High Glucose Concentrations Induce Oxidative Damage to Mitochondrial DNA in Explanted Vascular Smooth Muscle Cells

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Oxidative stress is considered to be one of the mechanisms leading to atherosclerosis. It occurs in response to injury or to altered metabolic state. Alterations in cell growth (proliferation or apoptosis) can also contribute to the pathogenesis of atherosclerosis and is influenced by oxidative stress. Smooth muscle cells (SMC) from aortic explants of JCR:LA-cp homozygous cp/cp corpulent rats who are genetically predisposed to develop atherosclerosis exhibit increased SMC proliferation, which can be attenuated by exercise and food restriction. This study was conducted to characterize the effects fo oxidative stress and high glucose media on cell growth and its relationship to mitochondrial DNA integrity and gene expression in explanted aortic SMC from corpulent and lean JCR:LA-cp rats. The results show that SMC from the cp/cp rat appear to be resistant to oxidant-induced cell death and that they accumulate mitochondrial DNA mutations, probably as a result of a reduction in apoptosis. These data suggest that susceptibility to age- and glucose-related atherosclerosis may be related to alterations in redox signaling. [Exp Biol Med Vol. 226(5):450–457, 2001]

Key words: smooth muscle cells; glucose; apoptosis; oxidative stress; mitochondrial DNA

Oxidative stress, defined as a disturbance in the balance between pro-oxidants (reactive oxygen species and reactive nitrogen species, ROS and RNS, respectively) and antioxidants in favor of the pro-oxidant state, is thought to be one of the mechanisms leading to specific diseases, including atherosclerosis, and perhaps to the general process of aging (1, 2). The role of the redox state in the initiation of vascular disease is still unclear (3), but oxidative stress may occur in response to injury or to altered metabolic state and may influence gene expression, cell growth, or apoptosis (4, 5).

The role of mitochondrial integrity and function in the balance between cell growth and apoptosis has recently received attention (6). Both mitochondrial integrity and function are influenced by aging and diabetes (7, 8), and excess ROS are associated with increased mitochondrial DNA (mtDNA) mutations, changes in mtDNA gene expression, and apoptosis (6, 7, 9).

The homozygous cp/cp JCR:LA-cp rat lacks the leptin receptor (10), and its associated vasculopathy is strongly associated with abnormal vascular smooth muscle cells (SMC) (11, 12). The propensity to cardiovascular disease (CVD) is clearly multifactorial and polygenetic, since related strains of rats do not develop CVD despite being insulin resistant (13). We have previously shown that aortic SMC explanted from male homozygous cp/cp JCR:LA-cp rats (cp/cp) exhibited enhanced proliferation of SMC in vitro compared with that of cells from lean normal rats (12, 14). Furthermore, exercise and diet initiated at weaning attenuated the proliferation of SMC of cp/cp rats in vitro (14). We also reported that aging and the cp/cp genotype confer susceptibility to mtDNA injury in vivo and that high glucose concentrations can induce mtDNA mutations in explanted SMC in vitro (15).

In this study we characterized the effects of oxidative stress and high glucose media on mtDNA integrity, gene expression, and cell growth in explanted aortic SMC from 17-month-old JCR:LA-cp rats. We chose to use high levels of glucose in these studies, as they may reflect glucose-related oxidative stress in vivo in hyperglycemic individuals who have a propensity to develop vascular disease, including atherosclerosis. We hypothesized that high glucose concentrations would induce oxidative stress, leading to mtDNA damage, and that the cp/cp genotype would confer resistance to oxidant-related apoptosis, leading to the accu-

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mulation of injured cells. The differential responses to the treatments tested may explain some of the differences between the cp/cp rat and its normal counterpart and may provide insight into possible relationships between cell growth or death and the accumulation of injury to mtDNA, both of which may play key roles in the pathogenesis of atherosclerosis.

Materials and Methods

Animals. Five male corpulent (cp/cp) and three lean (+/+) JCR:LA-cp rats 9 months of age were obtained from the established breeding colony maintained at the University of Alberta (Edmonton, Canada). The rats were bred and maintained as previously described (16). They were housed and fed an *ad libitum* diet at the University of Vermont College of Medicine until they were killed at 17 months of age, which is considered old for this strain and the age at which we previously found evidence of mtDNA injury (15). Food intake was approximately 30 to 50 g/day. All animal care and treatments were in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Vermont and the Canadian Council on Animal Care.

