Diabetes-related Changes in cAMP Response Element-binding Protein Content Enhance Smooth Muscle Cell Proliferation and Migration*

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We hypothesized that diabetes and glucose-induced reactive oxygen species lead to depletion of cAMP response element-binding protein (CREB) content in the vasculature. In primary cultures of smooth muscle cells (SMC) high medium glucose decreased CREB function but increased SMC chemokinesis and entry into the cell cycle. These effects were blocked by pretreatment with the antioxidants. High glucose increased intracellular reactive oxygen species detected by CM-H2DCFDA. SMC exposed to oxidative stress (H2O2) demonstrated a 3.5-fold increase in chemokinesis (p < 0.05) and accelerated entry into cell cycle, accompanied by a significant decrease in CREB content. Chronic oxidative challenge similar to the microenvironment in diabetes (glucose oxidation treatment) decreases CREB content (40–50%). Adenoviral-mediated expression of constitutively active CREB abolished the increase in chemokinesis and cell cycle progression induced by either high glucose or oxidative stress. Analysis of vessels from insulin resistant or diabetic animals indicates that CREB content is decreased in the vascular stroma. Treatment of insulin-resistant animals with the insulin sensitizer rosiglitazone restores vessel wall CREB content toward that observed in normal animals. In summary, high glucose and oxidative stress decrease SMC CREB content increase chemokinesis and entry into the cell cycle, which is blocked by antioxidants or restoration of CREB content. Thus, decreased vascular CREB content could be one of the molecular mechanisms leading to increased atherosclerosis in diabetes.

Diabetes is becoming epidemic in Western society (1). The majority of the morbidity and mortality in individuals with diabetes is secondary to macrovascular disease including atherosclerosis, acute MI, stroke, and amputation from peripheral vascular disease (1–7). Recent advances have greatly improved our understanding of vascular pathology, yet the mechanisms underlying the enhanced atherosclerosis in diabetes remain unclear (8–11). One aspect of vascular function that is altered in diabetes is smooth muscle cell (SMC) behavior. SMC undergo a change from a quiescent state to an activated proliferative and migratory phenotype. This change is termed phenotypic modulation and it is the hallmark of atherosclerosis (11).

Cyclic nucleotides have an important role in regulating vascular tone and maintaining the mature contractile phenotype in SMC. CREB, the Ca2+/cAMP response element-binding protein, is a transcription factor that is a downstream target of cyclic AMP signaling. β-Adrenergic stimulation of cyclic AMP-dependent signaling pathways and protein kinase A (PKA) in the vessel wall has antiproliferative effects. Inhibition of mitogen-activated protein (MAP) kinase activation is one aspect of this antiproliferative effect. Data presented by our group suggests that CREB (cAMP response element-binding protein) may be a downstream target for the antiproliferative effects of cyclic nucleotides (8–14). We have recently demonstrated that CREB protein content is important for regulation of SMC phenotype, specifically migratory and proliferative capacity. A significantly greater CREB content is found in proliferation-resistant SMC subpopulations in normal vessels, while much lower levels exist in proliferation-prone SMC and vessels exposed to hypoxia (15). Increasing CREB content and activity in SMC in culture by exogenous expression of wild type or constitutively active VP16CREB significantly decreased basal and PDGF-stimulated chemokinesis, DNA synthesis, and cell cycle progression (15).

Excess SMC proliferation and migration are features of diabetic vascular disease. In diabetes, there is an abnormal metabolic environment including high glucose, high free fatty acids, and either high insulin or insulin deficiency. Accumulation of reactive oxidant species (ROS) has been reported in human and rodent diabetic vessels (16–19). There is a suggestion in the neuronal literature that oxidative stress in a negative modulator of CREB DNA binding and also that ROS interferes with acute signaling to CREB. High glucose can increase intracellular ROS by numerous mechanisms including activation of PKC, AGE accumulation, and release form mitochondria (20). In this study, we examined the impact of chronic exposure to high glucose or ROS upon CREB content in SMC, both in

