous glucose tolerance tests (IVGTTs) were performed to assess β-cell responses to glucose challenge. Parameters measured included area under the curve for glucose and insulin, as well as K values for glucose disappearance rate [28].

2.3. Sample collection

Monkeys were sedated with ketamine (10-15 mg/kg intramuscularly) after an 18-hour fast for blood sampling at baseline and 4 weeks. Serum, plasma, and whole blood samples were stored at –80°C. At study end, monkeys were sedated and then anesthetized with sodium pentobarbital (80 mg/kg body weight intravenously) before exsanguination and collection of heart, aorta, and arteries.

2.4. Clinical chemistry analyses

Total plasma cholesterol, triglycerides, apolipoprotein B (Apo B) cholesterol (low-density lipoprotein + very low-density lipoprotein cholesterol), and HDLC were measured [29-31]. Plasma free fatty acids were measured by colorimetric assay (Wako Chemicals, Richmond, VA). Plasma glucose concentrations were determined by the glucose oxidase method (Roche Diagnostics, Indianapolis, IN), fructosamine concentrations by the Nitro Blue colorimetric methodology (Roche Diagnostics), and HbA_{1c} by automated affinity high-performance liquid chromatography (HPLC) (Primus, Kansas City, MO) as described [16,30,31]. Insulin and C-peptide were determined by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). Plasma lipid peroxides were measured by colorimetric assay (Zeptometrix, Buffalo, NY), and plasma oxidized low-density lipoprotein (oxLDL) concentrations were determined by an ELISA that detects oxidized Apo B antigen (Mercodia, Uppsala, Sweden). Plasma ferritin concentrations were determined by ELISA (AssayPro, St Charles, MO), and serum iron was measured using an Alfa Wassermann ACE analyzer (Direct TIBC; Reference Diagnostics, Bedford, MA).

2.5. Arterial superoxide levels

Lucigenin-enhanced chemiluminescence provided relative measures of superoxide levels [32]. Segments of carotid arteries were placed into microtiter plate wells containing phosphate-buffered saline with and without 1 mmol/L NADPH. The arteries were maintained at 37°C for 30 minutes, and then scintillation counts were obtained for 20 minutes in the presence of lucigenin (9,9′-bis(*N*-methylacridinium nitrate), 5 μmol/L) (Sigma-Aldrich, St Louis, MO) using a Fluostar Optima luminometer (BMG Labtech, Durham, NC). Background-corrected values were normalized to dry tissue weight (micrograms) and reported as either basal counts or NADPH-stimulated counts to basal counts.

Superoxide levels were also assessed by formation of 2-hydroxyethidium, modified from methodology based on the conversion of dihydroethidine (DHE) to 2-hydroxyethidium in the presence of superoxide [33]. A 1-cm segment taken near the abdominal/thoracic aorta junction was placed into ice-cold Krebs-HEPES buffer (KHB) and cut into thin rings (~2 mm). Rings were placed into wells with KHB containing 10 μmol/L DHE (Invitrogen, Carlsbad, CA), incubated at 37°C in 95% air/5% CO_2 for 20 minutes, washed with KHB, then incubated for 1 hour before transferring to methanol (1:10 wt/vol), and stored for analysis at –80°C. Before analysis, tissues were homogenized; and supernatant was retained for HPLC determination with a C-18 reverse phase column (Nucleosil 250, 4.5 mm, Sigma-Aldrich) equipped with UV and fluorescence detectors. The mobile phase consisted of 1% trifluoroacetic acid with increasing acetonitrile concentration (10%-70%) (Alltech SelectPro fluid processor; Alltech, Deerfield, IL) over 50 minutes at a flow rate of 0.5 mL/min to separate DHE, ethidium, and 2-hydroxyethidium. Formation of 2-hydroxyethidium was monitored with the fluorescence detector (emission, 580 nm; excitation, 480 nm), and DHE was detected by UV absorption at 355 nm.

2.6. Arterial cholesterol and lipid peroxides

A segment of carotid artery was removed and snap frozen in liquid nitrogen. Chloroform-methanol lipid extracts were prepared, and total and free cholesterol concentrations were determined enzymatically [29]. Esterified cholesterol was calculated as the difference between total and free cholesterol. Arterial lipid peroxide concentrations were measured in the same lipid extracts used for cholesterol determinations [30], and expressed as malondialdehyde equivalents.

2.7. Aortic iron and atherosclerotic lesion area

Sections of thoracic aorta were placed for 1 hour at 37°C in Perl solution containing 7% potassium ferrocyanide and 3% hydrochloric acid to stain ferric iron (Fe^{3+}). The sections were then incubated in 0.75 mg/mL diaminobenzidine for 15 minutes, followed by 15-minute incubation with 0.015% hydrogen peroxide. Perl iron staining was graded as 0, 1, 2, or 3 by a blinded assessor using photomicrographs assembled from high-magnification digital images using ImagePro Plus 5.1 (Media Cybernetics, Bethesda, MD). Sections were subsequently analyzed to determine atherosclerotic lesion area. The intimal (lesion) area (square millimeters) was determined by subtracting lumen area from total area within the internal elastic lamina.