Vascular SMC. Rats were anesthetized with pentobarbital before the performance of surgical procedures. The thoracic cavity was opened, and the rats were exsanguinated by cardiac puncture. Vascular SMC were isolated from the aortas as previously described (12). Briefly, explants were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 10% CO_2. SMC generally migrated from the tissue within 3 to 4 days. The explanted tissue fragments were then removed, and the cell monolayers were refed to allow growth to confluence. For propagation, the cells were seeded at 1×10^4 per cm^2 and thereafter subcultured at weekly intervals or stored in liquid nitrogen. Cells between four to eight passages were used. Preliminary studies were conducted to determine the best length of time to grow the cells prior to treatments in order to study them at similar levels of confluence. Both cp/cp and +/+ SMC were in the logarithmic phase of growth at the time of treatment. In all experiments, cell densities for both cell types ranged from 2 to 4×10^6 cells per flask. (Highly confluent cultures would have had approximately 8×10^6 cells per flask.) Cell behavior, appearance, or response to treatments did not differ between four to eight passages. Between-group comparisons of the responses to the various treatments were made between cells in the same passage. Cohort cultures were started at the same time, treated identically, and used to assess cell viability by trypan blue exclusion.

Treatment of SMC in Vitro. *Effects of free radical production on SMC.* Aortic SMC were seeded at a density of 1×10^4 per cm^2 on Day 1 in the complete DMEM containing 5 mM glucose and supplemented with 10% FBS and 1 U/mL penicillin/streptomycin. After 4 days of growth, the culture medium was changed to DMEM supplemented with 100 μM hypoxanthine (HX, Sigma Chemical Co., St. Louis, MO) and different doses (5, 10, 15, 20, and 25 mU/mL) of xanthine oxidase (XO, Sigma). Twenty-four hours later, DNA and RNA were extracted and analyzed as described below. In a parallel set of cultures, 25 units catalase (Sigma) per milliliter were added to degrade the hydrogen peroxide (H_2O_2) generated by the HX/XO treatment.

Effect of high glucose media on SMC. Aortic SMC were seeded on Day 1 in the same complete DMEM containing 5, 25, 62, or 100 mM glucose, respectively, or 5 mM glucose plus 20, 57, or 95 mM mannitol for iso-osmotic controls. These levels of glucose were selected to determine whether there was a dose-response effect, acknowledging that only 5 and 25 mM are physiologic. After 4 days, cells were harvested. DNA and RNA were extracted and analyzed as described below. In parallel sets of cultures containing 100 mM glucose, superoxide dismutase (SOD, 450 U/mL; Sigma) or catalase (25 units/mL, Sigma) were added to determine whether these antioxidants would attenuate the effects of high glucose.

Analytical Methods. *Extraction of DNA and RNA.* Total DNA was extracted with the use of a Genomic DNA Isolation Kit (Sigma). Cells grown in a 75-cm^2 flask were lysed by the addition of 350 μL of lysis solution, scraped, and collected into 1.5-mL tubes after which DNA was extracted following the manufacturer's procedures. Total RNA was extracted using Ultraspec RNA Isolation Systems (Biotecx Laboratories Inc., Houston, TX) following the manufacturer's procedure.

Polymerase chain reactions (PCR). Several primer pairs and PCR strategies were used in these experiments. The primers listed were designed based on the sequence of rat mtDNA previously published (17). L7683: 5'-CGAAGCTTAGAGCGTTAACCTTTTAAG-3', nucleotide (nt) 7683–7709; L7825: 5'-TTTCTTC-CCAAACCTTTCCT-3', nt 7825–7844; L-8526: 5'-ATTCAAGCCTATGTATTCACC-3', nt 8526–8546; L-12634: 5'-CAACATCCAGCCAACTAGG-3', nt 12,634–12,652; H-9130: 5'-CGAAATACTCTGAGGCTT-GTA-3', nt 9130–9110; H13117: 5'-AAGCCTGCTAG-GATGCTTC-3', nt 13,117–13,099; and H13753: 5'-GGAGTTGGTAGTGTTCTACTTGTTTAG-3', nt 13,753–13,727.

A nested long PCR was performed with rTth DNA Polymerase XL and XL BufferII Pack (PE Biosystems, Foster City, CA) (15). A hot start amplification was employed using Tth Start Antibody (Clontech, Palo Alto, CA). The 50-μL reaction mixture contained: 1 × buffer, 0.2 mM dNTPs, 1.0 mM Mg $(OAC)_2$, 2 μL rTth DNA polymerase XL (mixed with 0.25 μL Tth start antibody); 50 pmol of each primer, and up to 1.5 μg of DNA template.