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‡ The abbreviations used are: SMC, smooth muscle cells; CREB, cAMP response element-binding protein; CRE, cAMP response element; CBP, CRE-binding protein; FACS, fluorescence-assisted cell sorting; PKA, protein kinase A; MAP, mitogen-activated protein; ROS, reactive oxygen species; MEM, minimal essential medium; m.o.i., multiplicity of infection; ATF, activating transcription factor.
vessel walls and in culture. We observed high glucose-mediated depletion of CREB content in vitro, which correlated with increased chemokinesis and proliferation. These changes in SMC activation could be rescued either by pretreatment with antioxidants or by overexpression of constitutively active CREB protein. These studies suggest that one factor leading to atherosclerosis in diabetes could be loss of CREB content.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fetal bovine serum, glutamine, and penicillin/streptomycin were purchased from Gemini Bio Products, Inc. (Cabassas, CA). Trypsin/EDTA, LipofectAMINE Plus and Plus Reagent were purchased from Life Technologies, Inc. (Grand Island, NY). CREB and phospho-CREB-specific antisera were purchased from New England Biolabs (Beverly, MA). [3H]Thymidine was purchased from PerkinElmer Life Sciences (Boston, MA). Promoter-reporter constructs containing cyclic AMP regulatory elements driving expression of the gene encoding firefly luciferase (CRE-Luc) were obtained from Stratagene (La Jolla, CA). A plasmid containing the cDNA encoding the constitutively active CREB mutant DIEDML-CREB (DCREB) was graciously provided by Dr. Richard Goodman. Cells expressing luciferase and β-galactosidase following transfection with promoter-reporter plasmid constructs were extracted using diluted 5 × reporter lysis buffer obtained from Promega Corp. (Madison, WI). Reagents for assays of luciferase and β-galactosidase activity in cell extracts were purchased from Pharmingen (San Diego, CA). 12-Well Transwell Apparatus chambers with polycarbonate filters with 8-mm diameter were obtained from Costar, Corning, NY. Dif Quick Cell Staining Reagents were purchased from American Hospital Supply Corp., McGraw Park, IL. Minimal essential Eagle’s medium and all other reagents were purchased from Sigma.

**Cell Culture**

Aortic smooth muscle cells were isolated by media explant from bovine and rat aortas. Cells were passaged in culture and used for experiments between passages 2 and 5. Cells were maintained in modified Eagle’s medium containing penicillin and streptomycin, 2 mM glutamine, and 10% fetal bovine serum. For most of the experiments presented bovine cells were used, results were similar in all cases in rat SMC. Prior to experiments, cells were incubated in serum-reduced medium (MEM + 0.1% FBS) for 48 h. In experiments involving different glucose concentrations, glucose was added to MEM + 0.1%polyvinylpyrrolidone at final concentrations of 5 mM (low glucose) and 25 mM (high glucose) for 72 h prior to assay. Similarly, hydrogen peroxide and glucose oxido were added at varying concentrations (0–45 μM or 0–34 milligrams/ml, respectively) for 72 h prior to assay. For SMC chemokinesis studies, cells were plated on the upper membrane surface in a Boyden chamber (Transwells, Costar, Corning, NY) and chemokinesis allowed to continue for 3 h in the presence of low or high glucose or various concentrations of hydrogen peroxide as indicated in the individual figures.

**Assessment of Oxidative Stress in SMC**

SMC were labeled with 10 μM 5-(and 6)-chloromethyl-2′,7′-dichloro-rodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes, CA). This dye, in acetoacetoyl ester form, easily enters cells where it is trapped by the action of cellular acetylase. This dye, when exposed to an excitation wavelength of 485 nm, emits light at 530 nm only when it has been oxidized. Cells were exposed to ROS (using hydrogen peroxide) or altered medium glucose concentration in the absence or presence of t-buthionine (S,R)-sulfoximine (Sigma) which enhances oxidant stress in cells by blunting the synthesis of the intracellular antioxidant glutathione. Labeled SMC were examined using a fluorescence spectrofluorimeter for oxidized dye. A standard curve of fluorescence from green fluorescent protein is more robust. In either protein, proteins at a rate less than 100% of cells indicates that the impact of these proteins on SMC behavior may actually be understated by these discussions.