2.8. Immunoblotting

Frozen abdominal aorta was ground with a mortar and pestle in liquid nitrogen into a fine powder before protein extraction, gel electrophoreses, and transfer to nitrocellulose membranes as previously described [34]. The membranes were probed with antibodies to the various SODs (manganese SOD [MnSOD], copper/zinc SOD [Cu/ZnSOD], and extracellular SOD [EcSOD]) (Nventa Biopharmaceuticals, San Diego, CA), heme oxygenase-1 (Nventa Biopharmaceuticals), and catalase (EMD Chemicals, San Diego, CA). After labeling the membranes with the appropriate horseradish peroxidase–conjugated secondary antibody, they were developed using an ECL-plus detection reagent (GE Healthcare, Giles, United Kingdom) and quantified by expressing their respective densities as arbitrary units (AU) normalized to β-actin bands [34].

2.9. Statistical analysis

Values are presented as means ± SEM. Measures that were not normally distributed were log transformed to satisfy the normality assumption. Group differences were determined using Student *t* test or analysis of covariance (ANCOVA) (if baseline measures were available as covariates). Baseline plasma lipid peroxide concentrations were used as covariates for arterial superoxide measures because they significantly predicted the outcome measures. Differences between proportions of positive arterial Perl staining were assessed by the χ^2 statistic. Significance was set at $P < .05$. Analyses were done using SAS version 9.1.3 (SAS Institute, Cary, NC).

3. Results

3.1. Experimental diabetes induction

There were no significant differences between the control and STZ-DM groups with respect to age (17 ± 2 vs 12 ± 2 years, respectively; $P = .10$), body weight (6.8 ± 0.7 vs 6.0 ± 0.4 kg, respectively; $P = .30$), plasma total cholesterol (252 ± 17 vs 301 ± 43 mg/dL, respectively; $P = .28$), triglycerides (33 ± 5 vs 49 ± 9 mg/dL, respectively; $P = .12$), and glycemic measures at baseline (Table 1). The IVGTT performed 7 days after induction confirmed that STZ-DM monkeys had significantly reduced ability to clear glucose and secrete insulin (both Ps $< .001$; Fig. 1), indicative of β-cell destruction. As expected, plasma glucose, fructosamine, and HbA_{1c} levels were significantly higher in STZ-DM compared with control monkeys (Table 1). Plasma insulin and C-peptide concentrations were lower in STZ-DM compared with control monkeys (Table 1).

3.2. Plasma and arterial lipids

One month after induction, there were no differences in total cholesterol, Apo B cholesterol, HDLC, or free fatty acid concentrations between STZ-DM and control monkeys (Table 2). Furthermore, there were no differences in carotid artery total cholesterol, free cholesterol, or esterified cholesterol, and no differences in aortic intimal area between the STZ-DM and control monkeys (Table 2), consistent with the short time frame after diabetes induction. Plasma triglycerides were significantly increased in STZ-DM monkeys.

Table 1 – Effect of hyperglycemia on plasma glycemic indices in control and STZ-DM monkeys

		Control	STZ-DM	*P* value
Glucose (mg/dL)	Baseline	58 ± 1.1	63 ± 3.3	.13
	Study end	54 ± 3	291 ± 52	<.001
Insulin (IU/L)	Baseline	14 ± 2.7	16 ± 2.1	.54
	Study end	17.3 ± 3.9	8.8 ± 2.5	<.05
Fructosamine (μmol/L)	Baseline	192 ± 10	186 ± 13	.72
	Study end	193 ± 10	260 ± 19	<.05
HbA_{1c} (%)	Baseline	4.4 ± 0.17	4.5 ± 0.16	.44
	Study end	3.9 ± 0.12	6.8 ± 0.36	<.001
C-peptide (pmol/L)	Baseline	194 ± 23.5	224 ± 26.3	.42
	Study end	250 ± 43.9	173 ± 51.7	.05

Fasting blood samples were obtained from control and streptozotocin-treated monkeys 4 weeks after diabetes induction. Data are presented as the mean ± SEM (n = 10 animals per group) together with respective ANCOVA *P* values for study end parameter. Baseline parameters are assessed by Student *t* test.

3.3. Plasma oxidation measures

Plasma lipid peroxides were similar between STZ-DM and control groups at baseline (9.3 ± 1.0 vs 7.8 ± 0.6 nmol/mL, respectively), but increased 137% from baseline ($P < .01$) in STZ-DM compared with a 7% decrease from baseline in control monkeys (Fig. 2A). Similarly, oxLDL concentrations increased 67% from baseline ($P = .08$) in STZ-DM monkeys compared with a 6% reduction from baseline in control monkeys (Fig. 2A).