The PCR were run on PTC-100 Programmable Thermal Controller (M.J. Research, Watertown, MA). The primer pair L7683-H-13753 was used in the first round to amplify a 6071-bp fragment of rat mtDNA. The profile consisted of 14 cycles at 94°C for 1 min, 68°C for 1 min (−1°C/cycle),

and 72°C for 5 min; this was followed by 20 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 6 min. The different primer pair combinations were used in the second-round PCR to detect and confirm deletions. For example, the L7825–H13117 pair was used to amplify the 5293-bp fragment; the L7683-H13117 pair to amplify the 5435-bp fragment; and the L8526-H13117 pair to amplify the 4592-bp fragment. The second-round PCR profile was 34 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 5 min, followed by a final extension at 72°C for 7 min.

MtDNA probes. PCR was used to synthesize a 5.3-kb of mtDNA fragment from primer pair L7825-H13117, a coding region for cytochrome oxidase C subunit III (COIII) from primer pair L8526-H9130, and a coding region for NADH dehydrogenase subunit 5 (ND5) from primer pair L12634-H13753. The PCR products were gel purified and labeled with (α-^{32}P) dCTP (NEN Life Science Products, Boston, MA) using a Random Primers DNA Labeling System (Life Technologies, Grand Island, NY).

Northern blot analysis. Total RNA (15 μg) from normal or treated SMC were size-fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel and then transferred to Hybond N$^+$ membranes (Amersham, Piscataway, NJ). The membrances were prehybridized in Rapid-Hyb Buffer (Amersham) at 68°C for 15 min, and then hybridized with probes at 68°C for 1 hr. The membranes were then washed with 2 × SSC, 0.1% SDS at room temperature for 15 min, and with 0.1 × SSC, 0.1% SDS at 65°C for 15 min two times or more. Membranes were exposed to Reflection Autoradiography film (NEN Life Science Products) with intensifying screens at −80°C for 3 to 24 hr. Quantitative analysis of the autoradiograms was performed using a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). mRNA was normalized to 28S rRNA, and by rehybridization with 7S cDNA probe for further confirmation (18).

DNA fragmentation. Total DNA was loaded on a 1.8% agarose gel containing ethidium bromide and was visualized under UV light for evidence of DNA fragmentation.

Propidium iodide stain. Trypsin-digested cells were stained with propidium iodide (50 μg/mL) in 0.1% Triton X-100 (v/v in PBS) containing RNase A (0.3 mg/mL) for 20 min at 37°C. Cells were then analyzed on a flow cytometer for cell cycle distribution using the acquisition and analysis package provided by Coulter Epics Elite, Miami, FL. Cells were considered to be in apoptosis if they exhibited sub-G_1 DNA fluorescence and the same forward-angle light scatter as G_1 cells. Cellular debris was gated out using an electronic threshold.

Statistics. Analysis of variance with repeated measures (RMANOVA) was used to determine genotypic differences in the responses to different concentrations of glucose or XO in the presence of HX (Statistica Software, StatSoft Inc., Tulsa, OK). After significant effects were identified by RMANOVA, the Newman-Keuls test was used for *post hoc* analysis of group differences in response to the various treatments. This permits the identification of groups that differed within each treatment. Values are mean ± SE.

Results

Effect of Oxidative Stress on SMC Growth and mtDNA Integrity. As previously reported (14), aortic SMC from cp/cp compared to normal rats were characterized by increased proliferation in vitro. SMC were not confluent at the time of treatment or harvest. When exposed to oxygen free radicals generated by HX in the presence of different concentrations of XO, SMC from control (+/+) rats appeared to be more senitive than those from cp/cp rats (Fig. 1). Using trypan blue exclusion as an index of cell viability, there was a dose-dependent decline in viability with increasing concentrations of XO in cells from both groups ($P < 0.01$). The cp/cp cells were less sensitive than control ($P < 0.05$, Newman-Keuls) when exposed to HX/XO and grew at a higher density than control cells at all concentrations of XO in the presence of 100 μM HX.

To determine whether differences in apoptotic responses could account for these observations, total DNA extracted from the treated SMC was examined. Laddering of genomic DNA consistent with injury or apoptosis was found in all +/+ cell lines, but not in four of five cp/cp cell lines, further suggesting that SMC from cp/cp rats were resistant to free radical-associated cell death. Representative examples are shown in Figure 2. The addition of catalase to the highest concentration of XO prevented DNA fragmentation (laddering) in the cells from +/+ animals (data not shown). Increased mtDNA deletions in the region of the common deletion (15) were found with increased XO concentrations in explanted SMC from both +/+ and cp/cp rats when mtDNA integrity was examined using PCR. A representative sample from a cp/cp rat is shown in Figure 3. All other cell lines studied exhibited a similar response.