**CREB-luciferase Promoter Transactivation**

Early passage cultures (P1-P5) of either bovine or rat aortic SMC cells were plated in 6-well culture dishes at a density of 1.4 × 10^5 cells/cm². Cells were subsequently maintained for 18 h in growth medium, consisting of minimal essential medium (Sigma) containing 1 × non-essential amino acids, 0.4 mM glutamate, and 10% fetal bovine serum (Gemini Bio-Products Inc.). Transfection is performed using LipofectAMINE Plus Transfection Reagent (Life Technologies, Inc.) as described by the manufacturer. In addition to specific chimeric promoter-luciferase plasmid constructs, SMC were co-transfection with a constitutively active CREB plasmid and a luciferase reporter plasmid (Rsv-β-gal). As a control for the CRE-luc studies we employed a plasmid containing the enhancerless control plasmid pCIS-CR (Stratagene), identical to the CRE-luc construct but lacking CRE promoter elements. The LipofectAMINE Plus:DNA mixture in growth medium was left on cells for 3 h, and cells were allowed to recover in growth medium overnight. SMC cells were maintained in MEM (minimal essential medium containing 1 × non-essential amino acids and 0.4 mM glutamate) for 24 h. Agonist treatment was performed in 1 × MEM for durations of 4-24 h, and cells were subsequently extracted in 1 × reporter lysis buffer (Promega) for analysis of reporter gene expression. Luciferase reporter activity was corrected for differences in transfection efficiency, cell number, and extract recovery, using medicaid activity determined in the same cell extract. All results are expressed as luminescence as arbitrary light units/β-galactosidase activity in the sample extract.

**Adenovirus-mediated Protein Expression in Vascular Smooth Muscle Cells**

SMC were infected with recombinant, replication-deficient adenovirus under the regulation of the cytomegalovirus immediate early promoter regulating the ectopic expression of cDNA encoding constitutively active CREB mutant (VP16-CREB), DIEDML-CREB (DCREB), MCRCREB (MCREB), KCREB (KCREB), or β-galactosidase (β-gal). Constructs were assessed for enhanced expression of appropriate recombinant proteins in SMC following treatment of cultures with either different concentrations of crude virus (VP16-CREB or purified virus (at 2.5 × 10^6 m.o.i. per cell) using Western blot analysis. Additionally, all constructs were created using the same adenoviral DNA backbone containing a FLAG tag for assessment of expression in infected cells. Quality control experiments were performed to determine the number of cells expressing FLAG peptide following infection with purified CREB-mutant viruses (as described in Ref. 15). Results indicate that 42–70% of cells in these cultures expressed visually detectable FLAG peptide in cells infected with the same m.o.i. of different CREB-FLAG adenoviruses (data not shown). Cells with a adenoviral construct expressing green fluorescent protein, more than 90% of cells were infected. Immunohistochemical assessment of FLAG-epitope expression may underestimate the percent of cell infected as detection of fluorescence from green fluorescent protein is more robust. In either case, the observation that infection and expression of recombinant proteins at a rate less than 100% of cells indicates that the impact of these proteins on SMC behavior may actually be understated by these experiments.

**Endotoxin Blot Analysis for CREB, P-CREB, and ATF3 Content**

At the end of experimental treatments, SMC cultures were washed once with phosphate-buffered saline, and wells scraped in 1 × Laemmli sample buffer. Protein concentrations of samples were assessed by the Bradford protein assay, and 40 μg of cellular protein were run on PAGE-12% polyacrylamide gels for assessment of phospho-CREB, CREB, and ATF3 analyses. Resolved proteins were electrophoretically transferred to nylon membranes, and equivalence of protein loading was assessed by staining of membrane-bound proteins by Ponceau stain. CREB, phosphorylated CREB, and ATF3 content were evaluated immunologically using commercially available antisera (New England Biolabs), a horseradish peroxidase-coupled secondary antibody and subsequent enhanced chemiluminesence. Both Ponceau-stained membranes (for protein loading) and autoradiograph films (for specific protein signals) were analyzed densitometrically using a Fluor-S MultiImager and Quantity One software from Bio-Rad. In figures, results are expressed as arbitrary densitometric units with statistical differences (p < 0.05) as determined by Student’s t test.
chronic exposure of cultured SMC to high glucose leads to significantly decreased CREB protein content at 72 h. DNA Synthesis

Rates of DNA synthesis in cultured bovine or rat SMC cells were estimated by the rate of incorporation of \(^{3}H\)thymidine into cellular DNA. SMC plated in 12-well plates were subjected to experimental treatment, and pulsed with \(^{3}H\)thymidine (2.0 μCi/ml) for the final 3 h of treatment. Cells were fixed at 10 min in ice-cold 5% trichloroacetic acid, which also removed unincorporated \(^{3}H\)thymidine. DNA was isolated from the fixed cells using a DNA extraction procedure (Pierce, Rockford, IL) and the remainder of the sample subjected to liquid scintillation spectrometry. \(^{3}H\)Thymidine incorporation into SMC cell DNA is expressed as disintegrations per minute per μg of cell protein.