One month after diabetes induction, plasma ferritin concentrations were significantly higher in STZ-DM than in control monkeys ($P < .05$). Serum iron levels also tended to be higher ($P = .08$) but just failed to reach statistical significance (Fig. 2B).

3.4. Aortic iron deposition

Iron deposition within atherosclerotic lesions is thought to contribute to the oxidative stress common to the atherosclerotic lesion [35]. Perl staining of thoracic aortic segments revealed Fe^{3+} deposits in thoracic aortas from both control (Fig. 3A) and STZ-DM monkeys (Fig. 3B). Iron staining was considerably more prevalent in STZ-DM than control monkeys ($P < .05$).

The 2 representative aortic sections shown in Fig. 3 were chosen because they have comparable atherosclerotic lesion areas. To assess the relationship between iron deposits and lesion area among STZ-DM and control monkeys, we compared the atherosclerotic lesion size and grade of Perl staining for each arterial section. There was no relationship apparent

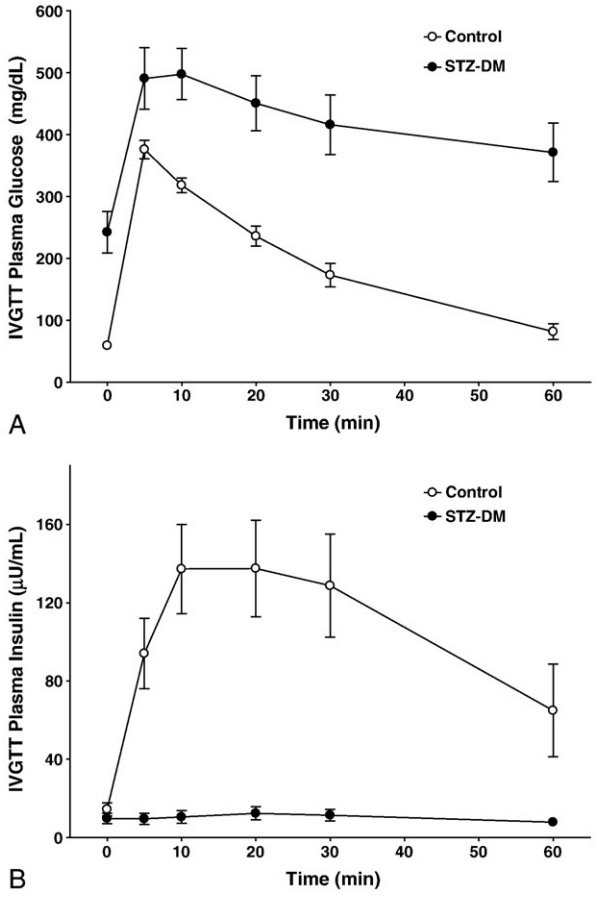

Fig. 1 – Effect of streptozotocin administration on β-cell responsiveness. Intravenous glucose tolerance tests were performed in both control monkeys (open circles) and STZ-DM monkeys (closed circles) 7 days after diabetes induction as described in "Methods." Values and vertical bars are means ± SEM (n = 10 animals per group) for plasma glucose (A) and insulin (B) concentrations measured at the indicated times after intervenous glucose bolus (750 mg/kg body weight) administration.

between lesion size and iron staining in either control or STZ-DM monkeys (Fig. 4). This suggests that increased iron deposition in STZ-DM arteries was not a consequence of atherosclerotic lesion development, but rather a consequence of diabetes.

3.5. *Arterial superoxide production*

Arterial superoxide levels were higher in STZ-DM monkeys as determined using 2 different methodologies (Fig. 5). Basal superoxide levels determined by 2-hydroxyethidium formation were 47% greater ($P < .05$) in aortas from STZ-DM compared with those from control monkeys (Fig. 5A). In carotid artery, basal superoxide levels determined by lucigenin-enhanced chemiluminescence were not significantly different ($P = .66$) between STZ-DM (2822 ± 647 AU/mg tissue) and control monkeys (3265 ± 745 AU/mg tissue), but were 56% greater ($P = .04$) in STZ-DM compared with control monkeys after stimulation with NADPH (Fig. 5B), suggestive of the involvement of the phagocyte NADPH oxidase.