Effect of High Glucose on SMC Growth and mtDNA Integrity. Figure 4 shows cell viability assessed by trypan blue exclusion at the four concentrations of glu-

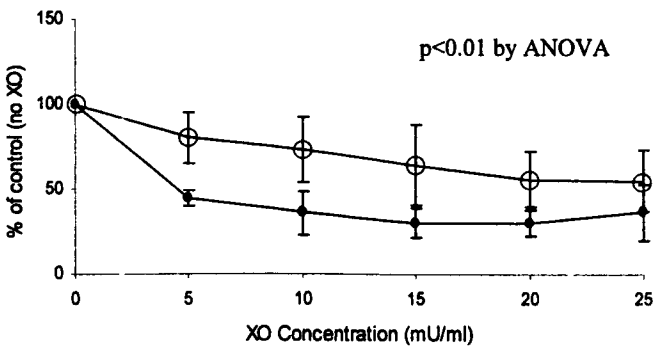

Figure 1. Effect of different concentrations of XO in the presence of 100 μM HX on cell viability assessed by trypan blue exclusion. Values are reported relative to control (no XO/HX). ○ represent cp/cp and ● +/+ rats. Values are means ± SE. Group difference in viability was significant at $P < 0.01$ by repeated measures ANOVA.

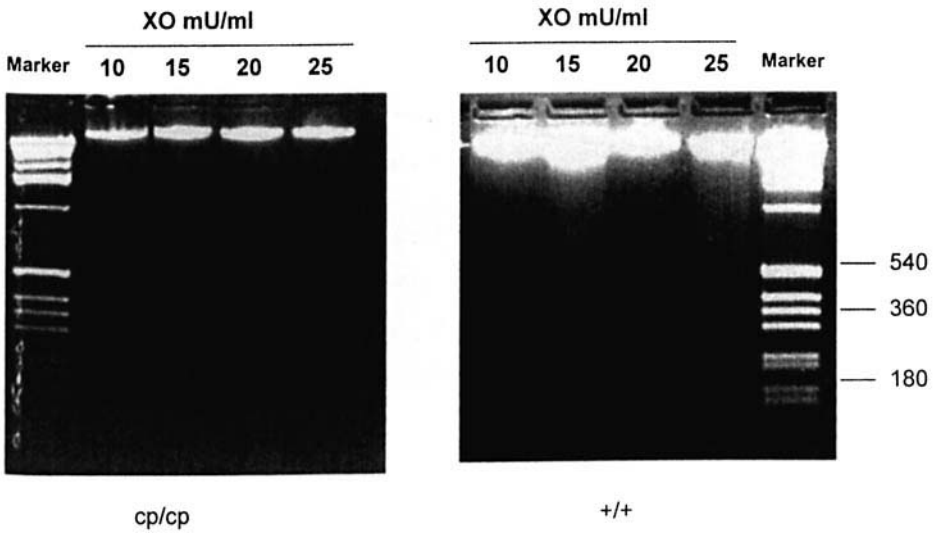

Figure 2. Genomic DNA extracted from SMC treated with different concentrations of XO in the presence of 100 μM HX and loaded onto agarose gels. Representative gel reveals classic "laddering" of DNA consistent with apoptosis in the +/+ rat, but not the cp/cp rat.

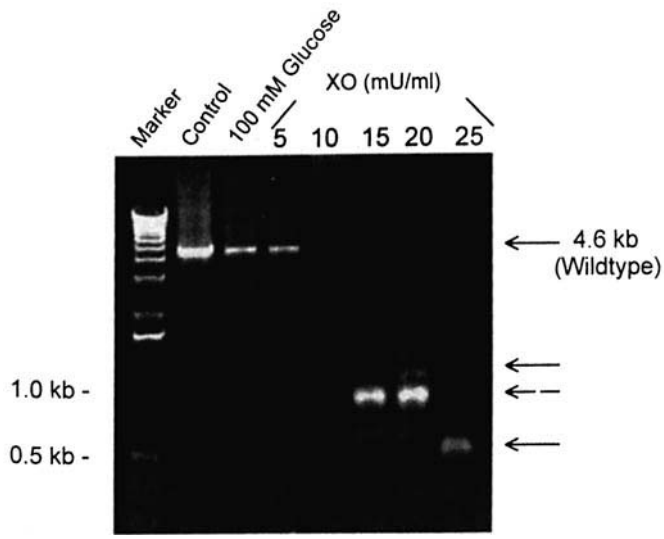

Figure 3. MtDNA integrity in the region of the "common deletion" assessed by LD-nested-PCR in SMC treated with 100 mM glucose or increasing concentrations of XO in the presence of 100 μM HX. A representative example from a cp/cp rat is shown. Multiple bands (←) ranging in size from 0.5 to 1.0 kb represent deleted fragments in the 4.6-kb region of mtDNA.

