Atherosclerosis is the leading cause of death in the Western world (http://www.cdc.gov/nchs-National). In the past few decades much has been learned about cardiovascular risk factors and their impact on the development of atherosclerotic plaque. The current theoretical model of atherogenesis includes a complex interplay between endothelial cells, vascular smooth muscle cells (VSMC), circulating inflammatory cells, and the vascular adventitia. The end result is a proinflammatory microenvironment that promotes a vicious cycle of plaque progression. Interruption of this cycle is necessary to slow atherosclerosis progression and stabilize existing plaque. Medications that lower low-density lipoprotein (LDL) cholesterol have revolutionized therapy for cardiovascular disease but do not fully normalize cardiovascular risk.

Our laboratory has identified the transcription factor cAMP-response element-binding protein (CREB) as a modulator of smooth muscle cell phenotype. CREB blunts mitogen-stimulated VSMC proliferation, migration, and matrix protein expression, and it protects smooth muscle cells from apoptosis. In previous reports, we observed decreased levels of CREB protein and the active form of CREB (phosphoserine 133 CREB) in medial VSMC in rodent models of insulin-resistant and insulin-deficient diabetes. Loss of VSMC CREB protein in this model was, in part, secondary to oxidant stress and could be modeled in vitro by exposure of aortic VSMC to H2O2 or glucose oxidase. Similarly, in a model of pulmonary vascular injury (hypoxia-induced pulmonary hypertension), we reported loss of CREB protein concurrent with pulmonary artery hypertrophy. This could be modeled in vitro by exposing pulmonary VSMC to platelet-derived growth factor, which induced CREB nuclear export and degradation by a pathway downstream of Akt and casein kinase 2 (CK2).

In this article we report on the response of VSMC in vivo to rodent models of traditional cardiovascular risk factors, such as hyperlipidemia, hypertension, and obesity, and in vitro in the context of hyperlipidemia. We present data demonstrating loss of aortic CREB content across rodent models of elevated cardiovascular risk factors. For example,
in an established model of atherosclerosis, LDL receptor-null (LDLR−/−) mice exposed to high-fat diet, we observed a significant loss of CREB protein in the vascular media. These observations suggest that loss of CREB content and function is a common pathogenic VSMC response to cardiovascular risk factors. In additional studies in vitro, 24- to 48-hour exposure of VSMC to LDL and oxidized LDL (oxLDL) revealed CREB downregulation by only oxLDL through a mechanism involving generation of reactive oxygen species (ROS) and ERK activation, but independent of Akt, leading to nuclear exclusion. This suggests that CREB downregulation in VSMC may be induced by several mechanisms, including generation of ROS, which are likely to be important for the consistent loss of CREB observed in rodent models of elevated cardiovascular disease risk.

Materials and Methods

All materials and methods used standard techniques and commercially available reagents. Animal studies were conducted under AAALAC guidelines and were approved by individual Institutional Animal Care and Use Committees. Methods and statistical analysis were performed as previously reported and are outlined in detail in the Supplemental Materials and Methods (available online at http://atvb.ahajournals.org).

Results

Loss of CREB Protein in Aging and Hypertension

We previously reported decreased vascular CREB protein content in animal models of diabetes and pulmonary hypertension.5,6 We queried whether loss of vascular CREB was also observed across the spectrum of other conditions known to induce vascular pathology, including aging, hypertension, and dyslipidemia. Figure 1 demonstrates significantly decreased CREB content in aortic lysates with aging in C57/B6 mice (Jackson Labs, Bar Harbor, Me) and Sprague-Dawley rats (Charles River Laboratory, Wilmington, Mass) (Figure 1A). We next examined spontaneously hypertensive heart failure in rats. This rat model has a hypertension phenotype in the lean line and hypertension, insulin resistance, obesity, and glucose intolerance in the obese line. We observed a significant age-dependent loss of CREB protein that was more pronounced in the obese rats (Figure 1B), as observed in the insulin-resistant Zucker rat.6

CREB Content Is Decreased in the Vasculature of High-Fat-Fed LDLR Knockout Mice

One of the best established models of rodent atherosclerosis is the LDLR−/− mouse that has a human pattern of dyslipidemia on a western diet. Aortic root sections were examined from 4 groups of animals (other results from this set of studies reported elsewhere7): (1) C57BL/6 mice fed a chow diet; (2) C57BL/6 mice fed the high-fat western diet; (3) genetically hypercholesterolemic LDLR−/− mice fed a chow diet; and (4) LDLR−/− mice fed a western diet. Specimens were analyzed from 10 animals in each group. The data are presented as representative photos (Figure 2A) and graphically as percent CREB-negative nuclei (Figure 2B). In the chow-fed animals, there was CREB staining in the majority of the nuclei of the medial VSMC in the aorta in control and LDLR−/− mice. The percentage of CREB-negative nuclei increased significantly in response to high-fat-feeding in both control and LDLR−/− mice (Figure 2A, B). Significant cholesterol elevation was only observed in the LDLR−/− mice.7 In the LDLR−/− mice, cholesterol levels were 5-fold higher in the western diet group than in the chow group, and lipids correlated significantly with loss of nuclear CREB staining (Figure 2C). These data suggest that both high-fat diet and elevated cholesterol contribute to decreased nuclear CREB expression in aortic VSMC.