Table 2 – Effect of hyperglycemia on plasma and artery lipids in control and STZ-DM monkeys

	Control (n = 10)	STZ-DM (n = 10)	P value
Plasma lipids			
Total cholesterol (mg/dL)	233 ± 25	270 ± 32	.91
Apo B cholesterol (mg/dL)	189 ± 29	227 ± 35	.94
HDLC (mg/dL)	45 ± 5	43 ± 5	.74
Triglycerides (mg/dL)	32 ± 4	152 ± 58	<.05
Free fatty acids (mEq/L)	0.68 ± 0.10	0.80 ± 0.16	.72
Carotid artery lipids			
Total cholesterol (μg/mg protein)	10.6 ± 3.2	14.2 ± 6.5	.63
Free cholesterol (μg/mg protein)	5.9 ± 1.0	6.4 ± 2.4	.85
Cholesterol ester (μg/mg protein)	5.5 ± 2.2	8.0 ± 4.1	.53
Lipid peroxides (nmol/mg)	5.06 ± 1.24	4.73 ± 1.72	.58
Aortic intimal area (mm^2)	0.86 ± 0.30	0.83 ± 0.33	.94

Fasting blood samples and carotid artery lipids were obtained from control and streptozotocin-treated monkeys 4 weeks after diabetes induction. Aortic intimal area was determined by computer morphometric analysis. Data are presented as the mean ± SEM (n = 10 animals per group) together with respective *t* test or ANCOVA *P* values.

3.6. *Vascular antioxidant enzyme levels*

In normal vascular tissues, antioxidant enzymes that degrade superoxide (Cu/ZnSOD, EcSOD, and MnSOD), hydrogen peroxide (catalase), and heme (heme oxygenase-1) are elevated in response to increased oxidative stress to decrease tissue levels of ROS [4,24]. However, protein levels of the SODs, catalase, and heme oxygenase-1 were not elevated in the aortas of STZ-DM monkeys when compared with the aortas of control animals (Table 3), even though plasma lipid peroxides (Table 2) and arterial superoxide levels (Fig. 5) were significantly elevated.

4. Discussion

Diabetes mellitus increases the incidence, severity, and mortality [1,36-38] of coronary artery disease independently from other traditional risk factors. Plasma markers of oxidant stress are increased in diabetic patients [39,40] and participate in progression of vascular disease [4,11,41,42]. However, as ROS production increases with atherosclerosis development, it is hard to determine whether the increased ROS is causative or coincident with the increased vascular disease in diabetes. Here we show that increases in systemic and arterial ROS production occur before development of increased atherosclerosis in streptozotocin-induced diabetic monkeys. Furthermore, we found evidence of increased whole-body iron stores as well as increased arterial iron deposition in diabetic monkeys that were fed a Western diet with controlled amounts of mineral and vitamin content.

The streptozotocin-induced diabetic model induces a hyperglycemic condition with diminished insulin production (Fig. 1). The significant but incomplete reduction in fasting