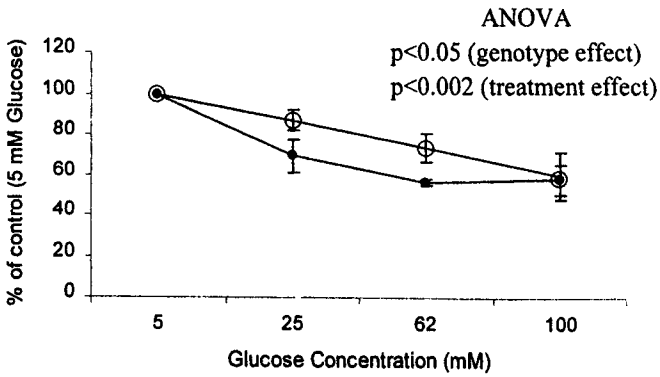

Figure 4. Effect of different concentrations of glucose on cell viability. Values are relative to 5 mM (control) glucose and are means ± SE. ○ represent cp/cp and ● +/+ animals. Dose-dependent decline in viability was significant at $P < 0.002$ by RMANOVA. A significant genotype effect ($P < 0.05$) was also found by RMANOVA.

cose tested. Both cp/cp and control rats showed a dose-dependent decline in viability with increasing concentrations of glucose. RMANOVA revealed significant genotype ($P < 0.05$) and treatment ($P < 0.002$) effects. Unlike treatment with HX/XO, varying the concentrations of glucose did not elicit DNA laddering. However, results after cell cycle analysis in cells from +/+ rats demonstrated a higher percentage of cells in the sub G_0G_1 (apoptotic) phase when grown in 25 mM glucose compared to control media (5 mM glucose) (Table I).

Multiple mtDNA deletions were found in SMC growth in 25, 62, and 100 mM, but not 5 mM, glucose from both +/+ and cp/cp rats. These represent only a small portion of total mtDNA deletions (19). Figure 5 and lane 3 in Figure 3 are representative examples of the different deletions found in cells exposed to different concentrations of glucose. The

Table I. Effect of Glucose on Cell Cycle Distribution (% cells)

Cell cycle	5 mM Glucose (control)	25 mM Glucose
Sub G_0G_1	2.0 + 0.2	3.2 ± 0.9[a]
G_1	78.9 + 3.0	76.2 ± 3.2
S	10.2 + 1.5	12.1 ± 1.2
G_2M	8.3 + 1.8	7.8 ± 1.2

Note. Values are means ± SE. $n = 4$ +/+ lines.
[a] $P < 0.05$.

5.3-kb fragment representing wild type mtDNA is visualized in both 5 and 25 mM glucose, but difficult to detect in 62 and 100 mM glucose using the methods described. This implies that only small amounts of intact mtDNA were present in cells grown in the higher concentratons of glucose and that our PCR approach, as in many situations, could more easily amplify smaller fragments of DNA.

Mitochondrial Gene Expression. In duplicate studies on the eight separate cell lines, probes including the ND5 and COIII genes were used to examine mtDNA expression normalized to 28S-rRNA. The results for COIII gene expression are summarized in Figure 6. Similar direc-

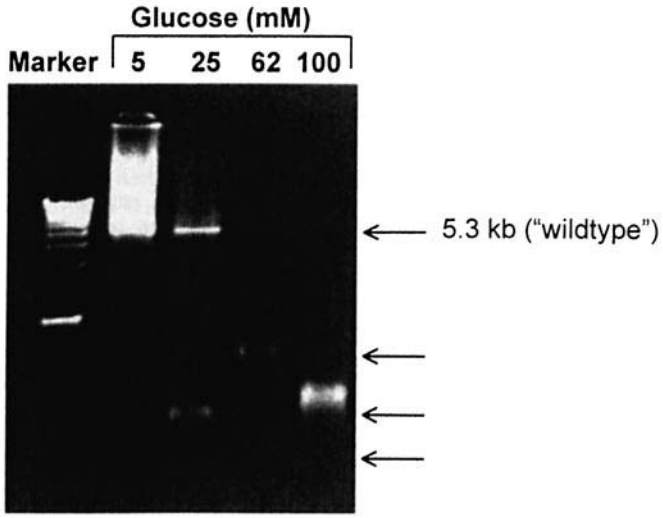

Figure 5. Representative example of results from a +/+ rat. PCR products of mtDNA in the area of the "common deletion" from SMC exposed to 5 (control), 25, 62, and 100 mM glucose for 4 days. Multiple deletions were found in 25, 62, and 100 mM glucose (←).