Cell Cycle Progression

Cell cycle progression was assessed using FACS. Cell cycle progression was assessed during which animals which had been injected had elevated blood glucose (>400 mg/dl), the animals were sacrificed with sodium pentobarbital at 45 mg/kg and aorta tissue removed. Tissue was frozen in liquid nitrogen until analysis.

Experiments in Insulin-resistant Animal Models—Aorta from female ob/ob and lean ob/+ littermate control mice (Jackson Labs, Bar Harbor, ME) were obtained in collaboration with Dr. Boris Draznin (Denver, CO). Ob mice were given ad libitum access to food and sacrificed at 12 weeks of age. Blood was collected by cardiac puncture and samples were analyzed for insulin and glucose concentrations. In separate experiments, C57 BL-6 male mice experimental obesity was created by intraperitoneal injection of gold thioglucose (Sigma: 0.5 mg/kg body weight) at 5 weeks of age (21). These and animals that matched the sham injected animals were given ad libitum access to food and sacrificed at 30 weeks of age. Age-matched Zucker obese (fa/fa) and lean control (fa–) rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were fed standard chow and sacrificed at 12 weeks of age. Aortic tissues from ob/ob, ob/+, gold thioglucose-obese, control, fa/fa, and fa/– animals were obtained from the laboratory of Dr. Boris Draznin, unrelated data on these animals has been previously published (22). For the rosiglitazone treatment studies, 40 male ob mice were purchased from Jackson Laboratories at 6 weeks of age. Mice with blood glucose greater than 15 mmol/liter were randomized to treatment via gavage with either 20 mg/kg/days of rosiglitazone or CMC vehicle. Glucose and body weight were assessed at baseline, days 4 and 8. Animals were sacrificed at day 11.

RESULTS

Chronic High Glucose Decreases CREB Protein Content and CREB Transcriptional Activity in Cultured SMC—Previous studies from our laboratory defined CREB as an antiproliferative protein in SMC. As mentioned in the Introduction, diabetes-mediated accumulation of ROS could negatively affect CREB function. Therefore, we examined the impact of high glucose on SMC CREB protein content and function. Chronic exposure (72 h) of SMC to high glucose (25 mM) results in a modest but statistically significant decrease in CREB protein content (Fig. 1). Chronic exposure to high glucose also leads to increased content of activating transcription factor 3, ATF3 (Fig. 1). ATF3 is a member of the CREB/ATF family of transcription factors which can inhibit CRE-mediated gene activation.

**Fig. 1.** Chronic exposure of cultured SMC to high glucose leads to significantly decreased CREB protein content at 72 h. SMC were cultured in serum-reduced medium. SMC were treated with glucose (5 mM or 25 mM glucose), harvested in LSB, and subjected to Western blot analysis with antisera specific for CREB and ATF3. Blots were subjected to autoradiography and densitometry as indicated under “Experimental Procedures.” CREB content results from four separate experiments, n = 3 for each experimental variable each experiment; ATF3 results are from three separate experiments, n = 2 for each variable in each experiment. Results are mean ± S.E.; #, increased relative to control; * increased relative to control; p < 0.05.