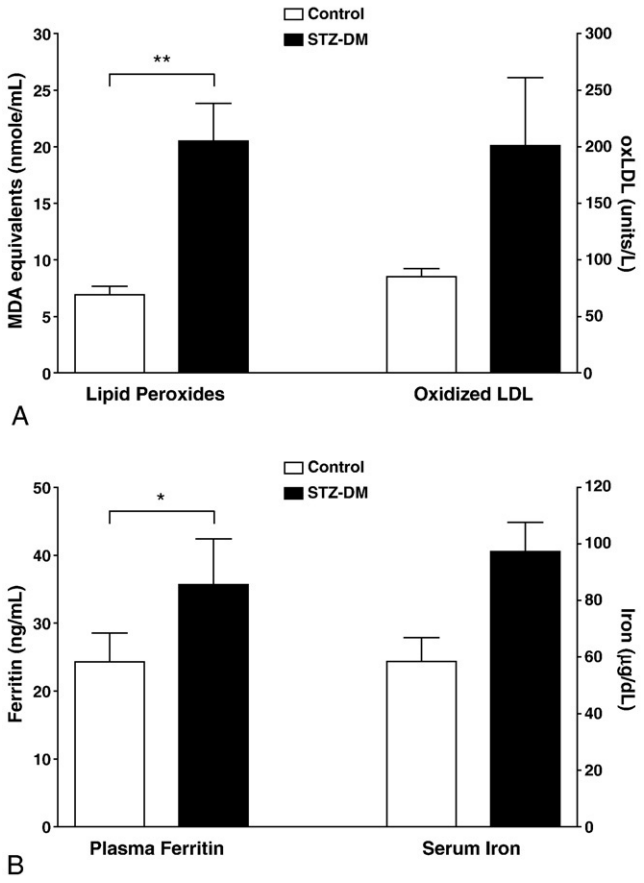

Fig. 2 – Plasma lipid peroxides, oxLDL, ferritin, and iron levels in control and streptozotocin-treated monkeys. Fasting blood samples were obtained from control and streptozotocin-treated monkeys 4 weeks after diabetes induction and assessed for lipid peroxides, oxLDL, ferritin, and iron levels as outlined in "Methods." A, Data are the mean ± SEM for plasma lipid peroxides and oxLDL for control (white bars; n = 10) and STZ-DM monkeys (black bars; n = 10). Plasma lipid peroxides, quantified as thiobarbituric-acid reactive substances, are expressed as malondialdehyde equivalents (nanomoles per milliliter) (P < .01, adjusted for baseline lipid peroxides). Plasma oxLDL is expressed as arbitrary units per liter (t test P = .08, adjusted for baseline oxLDL). B, Data are the mean ± SEM for plasma ferritin and serum iron concentrations for control (white bars; n = 10) and STZ-DM monkeys (black bars; n = 10). Plasma ferritin is expressed as nanograms per milliliter (*P < .05, adjusted for baseline ferritin levels). Serum iron is expressed as micrograms per deciliter (t test P = .08, adjusted for baseline iron levels).**

insulin and C-peptide levels in STZ-DM compared with control monkeys, together with their lack of insulin resistance and negligible insulin responses during IVGTT, is due to marked, albeit incomplete, β-cell loss after streptozotocin treatment [28]. This resembles the residual insulin production observed in humans with type 1 diabetes mellitus months or years after onset [43] and contrasts with insulin responses in either humans or cynomolgus monkeys with type 2 diabetes mellitus [44], indicating that changes in the pancreatic islets of STZ-DM monkeys are more characteristic of type 1 than type 2 diabetes mellitus [28,44].

In this study, we demonstrated that, within 1 month of hyperglycemia induction, at a time when there were no differences in plasma lipoprotein cholesterol, arterial cholesterol, and atherosclerosis extent between control and STZ-DM monkeys, circulating lipid peroxides and oxLDL were elevated, arterial basal superoxide and NADPH-stimulated superoxide concentrations were higher, plasma ferritin (an indicator of whole-body iron stores) and serum iron were elevated, and iron deposition within atherosclerotic lesions was more prevalent in STZ-DM compared with controls. The increases in ROS, particularly arterial superoxide, observed before differences in atherosclerosis are consistent with the hypothesis that elevated ROS production is an initiating event in diabetic vascular disease. Furthermore, the increase in iron stores and arterial iron deposition that occurred within 1 month of diabetes induction is also consistent with the suggestion that redox-active iron within the artery may play a role in catalyzing the production of hydroxyl radicals from elevated arterial superoxide, resulting in accelerated diabetic vascular damage.

Several mechanisms may account for the hyperglycemia-induced superoxide production. Hyperglycemia per se has been shown to stimulate mitochondrial superoxide overproduction in endothelial cells in vitro [8,14], and in vivo studies have suggested the importance of this pathway in vascular dysfunction [45] and atherosclerosis development [46] in diabetic animals. Activation of RAGE may also contribute to vascular superoxide production, as treatment of cultured human umbilical vein endothelial cells with advanced glycation end products has been shown to stimulate superoxide production and subsequent inflammatory gene expression [47].

Another pathway for generating reactive oxidant species involves nitric oxide, which is produced by endothelial cells to regulate vascular tone [6]. Nitric oxide can react with superoxide to produce peroxynitrite, a potent oxidant that can react with protein tyrosine residues to produce 3-nitrotyrosine, which is a marker of the reactive nitrogen pathway [4,6]. However, in previous studies in STZ-DM monkeys, we found that levels of 3-nitrotyrosine in arterial proteins were not elevated in STZ-DM monkeys relative to control animals [16], suggesting that hyperglycemia does not increase the production of reactive nitrogen species in artery walls of these diabetic animals.

Vascular macrophages can also generate superoxide and hydrogen peroxide through the action of NADPH oxidase. The hydrogen peroxide can be used by another macrophage enzyme, myeloperoxidase, to produce more potent cytotoxic oxidants, such as hypochlorous acid [4,6]. Indeed, human studies have demonstrated increased NADPH oxidase activity and protein levels in arteries from diabetic patients [48]. The higher arterial superoxide levels from STZ-DM monkeys after stimulation with NADPH also suggest the potential involvement of NADPH oxidase in diabetic vasculature. In our previous studies in STZ-DM monkeys where arterial proteins were isolated, levels of o,o'-dityrosine, a marker of myeloperoxidase activity, were significantly increased, but did not