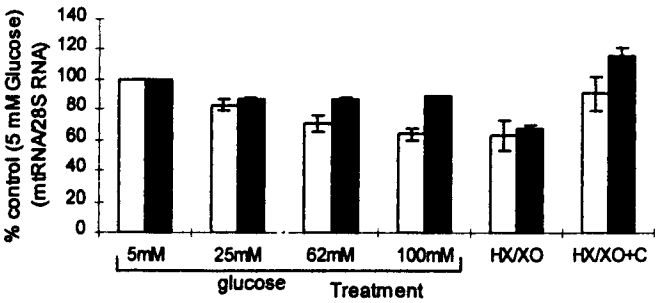

Figure 6. Mitochondrial COIII gene expression in response to 25, 62, or 100 mM glucose or HX (100 μM)/XO (20 mU/mL) with or without catalase (C) in cultured aortic SMC from five homozygous cp/cp (open bars) and three normal (dark bars) rats. Values are means ± SE. There was a significant glucose ($P < 0.0001$) and genotype effect ($P < 0.03$) by ANOVA. Reduction in expression by HX/XO was abrogated by the addition of C.

tional changes were found for the ND5 gene. Treatment with 20 mU/mL XO in the presence of 100 μM HX dramatically reduced mitochondrial gene expression with attenuation by the addition of 25 U/mL catalase (Fig. 7). As shown in Figure 6, the magnitude of the effect was similar in control (67% ± 2%) and cp/cp (63% ± 9%) cells with restoration of expression by catalase (116% ± 5% and 91% ± 11% in control and cp cells, respectively). Furthermore, as shown in Figures 6 and 8, all high (>5 mM) glucose concentrations reduced mitochondrial gene expression in cells from in both groups of animals ($P < 0.0001$). There was also a significant overall genotype effect ($P < 0.03$). A significant difference between +/+ and cp/cp animals at 100 mM glucose ($P < 0.006$) was found using *post hoc* analysis. Within genotype groups, the cp/cp cells had a more significant decline in gene expression compared to control when exposed to high glucose. Hence, these data support a glycemia-related effect on mitochondrial gene expression that is modulated by the genetic background of the animals.

To determine whether these data could be attributed to

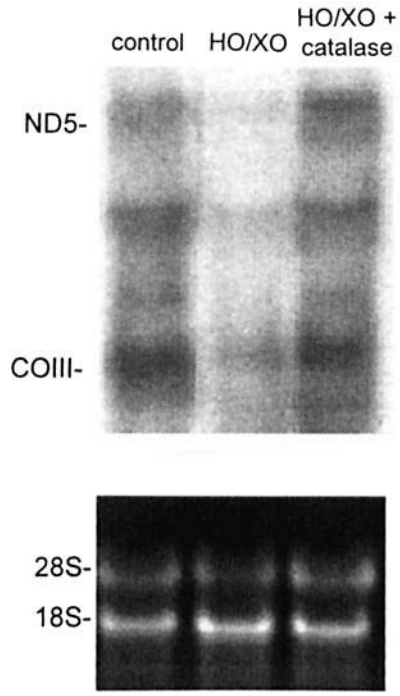

Figure 7. Mitochondrial gene expression (ND5 and COIII) in SMC from a cp/cp rat in response to 100 μM HX with 20 mU/mL XO without or with 25 U/mL catalase (upper panel). The lower panel shows 28S and 18S rRNA bands confirming equal loading of gel.

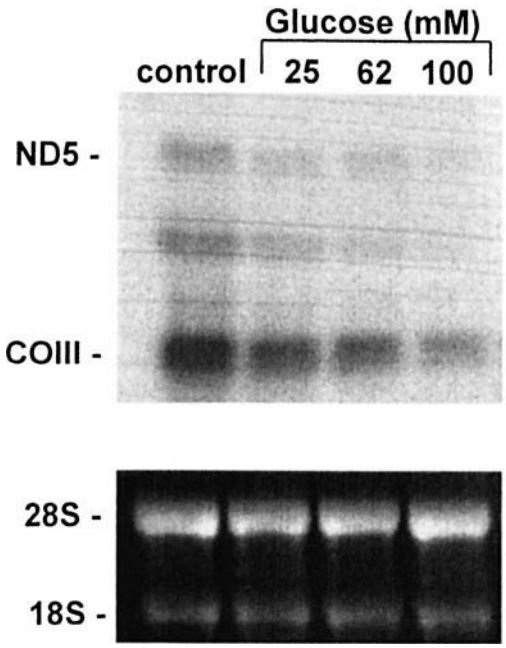

Figure 8. Mitochondrial gene expression (ND5 and COIII) in SMC from a cp/cp rat in response to 5 (control), 25, 62, and 100 mM glucose for 4 days. Data show increasing concentrations of glucose were associated with a reduction in gene expression of both genes. Lower panel shows 28S and 18S rRNA bands confirming equal loading of the gel.

oxidative stress, SOD or catalase was added to dishes with the highest concentration of glucose. As shown in the example in Figure 9, SOD appears to significantly attenuate the reduction in gene expression (165% ± 33%) with minimal effect of catalase.