**Fig. 2.** Loss of CREB protein in SMC following treatment with high glucose or ROS is accompanied by a significant decrease in basal CREB-mediated promoter transactivation. Chronic exposure of cultured SMC to high glucose or ROS 1. Induction of Diabetes in Rats with Streptozotocin—The animals were housed in the Denver Veterans Affairs Animal Facility and diabetes was induced in half of the animals via intraperitoneal injection of 45 mg/kg streptozotocin, with vehicle injected into control animals. Diabetes was be confirmed with tail vein blood (One Touch Glucometer, LifeScan, Newton, PA) 48 h after streptozotocin. Animals with blood glucose levels greater than 250 mg/dl were maintained for 8 weeks with
CREB is known to regulate cAMP response element (CRE)-dependent gene expression. To determine whether decreased CREB content led to decreased CREB-mediated gene expression we examined the effect of high glucose treatment upon CRE-dependent promoter activity. SMC were transfected with a consensus CRE-containing promoter-luciferase reporter plasmid (Stratagene). Transcriptional activity from this promoter was significantly diminished following treatment with chronic high glucose (Fig. 2). This decrease in transcription from the chimeric CRE-luc construct is a function of the CRE sequences in the construct, as luciferase production from the pCIS-CK construct, which lack CRE elements, was unaffected by ROS or glucose (Fig. 2). This was not a generalized effect on all transcription as experiments using two distinct promoters containing NF-κB regulatory elements indicated that transcription from these constructs was unaffected by exposure to glucose or ROS (data not shown). Acute NF-κB activation following treatment with ROS or glucose was not examined in these experiments, however, immunohistochemical examination of SMC following 30 min exposure to these agents indicates rapid and transient translocation of the p65 subunit of NF-κB to the nuclei of SMC (data not shown).

**Chronic High Glucose Accelerates Rates of DNA Synthesis and Cell Chemokinesis in SMC**—Exposure of either rat or bovine SMC to 25 mM glucose for 48 h results in a significant acceleration in the rates of DNA synthesis, cell cycle progression, and cell chemokinesis (Fig. 3). SMC were exposed to 5 or 25 mM glucose for 72 h. 

**Fig. 3.** Exposure of SMC to 25 mM glucose for 48 h results in a significant acceleration in the rates of DNA synthesis, cell cycle progression, and cell chemokinesis. SMC were exposed to 5 or 25 mM glucose for 72 h. A, cells used to assess rates of DNA synthesis had 4 μCi of [3H]thymidine for 3 h and harvested as described under “Experimental Procedures.” B, SMC exposed to glucose as described in A were fixed, stained for DNA, and subjected to FACS analysis. Results are expressed as cells in S + G2/M stages of cells cycle as compared with cells in G0 + G1. C, cells used for chemokinesis were trypsin-released from cultures and assessed for Boyden chamber chemokinesis as indicated under “Experimental Procedures.” Results were from three separate experiments, n = 3 for each experimental variable; mean ± S.E.; #, decreased relative to control; p < 0.05.

**High Glucose Induces Oxidative Stress in SMC**—We hypothesized that the glucose-mediated induction of ROS could be responsible for depletion of CREB in the SMC. This hypothesis was attractive because increased ROS has been reported in the vasculature of both insulin-resistant and insulin-deficient diabetes in rodents and humans (16–19). SMC were labeled with 10 mM CM-H2DCFDA (Molecular Probes), a dye which when exposed to an excitation wavelength of 485 nm, emits light at 530 nm only when it has been oxidized. Standard curves using hydrogen peroxide indicate the effectiveness of this approach in SMC (Fig. 4A). SMC exposed to increasing concentrations of glucose for 24 h demonstrate a significant dose-dependent increase in oxidation of intracellular dye relative to both mannitol and l-glucose controls (Fig. 4B). Additionally, the effect of
ROS Exposure Results in the Loss of CREB and Increase in Chemokinesis—We next assessed the impact of pretreatment with antioxidants upon glucose and ROS-mediated CREB depletion. Pretreatment of SMC with N-acetylcysteine, an antioxidant which will neutralize hydrogen peroxide, results in the prevention of high glucose- and ROS-mediated loss of CREB (Fig. 7). This suggests that high glucose-mediated CREB depletion is secondary to ROS generation.

Antioxidants and Active CREB Reduce Chemokinesis and Proliferation—Experiments were performed to determine whether the induction of progression through the cell cycle and increased chemokinesis induced by glucose or ROS were mechanistically related to the CREB depletion noted in Fig. 5. CREB content and function were manipulated by infection with a panel of adenoviral constructs encoding either active or dominantly negative CREB expression vectors (constitutively active CREB isoforms CREB-DIEDML (DCREB) and the chimeric VP16 CREB; dominant negative KCREB). For cell cycle experiments, cells were infected with either control adβGal or DCREB adenovirus, then incubated for 72 h in either 5 mM glucose, 25 mM glucose, 22.5 μM hydrogen peroxide or 12 milliunits/ml glucose oxidase. Cells were harvested, fixed in cold 50% ethanol, stained, and subjected to analysis for cell cycle progression using FACS analysis. Exposure to high glucose or ROS resulted in acceleration of the cell cycle, as indicated by increased migratory behavior (Fig. 6). In addition, SMC exposed to hydrogen peroxide demonstrated increased migratory behavior (Fig. 6), similar to that observed in SMC exposed to chronic high glucose.