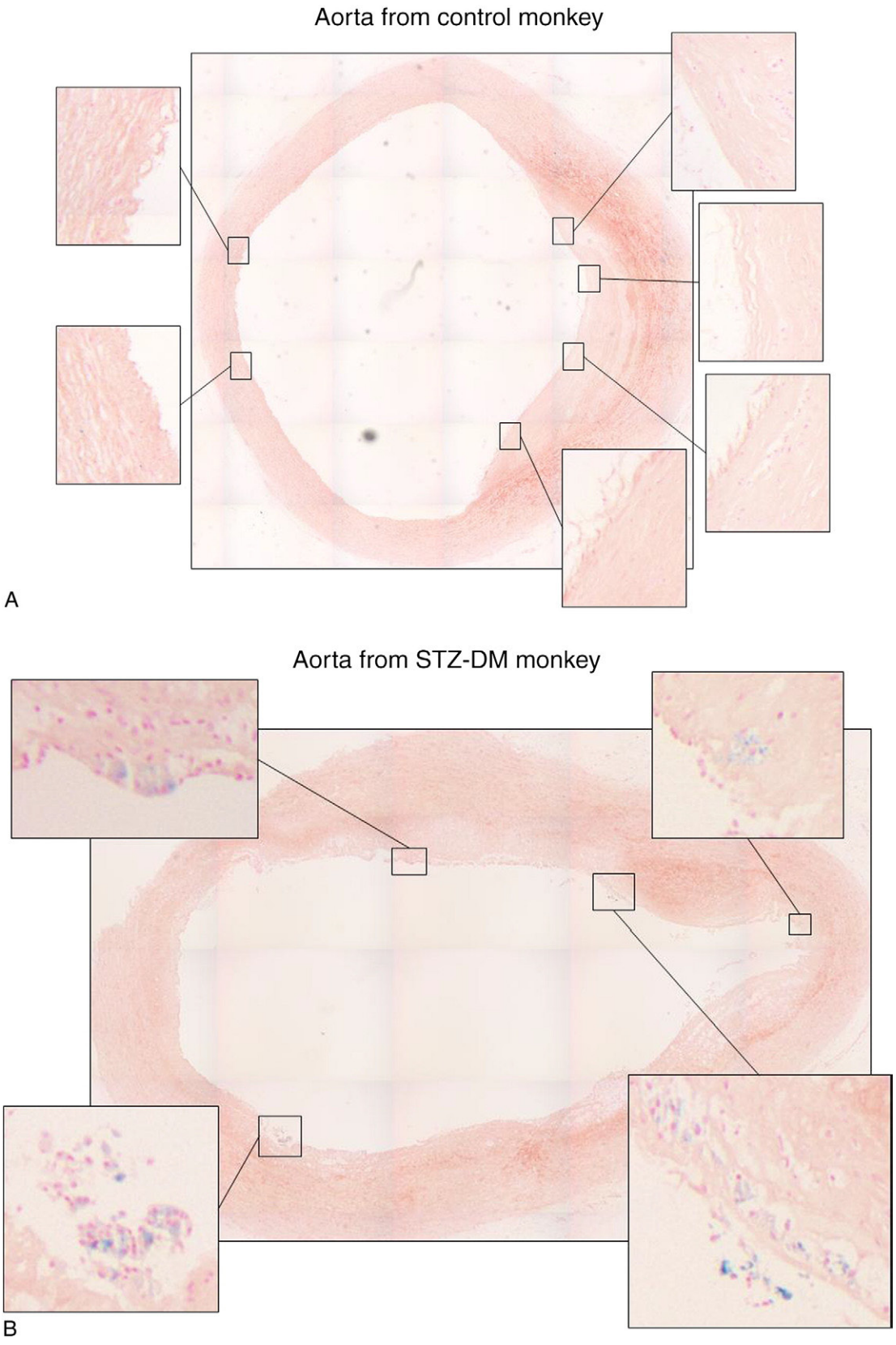

Fig. 3 – Iron deposition in thoracic aortas from control and streptozotocin-treated monkeys. Cross sections of thoracic aorta tissue were fixed for immunohistochemistry and stained with Perl stain for Fe^{3+} as described in "Methods." A, Aortic cross sections from control monkeys. B, Aortic cross sections from STZ-DM monkeys. Blue color indicates areas of iron staining. Inset pictures represent areas of staining that were expanded.