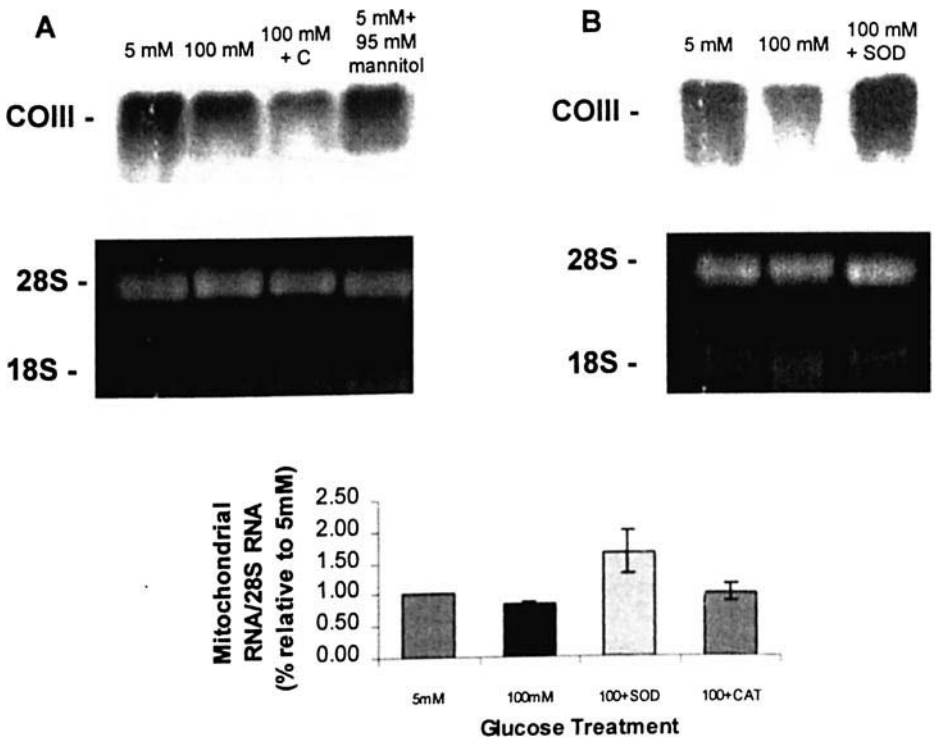

Figure 9. Expression of COIII gene in response to 5 (control) and 100 mM glucose. Panel A shows that addition of catalase (C; 25 U/mL) does not attenuate the reduction in gene expression and that osmolarity has minimal effect on gene expression. Panel B demonstrates that addition of SOD (450 U/mL) attenuates the high-glucose-induced effects.

Discussion

Effect of Oxidative Stress on Explanted SMC from Normal Rats. Although a large body of literature supports the pro-proliferative effects of ROS on SMC, this study demonstrates that after 24 hr of exposure to a combination of XO and HX, explanted aortic SMC from normal rats underwent apoptosis. These data lend support to the proposal that the redox state regulates mammalian cell growth, both at levels of mitogenesis and apoptosis (20, 21). As described by Papa and Skulachev (9), low ROS concentrations appear to stimulate mitosis; a further increase in ROS induces apoptosis, and very high levels of ROS results in necrosis. Li et al. (22) found that a single 1-hr exposure of growth-arrested SMCs to xanthine (X) and XO resulted in increases in [^{3}H] thymidine incorporation and cell number, both indices of cell proliferation. However, three consecutive exposures of growing SMC to XO/X every 24 hr led to gradual and dose-dependent decline of SMC viability. Griendling et al. (20) also concurred that although a certain level of oxidative stress appears to be growth promoting, more severe stress may lead to cell death.