OxLDL Induces a Time- and Dose-Dependent Downregulation of Nuclear CREB Protein

Previous work from our group indicates that oxidative stress is a potent stimulus for CREB downregulation.6,8,9 Published reports using LDL and modified LDL suggest that both directly increase VSMC oxidative stress.10 We explored the effects of both LDL and oxLDL on VSMC CREB expression in vitro. Rat aortic VSMC were serum-depleted for 48 hours and then exposed to either LDL or oxLDL for 0 to 48 hours. We observed a significant reduction in CREB protein level within 24 to 48 hours of exposure to oxLDL, whereas native LDL did not affect CREB protein content (Figure 3A). Downregulation of CREB was dose-dependent, occurring at oxLDL doses of ≈25 μg/mL after 24 hours of exposure.
ROS and ERK Contribute to CREB Downregulation by oxLDL

To characterize the signaling events critical for oxLDL-mediated CREB downregulation, we first assessed the signaling pathways activated in response to oxLDL. Consistent with previous reports in VSMC exposed to oxLDL, we observed acute transient activation of 2 stress MAP kinases (ERK and p38 MAPK) as detected by Western blot analysis (Figure 4), as well as a trend toward acute activation of another stress MAP kinase (JNK). In addition, we observe a modest activation of Akt. Both p38 MAPK and Akt activation are more persistent after oxLDL treatment than after LDL treatment. Our group recently reported that the mitogen/cytokine platelet-derived growth factor induces CREB nuclear export and degradation in pulmonary VSMC through persistent activation of Akt/CK2 and site-specific phosphorylation of CREB S103 and S107. In earlier studies, we also reported ROS-mediated CREB downregulation. To assess the importance of ROS activation of stress MAPK vs Akt/CK2 for CREB downregulation, we used studies in which primary rat VSMC were pretreated with a panel of pharmacological inhibitors or the ROS scavenger, N-acetylcysteine, before oxLDL exposure (Figure 5A). Pharmacological inhibition of PI3K, Akt, p38 MAPK, and JNK had no significant effect on oxLDL-mediated CREB downregulation (Figure 5A). Furthermore, experiments examining the Akt/CK2 pathway do not demonstrate induction of CK2 expression by either oxLDL or LDL (data not shown). Of interest, pharmacological inhibition of protein kinase A (H89), the original physiological activator of CREB, and protein kinase C (BIS), the mediator of fatty acid-induced CREB activation, also did not block oxLDL-mediated CREB downregulation. Only pharmacological inhibition of PI3K, Akt, p38 MAPK, and JNK had no significant effect on oxLDL-mediated CREB downregulation (Figure 5A). Furthermore, experiments examining the Akt/CK2 pathway do not demonstrate induction of CK2 expression by either oxLDL or LDL (data not shown). Of interest, pharmacological inhibition of protein kinase A (H89), the original physiological activator of CREB, and protein kinase C (BIS), the mediator of fatty acid-induced CREB activation, also did not block oxLDL-mediated CREB downregulation. Only pharmacological inhibition of PI3K, Akt, p38 MAPK, and JNK had no significant effect on oxLDL-mediated CREB downregulation (Figure 5A). Furthermore, experiments examining the Akt/CK2 pathway do not demonstrate induction of CK2 expression by either oxLDL or LDL (data not shown). Of interest, pharmacological inhibition of protein kinase A (H89), the original physiological activator of CREB, and protein kinase C (BIS), the mediator of fatty acid-induced CREB activation, also did not block oxLDL-mediated CREB downregulation. Only pharmacological inhibition of PI3K, Akt, p38 MAPK, and JNK had no significant effect on oxLDL-mediated CREB downregulation (Figure 5A). Furthermore, experiments examining the Akt/CK2 pathway do not demonstrate induction of CK2 expression by either oxLDL or LDL (data not shown). Of interest, pharmacological inhibition of protein kinase A (H89), the original physiological activator of CREB, and protein kinase C (BIS), the mediator of fatty acid-induced CREB activation, also did not block oxLDL-mediated CREB downregulation. Only pharmacological inhibition of PI3K, Akt, p38 MAPK, and JNK had no significant effect on oxLDL-mediated CREB downregulation (Figure 5A).