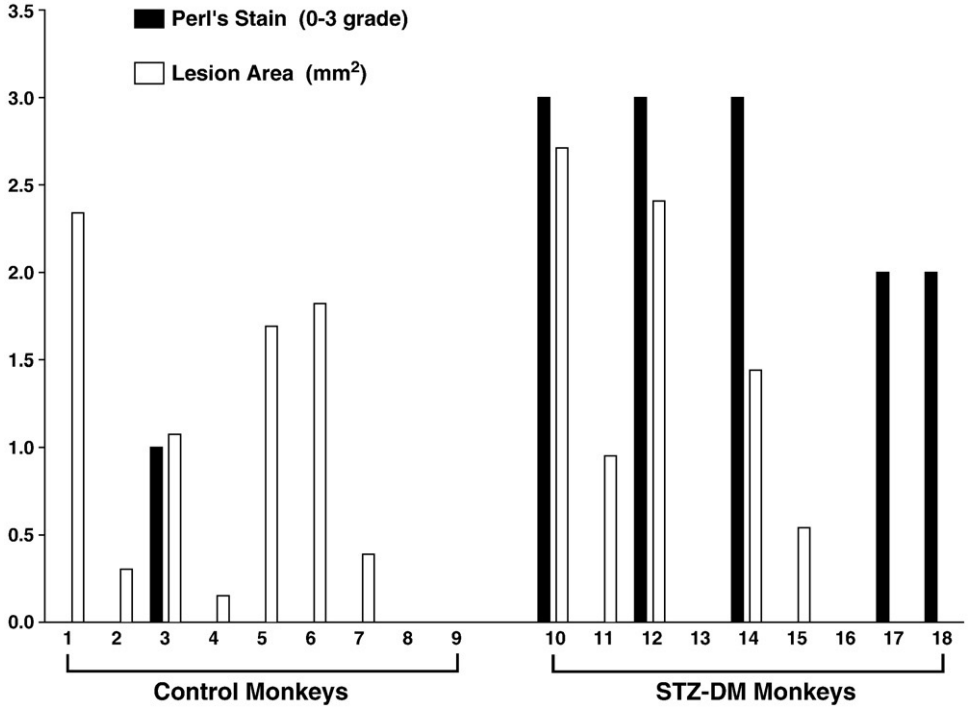

Fig. 4 – Atherosclerotic lesion area and iron staining in thoracic aortas from control and STZ-DM monkeys. Cross sections of aorta tissue were fixed for immunohistochemistry and stained with Perl stain for Fe^{3+} as described in "Methods." Morphometric analysis was used to determine atherosclerotic lesion area (white bars). Perl stain for Fe^{3+} (black bars) was quantified by assigning a grade of 0, 1, 2, or 3 based on presence or absence of blue staining as well as the number of stained regions within a section.

correlate with degree of hyperglycemia [16]. Additional studies using inhibitors of NADPH oxidase, such as apocynin, could help to explore the potential for a role of phagocyte NADPH oxidase vs myeloperoxidase.

It is also interesting that arterial superoxide levels were increased but not the less specific arterial lipid peroxidation (Table 2), suggesting that superoxide production is a specific glucose-induced ROS mechanism. Importantly, protein levels of antioxidant enzymes (SOD, catalase, and heme oxidase) responsible for the conversion of superoxide to less harmful species were unchanged in aortic tissue from STZ-DM monkeys, suggesting that higher superoxide levels equate to higher arterial damage.

Increases in circulating ferritin and iron levels in insulin-resistant states such as the metabolic syndrome, gestational diabetes, polycystic ovary syndrome, and familial hemochromatosis are thought to play a role in increased risk of type 2 diabetes mellitus [24]. This concept is supported by the observations that reductions in whole-body iron load, through repetitive phlebotomy and/or iron chelation therapy using deferoxamine, result in improvements in insulin sensitivity and glycemic control and reduce the risk of diabetes in patients with familial hemochromatosis [24]. Furthermore, the strong prooxidant effects of iron are associated with increased levels of oxidative stress thought to play a role in diabetic vascular complications [24]. Similar to findings in diabetic [25,26] and insulin-resistant nondiabetic people [27], plasma ferritin levels were higher in STZ-DM monkeys (Fig. 2). In addition, serum iron concentrations tended to be greater. These measures indicate that whole-body iron stores are likely increased. In addition to these indirect systemic indicators of iron stores, Fe^{3+} deposition was more prevalent in aortic sections from STZ-DM monkeys (Figs. 3 and 4). The staining pattern appeared to be lumen associated and nonuniform, suggesting involvement of macrophage-associated hemosiderin. Furthermore, hemoglobin may be the source of this iron, derived either from intravascular hemolysis or from erythrocyte attachment to the endothelium via RAGE [49].

It has been proposed that the overproduction of superoxide that occurs in response to mitochondrial dysfunction may be the unifying mechanism by which hyperglycemia exerts its role in producing vascular damage, via its dismutation to produce hydrogen peroxide, which in the presence of redox-active transition metals, such as iron, produces hydroxyl radicals that act locally within arteries to produce damage [4,8]. As mentioned above, tissue hydroxyl radical production depends directly on both the availability of free iron and the balance between production of hydrogen peroxide, primarily via dismutation of superoxide, and degradation by antioxidant enzymes such as catalase [4]. The increase of both superoxide and iron content in the artery wall would likely lead to increased hydroxyl radical formation [4,17]. This is consistent with our previous study where we found increases in 3 protein modifications, *ortho*-tyrosine, *meta*-tyrosine, and *o,o'*-dityrosine, but not 3-nitrotyrosine, in aortic tissue of hyperglycemic