For cell chemokinesis (A), cells were fixed in cold ethanol and stained for DNA content and function were manipulated by infection with a panel of adenoviral constructs encoding either active or dominantly negative CREB expression vectors (constitutively active CREB isoforms CREB-DIEDML (DCREB) and the chimeric VP16 CREB; dominant negative KCREB). For cell cycle experiments, cells were infected with either control adβGal or DCREB adenovirus, then incubated for 72 h in either 5 mM glucose, 25 mM glucose, 22.5 μM hydrogen peroxide or 12 milliunits/ml glucose oxidase. Cells were harvested, fixed in cold 50% ethanol, stained, and subjected to analysis for cell cycle progression using FACS analysis. Exposure to high glucose or ROS resulted in acceleration of the cell cycle, as indicated by increased migratory behavior (Fig. 6A), similar to that observed in SMC exposed to chronic high glucose.

Results indicate that exposure to ROS increases the number of cells progressing through the cell cycle, similar to the effects seen with chronic glucose exposure (Fig. 6A). In addition, SMC exposed to hydrogen peroxide demonstrated increased migratory behavior (Fig. 6B), similar to that observed in SMC exposed to chronic high glucose.

ROS Exposure Results in the Loss of CREB and Increase in ATF3 Protein, Similar to That Observed with High Glucose—SMC were exposed to two methods commonly employed for induction of ROS, hydrogen peroxide (H$_2$O$_2$) and glucose oxidase; which generates a constant level of H$_2$O$_2$). Increased ROS exposure to SMC resulted in the loss of CREB and increase in chemokinesis (see “Experimental Procedures”). Increased CREB activity following DCREB expression resulted in a significant reduction of the number of cells migrating. Results were from two separate experiments, n = 3 for each experimental variable each experiment. Results are mean ± S.E.; *, increased relative to control; #, decreased relative to adβGal treatment within experimental group; p < 0.05.

5-glucose to increase dye oxidation was enhanced by treatment with L-buthionine-(S,R)-sulfoximine, which blocks production and accumulation of the intracellular anti-oxidant glutathione (Fig. 4B).

ROS Exposure Results in the Loss of CREB and Increase in ATF3 Protein, Similar to That Observed with High Glucose—SMC were exposed to two methods commonly employed for induction of ROS, hydrogen peroxide (H$_2$O$_2$) and glucose oxidase; which generates a constant level of H$_2$O$_2$). Increased ROS mimicked the effects of chronic high glucose treatment on CREB (Fig. 5, top and center) as well as increased the content of ATF3 (Fig. 5, bottom). These results are consistent with the hypothesis that decreases in CREB content seen with chronic hyperglycemia result from glucose-induced oxidative stress.

ROS Exposure Results in Increased SMC Proliferation and Chemokinesis—SMC were exposed to chronic ROS as detailed in the figure legends. After 72 h, cells were assessed for cell cycle progression by FACS analysis as described under “Experimental Procedures.” Results indicate that exposure to ROS increases the number of cells progressing through the cell cycle, similar to the effects seen with chronic glucose exposure (Fig. 6A). In addition, SMC exposed to hydrogen peroxide demonstrated increased migratory behavior (Fig. 6B), similar to that observed in SMC exposed to chronic high glucose.
CREB and Smooth Muscle Cell Dysfunction with Diabetes