Antagonism of LDLR Does Not Interfere With oxLDL-Mediated CREB Degradation

To determine whether the oxLDL-mediated CREB degradation required the LDLR, cells were pretreated with antibodies to the LDLR (R&D Systems) for 30 minutes as outlined in Figure 6. Acute CREB activation and later CREB downregulation and HO-1 induction by oxLDL were not prevented by pretreatment with anti-LDLR antibodies (Figure 6 and data not shown), suggesting that these events do not require interaction with LDLR, consistent with a recent report by Hansen-Hagge.
Figure 3. Effect of LDL and oxLDL on CREB content, activation, and mRNA in primary culture rat VSMC. CREB and phosphoserine 133 CREB (PCREB) proteins were quantified by densitometric analysis of Western blots. CREB mRNA was quantified by quantitative reverse-transcriptase polymerase chain reaction and normalized to 18S rRNA. A, Time course for CREB content with exposure to 100 μg/mL LDL or oxLDL. Error bars represent SD from 2 separate experiments with total n=4, except for duplicate samples at 6 hours. LDL, P=not significant; oxLDL, P for trend <0.001; *P<0.05 vs 0 time. B, Dose response for CREB protein (first 5 bars) and CREB mRNA (last 2 bars) content at 24 hours of exposure to oxLDL. Error bars represent SD from 2 separate experiments with total n=3 to 5 for protein levels and n=5 for mRNA levels. P for protein trend <0.001; *P<0.05 vs 0 μg/mL; P for mRNA difference=0.17. C, Time course for CREB activation with 100 μg/mL LDL or oxLDL. Error bars and sample number as in (A); oxLDL and LDL, P for trends <0.001; *P<0.05 vs 0 time. D, Dose response for CREB activation at 15 minutes of exposure to oxLDL. Error bars and sample number as in (B); P for trend <0.01; *P<0.05 vs 0 μg/mL. E and F, CREB localization after 24-hour exposure to LDL or oxLDL. Cy3 (red) indicates CREB; DAPI (blue), nuclei. Representative Western blots are shown. Results shown are representative of >10 separate experiments. Values are mean±SD. P for trends were calculated by 1-way ANOVA, and P for comparison to control were calculated by ANOVA with Dunnett post test.
Discussion

Atherosclerosis is a complex process in which long-term exposure of the vessel wall to vascular insults induces the vicious cycle of vascular pathology. Regardless of the etiologic factor or factors, most atherosclerotic lesions share overlapping features, including endothelial dysfunction, neointimal proliferation, lipid accumulation, inflammation, and necrosis. Risk factors such as dyslipidemia, hypertension, diabetes, smoking, and age likely have both common and unique effects on the cellular and extracellular components of the vessel wall. We previously have reported that oxidant stress, mitogens, and cytokines lead to CREB downregulation in pulmonary and aortic VSMC, as well as in neurons and beta cells. Here, we present evidence that diminished aortic medial CREB expression is a common deleterious response to a variety of atherosclerotic risk factors. This series of experiments demonstrates significant age, hypertension, insulin resistance, and obesity-induced aortic VSMC CREB downregulation. Importantly, we directly demonstrate decreased aortic nuclear CREB expression in response to high-fat-feeding in both C57BL/6 and LDLR−/− mice. Furthermore, we demonstrate that in vitro exposure of rat primary VSMC to oxLDL leads to decreased CREB protein expression, specifically via a ROS/ERK-dependent pathway. These data support a model wherein loss of VSMC CREB expression is a common pathological response to vascular injury, likely attributable to oxidant stress, which renders the vessel wall more susceptible to proliferation, migration, inflammation, and apoptosis, and potentially contributes to plaque progression.
Others and we\textsuperscript{4,14–17,18} have reported that CREB is critical for oxidant defense and survival of VSMC, cardiac myocytes, neurons, and adipocytes. Cardiac-selective expression of dominant-negative CREB leads to accelerated heart failure and blocks the adaptive cardiac response to exercise training.\textsuperscript{14,19} In this context, cardiac CREB appears to regulate not only myocardial apoptosis but also metabolic adaptation by affecting mitochondrial biogenesis and efficiency.\textsuperscript{17} We have further reported\textsuperscript{8,9} that overexpression of CREB in cultured neurons and beta cells can protect these cell types from oxidant and cytokine-mediated apoptosis. It seems likely that CREB plays a similar protective role in the vessel wall, where inflammation and oxidant stress accelerate atherosclerosis.

CREB protects cells from oxidant injury through transcriptional regulation of numerous targets, including genes involved in antiapoptotic mechanisms, upregulation of antioxidant defense, and augmentation of growth factor signaling.

First, CREB inhibits apoptosis by stabilizing mitochondria through induction of bcl-2 and by augmenting mitochondrial biogenesis via peroxisome proliferator-activated receptor gamma coactivator-1\textsuperscript{\textgamma} (PGC-1). In addition, oxidative stress leads to the induction of CREB-mediated antioxidant defenses on 2 levels, by inducing expression of antioxidant enzymes and by promoting mitochondrial biogenesis.