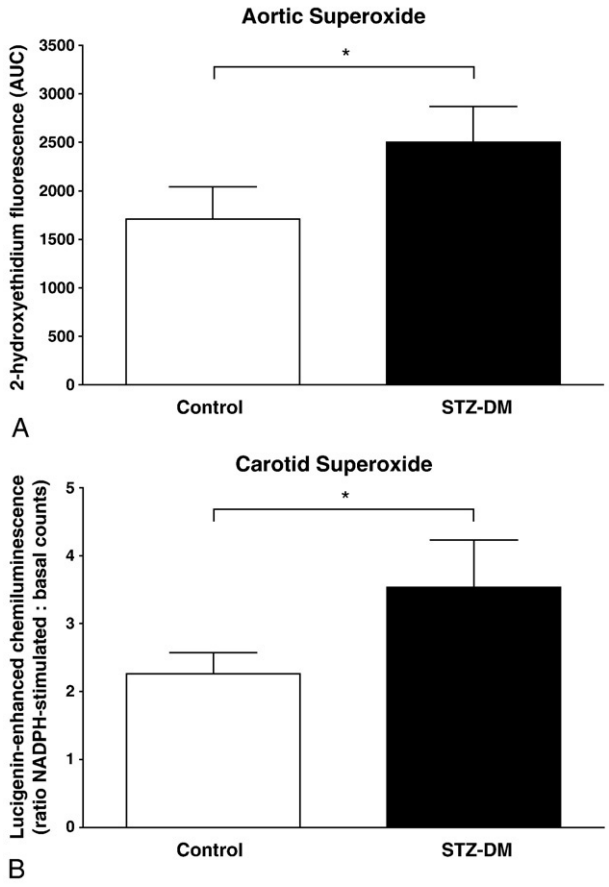

Fig. 5 – Arterial superoxide levels in control and streptozotocin-treated monkeys. Segments of carotid and aortic arteries were assessed for superoxide levels as outlined in "Methods." A, Data are the mean ± SEM for superoxide anion-induced 2-hydroxyethidium fluorescence in aortic segments isolated from control (white bars; n = 9) and STZ-DM monkeys (black bars; n = 9). Arterial rings were incubated in DHE and subjected to HPLC separation. Formation of 2-hydroxyethidium in the presence of superoxide anion was quantified by fluorescence area under the curve. B, Data are the mean ± SEM for the ratio of NADPH-stimulated to basal chemiluminescence in carotid arteries isolated from control (white bars; n = 9) and STZ-DM monkeys (black bars; n = 9). Luminescence is produced by the reaction of lucigenin with superoxide anion in the presence of NADPH (*P < .05).

animals [16]. In these studies, various oxidative agents were tested in arterial samples; and only hydroxyl radical mimicked the pattern of oxidized amino acids [16]. Moreover, glycemic control was strongly correlated with the tissue concentration of both *ortho*-tyrosine and *meta*-tyrosine, further suggesting that glucose levels were promoting specific oxidative pathways [16].

In summary, the increase in superoxide production in diabetes before differences in atherosclerosis suggests that increased ROS is an important initiating event in vascular disease. The increase in whole-body iron stores (ferritin and serum iron) and particularly arterial iron in the presence of higher superoxide levels may catalyze production of hydroxyl

Table 3 – Antioxidant enzyme levels in aortas from control and STZ-DM monkeys

	Control	STZ-DM
EcSOD	2.84 ± 0.31	2.52 ± 0.15
MnSOD	4.09 ± 0.85	4.49 ± 0.90
Cu/ZnSOD	0.41 ± 0.05	0.38 ± 0.05
Catalase	0.065 ± 0.011	0.067 ± 0.010
Heme oxygenase-1	0.028 ± 0.002	0.030 ± 0.004

Aortic tissue was frozen in liquid nitrogen; and protein fractions containing the SODs, catalase, and heme oxygenase-1 were isolated and subjected to gel electrophoresis and immunoblotting as outlined in "Methods." Membranes were developed using an ECL-plus detection reagent and quantified by expressing their respective densities as arbitrary units normalized to those of β-actin bands as outlined in "Methods." Values are means ± SEM (n = 10 animals per group).

radicals, resulting in vascular damage. Furthermore, in our previous studies [16,17] and in this report, hyperglycemia results in activation of specific pathways of ROS generation and not in generalized lipid oxidation. A limitation of this study is that the exact mechanisms through which hyperglycemia increases iron stores could not be determined. In this regard, the iron chelator, deferoxamine, an agent used to treat iron overload in humans [50], has been reported to reduce atherosclerosis both in cholesterol-fed rabbits [51] and in C57BL6J mice [52] and to up-regulate glucose uptake and insulin signaling in rat liver [53]. Further studies with iron chelation agents, such as deferoxamine, in diabetic monkeys could provide additional mechanistic insight into the role of iron in the development of diabetic vascular disease.

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