Vascular CREB Protein Content Is Decreased in Animals with Insulin Resistance or Diabetes—As CREB affects SMC activation and is depleted secondary to both high glucose and ROS, we examined CREB content of aortic tissue harvested from animals with either insulin resistance that were not overtly diabetic (Ob/Ob mice, Zucker rats, and thiglucose-treated rats) or with diabetes for 8 weeks (streptozotocin rats; average blood glucose 400–500 mg/dl). Analysis of aortic homogenates from these animals indicates that CREB protein content was significantly diminished in the vessel wall (Fig. 10, A and C). Quantitation of CREB content revealed a 49 ± 8% (n = 5, p < 0.05) decrease in ob/ob versus control; 38 ± 9% (n = 7, p < 0.05) decrease in Zucker versus control; and a 50 ± 11% (n = 4, p < 0.05) decrease in thioglucose-injected animals. Six-week-old ob/ob diabetic mice were treated with the insulin sensitizer rosiglitazone (20 mg/kg/day) for 11 days (Table I). These animals demonstrated a reduction in the levels of glycemia accompanied by a restoration of aortic CREB content toward baseline (p < 0.05, n = 3; Fig. 10B). A similar magnitude of decline in CREB content was noted in insulin-deficient streptozotocin animals (Fig. 10C).

**DISCUSSION**

The transition of arterial smooth muscle cells from a contractile to a synthetic, proliferative state (phenotypic modulation) appears to be an early event in the pathogenesis of atherosclerosis (8–11) and in angioplasty-induced restenosis. Dysregulation of this remodeling process is observed in atherosclerosis and exaggerated by diabetes. Numerous studies using SMC from diabetic animals and humans have demonstrated an increased migratory and proliferative phenotype (25–28). Despite extensive study in this area the mechanism of enhanced SMC activation by diabetes remains elusive (25, 29, 30). In diabetes elevation of PKC activity in the vessel wall increase SMC proliferation. It has recently been reported that glucose can enhance cellular content of reactive oxygen species by overwhelming mitochondrial capacity (20). Additionally, elevation of PKC activity and accumulation of advanced glycation end-products (known mediators of glycemic injury) have been described in the vasculature in diabetes. Accumulation of AGEs and activation of PKC is increased in the presence of ROS. The studies presented in this paper define an additional molecular change in SMC in response to glucose and ROS that is observed in rodent models as well, depletion of vascular CREB content.

CREB promotes gene transcription through its association with a specific target sequence, the CRE (5′-TGACGTCA-3′) in the promoter regions of CRE-regulated genes. CREB transcription is stimulated through phosphorylation at serine 133, which increases its association with transcriptional adapter proteins like CREB-binding protein or P300. These transcription coactivators interact with the basal transcriptional machinery and increase the rate of transcription (31–35). The specific upstream signaling events that are activated by chronic exposure to high glucose and their impact upon CREB-mediated transcriptional events is also the focus of much attention. CREB’s serine 133 phosphorylation state is determined by the level of activity of numerous intracellular signaling cascades. In addition to PKA, a number of signaling activities have been linked to CREB phosphorylation. These include extracellular regulated kinase 1/2 MAP kinase, p38
Fig. 9. Pretreatment with the antioxidant N-acetylcystiene or increased CREB activity prevents high glucose-mediated increases in cell cycle progression and chemokinesis in SMC. A, SMC in culture were treated with low and high glucose and pretreated with N-acetylcystiene as described in the legend to Fig. 3. Cell chemokinesis was assessed as described in the legend to Fig. 5. Results are mean ± S.E.; *, increased relative to control; #, increased relative to N-acetylcystiene treatment; p < 0.05. B, SMC in culture were infected with recombinant adenovirus (1 × 10^10 m.o.i./400,000 cells) expressing constitutively active (DCREB, VP16CREB) or dominant-negative (KCREB) forms of CREB. Cells were subsequently treated with glucose as in A for 72 h. Cells were harvested by trypsinization and plated in Boyden chambers for assessment of cell chemokinesis (see “Experimental Procedures”). Overexpression of either constitutively active form of CREB (adVP16CREB or adDCREB) in SMC dramatically blunted high glucose-induced cell chemokinesis. Dominant-negative KCREB accentuated glucose-induced cell chemokinesis. Results are mean ± S.E.; *, increased relative to β-gal control; #, increased relative to β-gal control; p < 0.05. C, SMC were incubated for 72 h in 15 μM hydrogen peroxide. Prior to treatment, cells were infected with either control abBetaGal or adDCREB. Cells were harvested and analyzed for cell chemokinesis as in B. Increased CREB activity following DCREB expression resulted in a significant reduction of the number of cells migrating. Results from three separate experiments, n = 3 for each experimental variable each experiment. Results are mean ± S.E.; *, increased relative to control; #, decreased relative to adBetaGal treatment within experimental group; p < 0.05.