Figure 5. Involvement of signaling pathways in the effects of oxLDL on primary culture rat VSMC. Cells were pretreated for 30 minutes with or without (control [CON]) inhibitors of signaling pathways: SB-203580 (SB; 20 \textmu mol/L, p38 MAPK), SP-600125 (SP; 20 \textmu mol/L, JNK), U-0126 (U0; 10 \textmu mol/L, ERK), Wortmannin (WORT; 100 nM, PI3K), H89 (10 \textmu mol/L, protein kinase A [PKA]), bisindolyl maleimide (BIS; 4 \textmu mol/L, protein kinase C [PKC]), N-acetyl cysteine (NAC; 30 mmol/L, ROS), and then incubated with (+) or without (–) oxLDL (40 \textmu g/mL) as indicated. CREB at 24 hours (A), phosphorylated CREB at 6 hours (B), and HO-1 at 6 hours (C) were quantified and normalized to \beta-actin by densitometric analysis of Western blots. D, Representative Western blots are shown for CREB, phosphorylated CREB, and \beta-actin at 6 and 24 hours. Individual portions of Western blots were from 2 gels from a single experiment. Control extracts (0, C) were loaded on all gels to allow gel to gel comparison, and blots of comparable exposure are shown. E, Representative Western blots are shown for HO-1. Blot portions shown were from the same experiment but from multiple gels as in (D). Note that inhibitor order is different from that shown in the graphs. Graphs represent data combined from 3 experiments, each with duplicate or triplicate independent samples. Because some experiments did not include all inhibitors, 3 to 7 independent samples are represented in each data point. Values are mean \pm SD; \textsuperscript{\textast}P < 0.05 vs corresponding no oxLDL control.

Figure 6. OxLDL-mediated CREB downregulation and HO-1 induction are not LDLR-dependent. Rat VSMC cells pretreated with or without LDLR-blocking antibody (5 \textmu g/mL) for 30 minutes and then exposed to oxLDL for 24 hours at the indicated doses. CREB is shown on top graph and HO-1 is shown on bottom graph. Bar heights represent means for independent duplicate samples, with error bars illustrating maximum values. Data shown are representative of 2 separate experiments, each performed with independent duplicate samples. One of the corresponding Western blots is shown below the graphs with a normalization control lane spliced out centrally.

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enzymes, such as manganese superoxide dismutase, thioredoxin, and HO-1, and by increasing mitochondrial biogenesis via upregulation of PGC-1. Finally, CREB enhances appropriate growth factor signaling through regulation of brain-derived neurotrophic factor and insulin receptor substrate-2 (IRS)-2. Thus, loss of VSMC CREB function may contribute to disruption of vascular growth regulation and antioxidant and antiapoptotic defenses.

In this report, we demonstrate dynamic upregulation of the protein level of 1 antioxidant CREB target, HO-1, in response to oxLDL treatment. We hypothesize that this upregulation of HO-1 is, at least in part, a response to CREB activation and is a beneficial effect of CREB activation that could be lost with CREB downregulation. The persistence of HO-1 protein after CREB downregulation is not inconsistent with a CREB role in HO-1 activation, because the half-life of HO-1 mRNA is increased to up to 11 hours under certain types of oxidative stress. However, HO-1 regulation is complex and multifactorial, involving many signaling pathways and transcriptional factors other than CREB, in addition to nontranscriptional regulatory mechanisms. Overall, the HO-1 activation we demonstrate is a marker of severe oxidative stress imposed by oxLDL treatment, and our studies do not rule out a role for other regulatory pathways. In fact, the oxLDL resistance inferred by ERK inhibition with UO126 may be the result of early HO-1 activation and represent a preconditioning that allows cells to cope with the oxidant stress imposed by oxLDL.

The hypothesis that CREB downregulation contributes to vascular pathology in atherosclerosis implies that CREB activity is important for maintaining normal vascular physiology. CREB is a differentiation factor in VSMC, but the function of CREB in normal physiology is not as clearly delineated in the vasculature as it is in the nervous system. Acute CREB activation by a broad array of toxins may be a cytoprotective response to injury. Recently, a number of observations have been published demonstrating acute CREB activation by established mediators of vascular injury, such as fatty acids, LDL cholesterol, very low density lipoprotein cholesterol, thrombin, endothelin-1, angiotensin II, and tumor necrosis factor-α in endothelial cells and VSMC, both in vitro and in vivo. We postulate that acute CREB activation by vascular toxins is a healthy response that is intended to decrease oxidant injury and inflammation and maintain VSMC survival and function. However, we acknowledge that there are conflicting data on the role of CREB in acute vascular injury. CREB activity was found to be increased in carotid arteries after wire injury. Introduction of a dominant-negative CREB adenovirus at the time of wire injury blunted neointimal lesion formation and led to increased VSMC apoptosis and decreased proliferation. The explanation for these differences from our studies of CREB and VSMC phenotype remains unclear. They are likely dependent on intracellular pathway activation profiles and coadaptor/corepressor recruitment to CREB target genes that are unique depending on the external stimulus and cell context.