The effects of oxidative stress on mtDNA and the accumulation of mtDNA deletions with advancing age are well accepted (7, 9). It has also been reported that aging is associated with a decline in the levels of mitochondrial transcripts and associated enzyme activities (23, 24), as well as with deleted mtDNA (25). The effect of the apoptotic process on mtDNA is unknown. The present results are consistent with findings of Vincent et al. (26) who also found that oxidative stress generated by HX/XO induced a transient decrease in the levels of both ND5 and 12S rRNA and that these RNA changes were not associated with modifications in the structure or the copy number of the mitochondrial genome. The experimental results obtained by our PCR detection system enabled us to directly visualize mtDNA deletions and fragmentation induced by ROS in a dose-dependent manner. Unlike nuclear DNA, which fragments into a 180-bp DNA ladder, mtDNA damage based on double-strand breaks by -OH attacks may rejoin into smaller circular DNA (ΔmtDNA). We found that the reduction in mitochondria mRNA levels paralleled the extent of mtDNA fragmentation, providing evidence that mtDNA damage potentially leads to loss of components of the mitochondrial respiratory chain. This may induce an energy crisis and may affect mitochondrial membrane function (19). Therefore, these data appear to link oxidative stress with mtDNA damage and cell injury or apoptosis in explanted aortic SMC. We speculate that an acute oxidative insult would injure mitochondria, including mtDNA, and trigger the apoptotic cascade to remove injured cells. The cp/cp genotype appears to confer resistance to apoptosis in response to increased oxidative stress, possibly accounting for certain aspects of the phenotype, including the development of vascular disease. Hyperinsulinemia is likely to play a complementary role (14) and may also contribute to oxidative stress via a reduction in catalase synthesis (27).

Effect of High Glucose on Explanted SMC. Although the pathogenesis of diabetic vasculopathy is multifactorial, high glucose may induce oxidative stress either directly or through advanced glycation end products (AGEs). In this study we compared the effects of high glucose with the HX/HO free radical generation system on explanted aortic SMCs. The data suggest that high glucose can mimic the effects of HX/XO in several ways by reduc-

ing cell viability, by inducing mtDNA deletions, and by reducing mtDNA gene expression. However, differences do exist. For example, we were unable to elicit DNA laddering in cells grown in high glucose. In addition, the changes in mitochondrial gene expression induced by high glucose could not be attenuated by catalase, but could be diminished by SOD.

Previous studies have reported that high glucose stimulates SMC growth (28–31). Oikawa et al. (29) reported that human arterial SMC proliferated when grown in media conditioned with serum from diabetic patients, suggesting that the diabetic state results in serum alterations contributing to SMC proliferation. In contrast to other reports (28–31), we did not find an increase in SMC proliferation, but detected reduced cell viability with higher concentrations of glucose. This apparent discrepancy may be explained by species differences, differences in the length of exposure, and differences in the antioxidant capacity of the cells. Our present results suggest that the time of exposure, endogenous antioxidant systems, and mtDNA integrity play an important role in the balance between proliferation and cell death.

High Glucose and cp/cp Genotype Contribute to Atherosclerosis. Compared to early reports that focused on vascular SMC proliferation in the development of atherosclerosis, later findings show that apoptosis was also observed in human atherosclerosis and restenosis (4, 5). This suggests that an imbalance between proliferation and apoptosis may be key to understanding the pathogenesis of atherosclerosis. The concept that atherosclerosis is an inflammatory disease has gained momentum. Alterations in the balance between reduction and oxidation (redox) state is emerging as a general theme in variety of diseases, including cell growth, cell death, and generation of inflammatory responses (3). Glucose itself induces oxidative stress, glycation of low density lipoproteins (LDL) (32), and formation of AGEs (33). These may all contribute to vascular oxidative stress and the stimulation of inflammatory responses, providing a link between diabetes mellitus and its major complication, atherogenesis.

In this study we found that SMC derived from the cp/cp rat appear to be resistant to oxidative stress induced cell death by XO in the presence of HX. The cp/cp cells accumulated mtDNA damage, having mtDNA changes, but retaining the capacity to proliferate. This is consistent with the persistently higher cell density and greater reduction in mitochondrial mRNA expression in explanted aortic SMC derived from cp/cp rats. It appears that SMC from cp/cp rats accumulated deleted mtDNA, leading to reduced gene expression, but the functional consequences were not sufficient to induce apoptosis. It is also possible that the cp/cp genotype carries a yet unknown gene that inhibits the apoptotic process. It would be of great interest if the known defect in the leptin receptor were related to these findings. Normalization of SMC behavior in response to exercise and caloric restriction (14) suggests that the metabolic milieu is an important determinant of cell growth, perhaps through its influence on oxidative stress. Leptin may be a link between signaling of the metabolic state and cell growth (proliferation or apoptosis). The contribution of resistance to apoptosis in cp/cp cell lines and consequent enhanced accumulation of cells with mtDNA injury may contribute to the susceptibility to age-related and glucose-related atherosclerosis (34). Detailed mechanistic studies can now be conducted to clarify the role of redox signaling in the pathogenesis of vascular disease.

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