Fig. 10. CREB content of aortic tissue harvested from animals with either insulin resistance (Fig. 1A; thioglucose-treated rats and Zucker rats; Fig. 1B, ob/ob mice) or diabetes (Fig. 1C; STZ rats). Treatment of ob/ob mice with the insulin-sensitizing agent rosiglitazone partially restores CREB content in aortic tissue toward that seen in non-insulin-resistant control animals. Aortic tissue was taken from animals and frozen in liquid nitrogen. Samples were homogenized in lysis buffer and cellular debris pelleted by centrifugation. Following protein assay, equal quantities of protein were added to 2 × Laemmli sample buffer and subjected to gel electrophoresis (12% polyacrylamide-SDS). Proteins were transferred to nylon membranes and subjected to Western blot analysis as described under “Experimental Procedures.” Analysis indicates that CREB protein content was significantly diminished in the vessel wall (Fig. 1). n = 4 for each experimental variable each experiment. Results are mean ± S.E.; *, increased relative to control; #, decreased relative to control; p < 0.05.
MAP kinase, PKC, and phosphatidylinositol 3-kinase in response to multiple growth factors, calcium, IgGs, and oxidant stress (36–44). Multiple CREB kinases activated by these upstream signaling pathways have been identified. These include PKA, RSK 1,2, MAPKAP kinase 2 and 3, MSK1 and 2, and calmodulin kinase. Phosphorylation of CREB on serine 133, which is one determinant of CREB transcriptional activity, can be viewed as a nuclear read out of the convergence of multiple signaling pathways.

Multiple lines of evidence define a role for CREB in proliferation and differentiation of certain cells and tissues (45–47). In neuronal tissue, CREB regulation by nerve growth factor and insulin-like growth factor-1 is essential for neuronal plasticity, full axonal development, memory consolidation, and neuroprotection (43, 47, 48). Disruption of CREB activity, using expression of a dominant-negative CREB, slows neurite outgrowth and blocks adipocyte differentiation (48, 49). In 3T3-L1 adipocytes, we have demonstrated that CREB is necessary and sufficient for differentiation (50).

Much confusion exists in the literature about the importance of hyperglycemia as a mediator of enhanced atherosclerosis. In a recent report using porcine explants from a new model of atherosclerosis, high glucose alone was not sufficient to increase SMC proliferation (51). In contrast, numerous groups have observed (as we do) a modest increase in proliferation in animals. The point has been made that endothelial cells are a potential target that is diminished by high glucose, which permits a proliferative response to glucose and other mitogenic stimuli. If it is quite possible that glucose is not the primary source of ROS that leads to CREB down-regulation in vivo. It is likely that vascular ROS accumulation in response to free fatty acids, PKC activation, and AGE accumulation and activation of receptor for AGE, RAGE, all contribute to this response in animals. The point has been made that endothelial cells are much more sensitive to glycemic injury that SMC. A similar diminution in CREB content is observed in endothelial cells (data not shown) but the phenotypic importance of this observation has not yet been characterized. ROS generation by AGE’s, PKC, and oxidized lipoproteins is widely reported in the literature. Accumulation of AGEs in vascular lesions of apolipoprotein-E knockout mice was ameliorated by treatment with a soluble receptor for AGE (RAGE). Ligand binding to RAGE leads to activation of MAP kinase signaling as well as generation of ROS and activation of the nuclear transcription factor NF-κB (52). In at least two reports NF-κB activation has been demonstrated to interfere with CREB activation, presumably via competition for CREB-binding protein (54). Additionally, NF-κB activation is seen in SMC exposed to hyperglycemia (55). Additionally high glucose and ROS increase the expression of ATF3, a negative modulator of CRE activity. The precise mechanism of CREB depletion in SMC still needs to be elucidated and is a current area of investigation in the laboratory.

In summary, these studies define the ability of glucose and ROS to decrease CREB content and activity in SMC. CREB is a determinant of SMC proliferative capacity. Depletion of CREB is also noted in vascular stroma of diabetic and insulin-resistant rodents. Thus, loss of CREB may play a permissive role in enhancing the atherosclerosis observed in diabetes.

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Diabetes-related Changes in cAMP Response Element-binding Protein Content Enhance Smooth Muscle Cell Proliferation and Migration
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