The mechanism of CREB downregulation in the vasculature also remains unclear. Numerous mechanisms have been shown to contribute to the CREB dysfunction observed in Alzheimer disease, human immunodeficiency virus, cocaine addiction, and pulmonary hypertension. It is likely that decreased vascular CREB content across the different rodent models presented in this article is also the consequence of multiple mechanisms. First, CREB is a self-regulating protein, such that loss of upstream signaling to CREB can result in decreased CREB expression. For example, depletion of cyclic nucleotides and decreased protein kinase A/protein kinase G activation decreases CREB expression. The loss of endothelial nitric oxide synthase-stimulated cyclic nucleotide accumulation in endothelial dysfunction results could thus contribute to decreased vascular CREB expression. Another mechanism is suggested by our previous finding that platelet-derived growth factor induces an Akt/CK2-mediated ubiquitination and degradation of CREB in pulmonary VSMC. Since expression of platelet-derived growth factor and its receptor are increased in models of atherosclerosis, this mechanism could also occur in the systemic vasculature. It is probable that generation of ROS is the major underlying contributor to CREB downregulation across vascular disease models. ROS have been shown to disrupt signaling to CREB and to promote CREB degradation in Alzheimer disease. We have reported downregulation of CREB in aortic VSMC, neurons, and beta cells in response to direct oxidant stress. In further support of this hypothesis, we now report that the potent cardiovascular risk factor, oxLDL, directly induces CREB downregulation in VSMC in vitro. In cell culture, simple screening with pharmacological inhibitors indicates that generation of ROS and activation of ERK, but not protein kinase A or Akt, are critical for oxLDL-mediated loss of CREB expression. Another recent report has also suggested that induction of oxidant defense can block effects of LDL on VSMC. A more exhaustive series of studies will be needed to fully define the oxidant-induced signaling pathways important for CREB downregulation.

Loss of vascular medial CREB expression appears to be an early response of the vessel wall to cardiovascular risk exposure; as such, interventions aimed at restoring or maintaining vascular CREB are an appealing goal. Lifestyle interventions demonstrated to improve longevity and decrease cardiovascular risk also increase CREB function. In the central nervous system, exercise training enhances neural nitric oxide synthase expression, leading to induction of CREB. Furthermore, exercise increases active CREB and contributes to improved recovery from ischemia, stroke, and memory tasks after seizure. Preliminary observations indicate that age-related decrease in aortic CREB protein content can be abrogated by caloric restriction (Reusch et al, unpublished observations 2009). Watson et al also recently reported increased cardiac CREB content with modest exercise training in a rodent model of heart failure. Unfortunately, the compensation for VSMC CREB deficiency is not as easy as adding CREB to the vessel wall, because inflammatory cell differentiation also uses CREB (lumen in Figure 2). Thus, interventions aimed at restoring CREB likely will need to be directed at the underlying mechanisms of CREB loss and will require a better understanding of these mechanisms. Interestingly, thiazolidinediones, agents that increase insulin sensi-
tivity have also been found to increase CREB content in the heart and blood vessels of the insulin-resistant ob/ob mouse model (Reusch, unpublished observations). In an Alzheimer disease model, blocking CREB degradation with an inhibitor of ubiquitin 1 ligase led to increased CREB expression and improved cognition. Overall, clarification of the mechanisms of CREB loss in vascular disease may suggest novel therapeutic targets for vascular intervention and augmentation of CREB-dependent vascular defenses.

Conclusion

In summary, we report loss of CREB expression across a spectrum of rodent models of vascular risk, including insulin resistance, aging, and high-fat diet. The relevance of oxLDL to the in vivo vascular CREB depletion model is supported by the loss of CREB on direct challenge of VSMC with oxLDL in vitro. Simple analysis of critical pathways reveals that blockade of ROS generation or ERK activation protects cells from CREB depletion and from oxLDL toxicity. This in vitro model will allow further analysis of the mechanism of oxLDL-mediated CREB loss in VSMC. These experiments provide new information on a common response to vascular injury that may be an early event in atherosclerosis and is a potential new target for cardiovascular disease intervention.

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CREB Downregulation in Vascular Disease: A Common Response to Cardiovascular Risk
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Materials and Methods:

Chemicals and Reagents

Fetal bovine serum, L-glutamine, and penicillin/streptomycin were purchased from Gemini Bio-Products (Cabassas, CA). Dulbecco’s Modified Eagle’s media, Hams F-12 nutrient mixture and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). The trypsin/EDTA was purchased from Life Technologies (Grand Island, NY). LDL and oxLDL were purchased from Intracel Resources (Fredrick, MD). A non-essential amino acid solution, DAPI, bovine serum albumin (BSA), fatty acid free low endotoxin BSA, β-Actin antibody and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to CREB, phospho-CREB, phospho-ERK, phospho-p38, phospho-JNK, phospho-Akt and anti-rabbit and anti-mouse IgG alkaline phosphatase-linked antibodies were from Cell Signaling Technology (Danvers, MA). SB-203580 (SB), SP-600125 (SP), U-0126 (U0), Wortmannin (WORT) and H-89 were purchased from BIOMOL International (Plymouth Meeting, PA). Bisindolyl maleimide (BIS) is from Calbiochem (San Diego, CA). N-acetyl cysteine (NAC) is from Sigma (St. Louis, MO). The Cy3 AffiniPur Donkey Anti-Mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). CDP-Star reagent kit was purchased from New England BioLabs (Ipswitch, MA).

Animals

Archived aorta samples were procured through collaborations in order to assess an array of disease states. Aortas from C57/B6 (3,12,24 months) were generated in collaboration with Dr. M. Levi (University of Colorado, Denver, CO); aortas from Sprague-Dawley rats (4-month and 14-month old females) were harvested in collaboration with Dr. M. Levi (University of Colorado, Denver, CO); aortas from spontaneous hypertensive heart failure (SHHF) rats were donated by Dr. S. McCune (University of Colorado, Boulder, CO).
Six- to 8-week old female LDL-receptor-null (LDLR-/-) mice bred onto a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) and wild-type C57BL/6 mice were fed ad libitum chow diets (Wayne Rodent BLOX, 4% fat (wt/wt), 0.04% cholesterol) or “Western” diets high in saturated fat (21%), with added cholesterol (0.15% ) for 10 weeks. All diets were purchased from Harlan-Teklad, Madison, WI, and all percentages are weight/weight. Aortas were collected from LDLR-/- mice and controls as previously described 1.

All animals were housed in temperature controlled rooms, allowed unrestricted access to food and water and maintained on a 12-hour light/dark cycle. Animal studies were conducted under Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and were approved by individual Institutional Animal Care and Use Committees.

**Tissue processing of aortic lysates**

Following termination, aortic tissue was taken from animals and immediately frozen in liquid nitrogen. Samples were homogenized in Mammalian Protein Extraction Reagent (M-PER) lysis buffer (Thermo-Scientific, Rockford, IL) and cellular debris was pelleted by centrifugation. Protein concentrations in these extracts were quantified by Bradford protein assay.

**Cell Culture**

Primary culture rat vascular smooth muscle cells (VSMC) were isolated from aortas of Sprague-Dawley rats as previously described 2. All experiments used cells within passages 4 - 10. Cells were expanded in a 10% fetal bovine serum (FBS) media that consisted of high glucose (4.5 g/L) DMEM:Hams F-12 nutrient solution (1:1) plus L-glutamine, non-essential amino acids, penicillin and streptomycin. When cells reached 50-75% confluency, they were serum-deprived in 1:1 DMEM:Ham’s F-12 with 0.1% FBS for 48 hours before each experiment. After serum deprivation the cells were treated with reagents and pharmacological inhibitors as noted in the
text or figure legends. Cells were either fixed and stained for immunocytochemistry or harvested in 1x LSB containing BME or 1x mammalian lysis buffer (MLB,: 150mM NaCl, 1mM EDTA, 1mM EGTA, 5mM sodium pyrophosphate, 1mM sodium orthovandadate, 20mM sodium fluoride, 500mM okadaic acid, protease inhibitor cocktail p8340 (Sigma-Aldrich), M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL)) for Western blot analysis.

**Tissue processing for immunohistochemistry**

Aortas were collected from LDLR-/- mice and controls as previously described. After 10 weeks of diet treatment, mice were euthanized by cervical dislocation, and the heart and aorta were removed and perfusion-fixed with 10% formalin. All data regarding lipids and atherosclerosis burden are as reported previously.

**Immunohistochemistry**

A. Aortic sinus immunohistochemistry. Single-label immunohistochemistry was performed on murine aortic sinuses using procedures described in detail previously. VSMC were detected using a mouse monoclonal antibody against α-actin (titer 1:1,000; Sigma St Louis, MO). CREB was detected using a mouse monoclonal Ab (titer 1:50; Cell Signaling, Danvers, MA, State). Nova Red (Vector Laboratories) was used as the peroxidase substrate to yield a red-brown reaction product; cell nuclei were identified by counterstaining with hematoxylin. Quantification of medial VSMC nuclear CREB expression was performed by manual counting of positively- and negatively-stained nuclei from an average of 798 nuclei for C57BL/6 and an average of 677 nuclei for LDLR -/- by an operator (TM) blinded to treatment group.

B. Immunohistochemistry of cultured VSMC. Cells were grown on 8-well Poly-D-lysine/laminin treated glass slides (BD Biosciences, Bedford, MA), serum starved for 48 hours and treated with LDL or oxLDL. After treatment cells were fixed with 4% paraformaldehyde and
permeabilized (5% BSA/0.2% Triton X-100/PBS). They were then incubated with an anti-CREB antibody (mouse monoclonal, 1:500) followed by a Cy3-tagged anti-mouse IgG antibody (1:500). Cells were also incubated with DAPI (1:500) in order to visualize the nuclei. Photographs were taken using a Zeiss Axioplan 2 EPI Fluorescence up right microscope (Carl Zeiss, Maple Grove, MN). All images were digitally captured with a Cooke Sensicam QE high resolution (1376 x 1024 resolution) black and white super-cooled CCD camera. The digital images were then assigned colors by the software interface, SlideBook (Intelligent Imaging Innovations, Inc, Denver, CO).

**LDL/oxLDL treatment**

Each individual lot of oxLDL was assessed for potency. Cell cultures were serum-starved with DMEM:Ham’s F-12 + 0.1% fetal bovine serum for 48 hours and then treated with 10-100 μg/ml LDL or oxLDL Intracel Resources (Fredrick, MD) in the same medium. After 24 hours of exposure, the health of the cells was visually ascertained by shape, adherence, and granularity. The concentration that caused morphological change, without significant cell or protein loss and induced a ≥ 70% reduction in CREB protein at 24 hours was used during all experiments treated with each characterized lot of oxLDL. LDL treatment was always with 100 μg/ml LDL.

**Western Blot Analysis**

Protein content was assessed using a Bradford analysis protocol (3). Equal protein or equal volume samples were run on SDS 12%-polyacrylamide gels. In large experiments where more than one gel was required, a sample of a large volume control extract was run on each gel to allow normalization to a common control band. This allowed quantitation and comparison between gels for any gels loaded with the same batch of control extract. Following electrophoresis, proteins were transferred from the gels to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in a 5%milk/TBST solution and incubated with primary
antibodies diluted in 5% BSA in TBST. The membranes were then incubated with anti-rabbit or anti-mouse IgG alkaline phosphatase-linked antibodies. An alkaline phosphatase-based chemiluminescent detection assay (CDP Star, New England BioLabs, Ipswitch, MA) was used, and membranes were exposed to Kodak scientific imaging film. Films were quantitatively evaluated by densitometry with Quantity One software by Bio-Rad (Hercules, CA). For probing with multiple antibodies with targets in the same MW range, blots were stripped between antibodies for 15 minutes at 55°C in TBST. To control for actual protein loading, blots were last probed with anti-β-actin antibody. If β-actin signal varied, experimental signals were normalized to β-actin. If between gel comparisons or averages were to be performed experimental signals were normalized to corresponding to control extract signals.

**CREB mRNA Quantification**

Cells were grown in 6-well plates to 50-60% confluency and then serum starved for 24 hrs. After 24 hours of exposure to 0.1 mg/ml oxLDL, media was removed and cells were washed with ice cold PBS. 1ml of Trizol reagent (Invitrogen) was applied directly to each well and RNA extraction was followed according to manufacturer’s protocol up to chloroform extraction. RNA cleanup was done using a Qiagen RNeasy Mini Kit. RT-PCR was run by the University of Colorado Cancer Center PT-PCR core for CREB mRNA and normalized to 18S rRNA using primers and probes from ABI on an ABI 7900. Control and exposed samples were done in quintuplicate.

**Statistical Analysis**

Values shown are mean ± SEM or SD as indicated in figure legends. Significance of differences between means was assessed by Student’s t test or one way ANOVA with post test for multiple comparisons (Dunnett’s test for comparison to one control or Bonferroni correction for pairwise
comparisons). Significant relationships were analyzed by multiple linear regression analyses. Probability values with $p<0.05$ were considered statistically significant.

**Materials and Methods:**

**Chemicals and Reagents**

Fetal bovine serum, L-glutamine, and penicillin/streptomycin were purchased from Gemini Bio-Products (Cabassas, CA). Dulbecco’s Modified Eagle’s media, Hams F-12 nutrient mixture and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). The trypsin/EDTA was purchased from Life Technologies (Grand Island, NY). LDL and oxLDL were purchased from Intracel Resources (Fredrick, MD). A non-essential amino acid solution, DAPI, bovine serum albumin (BSA), fatty acid free low endotoxin BSA, β-Actin antibody and N-Acetyl-L-cysteine (NAc) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to CREB, phospho-CREB, phospho-ERK, phospho-p38, phospho-JNK, phospho-Akt and anti-rabbit and anti-mouse IgG alkaline phosphatase-linked antibodies were from Cell Signaling Technology (Danvers, MA). SB-203580 (SB), SP-600125 (SP), U-0126 (U0), Wortmannin (WORT) and H-89 were purchased from BIOMOL International (Plymouth Meeting, PA). Bisindolyl maleimide (BIS) is from Calbiochem (San Diego, CA). N-acetyl cysteine (NAC) is from Sigma (St. Louis, MO). The Cy3 AffiniPur Donkey Anti-Mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). CDP-Star reagent kit was purchased from New England BioLabs (Ipswitch, MA).

**Animals**

 Archived aorta samples were procured through collaborations in order to assess an array of disease states. Aortas from C57/B6 (3,12,24 months) were generated in collaboration with Dr. M. Levi (University of Colorado, Denver, CO); aortas from Sprague-Dawley rats (4-month and 14-month old females) were harvested in collaboration with Dr. M. Levi (University of Colorado, Denver, CO); aortas from spontaneous hypertensive heart failure (SHHF) rats were donated by Dr. S. McCune (University of Colorado, Boulder, CO).
Six- to 8-week old female LDL-receptor-null (LDLR-/-) mice bred onto a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) and wild-type C57BL/6 mice were fed ad libitum chow diets (Wayne Rodent BLOX, 4% fat (wt/wt), 0.04% cholesterol) or “Western” diets high in saturated fat (21%), with added cholesterol (0.15% ) for 10 weeks. All diets were purchased from Harlan-Teklad, Madison, WI, and all percentages are weight/weight. Aortas were collected from LDLR-/- mice and controls as previously described 1.

All animals were housed in temperature controlled rooms, allowed unrestricted access to food and water and maintained on a 12-hour light/dark cycle. Animal studies were conducted under Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and were approved by individual Institutional Animal Care and Use Committees.

Tissue processing of aortic lysates
Following termination, aortic tissue was taken from animals and immediately frozen in liquid nitrogen. Samples were homogenized in Mammalian Protein Extraction Reagent (M-PER) lysis buffer (Thermo-Scientific, Rockford, IL) and cellular debris was pelleted by centrifugation. Protein concentrations in these extracts were quantified by Bradford protein assay.

Cell Culture
Primary culture rat vascular smooth muscle cells (VSMC) were isolated from aortas of Sprague-Dawley rats as previously described 2. All experiments used cells within passages 4 - 10. Cells were expanded in a 10% fetal bovine serum (FBS) media that consisted of high glucose (4.5 g/L) DMEM:Hams F-12 nutrient solution (1:1) plus L-glutamine, non-essential amino acids, penicillin and streptomycin. When cells reached 50-75% confluency, they were serum-deprived in 1:1 DMEM:Ham’s F-12 with 0.1% FBS for 48 hours before each experiment. After serum deprivation the cells were treated with reagents and pharmacological inhibitors as noted in the
Cells were either fixed and stained for immunocytochemistry or harvested in 1x LSB containing BME or 1x mammalian lysis buffer (MLB; 150mM NaCl, 1mM EDTA, 1mM EGTA, 5mM sodium pyrophosphate, 1mM sodium orthovanadate, 20mM sodium fluoride, 500mM okadaic acid, protease inhibitor cocktail p8340 (Sigma-Aldrich), M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL)) for Western blot analysis.

Tissue processing for immunohistochemistry
Aortas were collected from LDLR-/- mice and controls as previously described. After 10 weeks of diet treatment, mice were euthanized by cervical dislocation, and the heart and aorta were removed and perfusion-fixed with 10% formalin. All data regarding lipids and atherosclerosis burden are as reported previously.

Immunohistochemistry
A. Aortic sinus immunohistochemistry. Single-label immunohistochemistry was performed on murine aortic sinuses using procedures described in detail previously. VSMC were detected using a mouse monoclonal antibody against α-actin (titer 1:1,000; Sigma St Louis, MO). CREB was detected using a mouse monoclonal Ab (titer 1:50; Cell Signaling, Danvers, MA, State). Nova Red (Vector Laboratories) was used as the peroxidase substrate to yield a red-brown reaction product; cell nuclei were identified by counterstaining with hematoxylin. Quantification of medial VSMC nuclear CREB expression was performed by manual counting of positively- and negatively-stained nuclei from an average of 798 nuclei for C57BL/6 and an average of 677 nuclei for LDLR -/- by an operator (TM) blinded to treatment group.

B. Immunohistochemistry of cultured VSMC. Cells were grown on 8-well Poly-D-lysine/laminin treated glass slides (BD Biosciences, Bedford, MA), serum starved for 48 hours and treated with LDL or oxLDL. After treatment cells were fixed with 4% paraformaldehyde and
permeabilized (5% BSA/0.2% Triton X-100/PBS). They were then incubated with an anti-CREB antibody (mouse monoclonal, 1:500) followed by a Cy3-tagged anti-mouse IgG antibody (1:500). Cells were also incubated with DAPI (1:500) in order to visualize the nuclei. Photographs were taken using a Zeiss Axioplan 2 EPI Fluorescence up right microscope (Carl Zeiss, Maple Grove, MN). All images were digitally captured with a Cooke Sensicam QE high resolution (1376 x 1024 resolution) black and white super-cooled CCD camera. The digital images were then assigned colors by the software interface, SlideBook (Intelligent Imaging Innovations, Inc, Denver, CO).

**LDL/oxLDL treatment**

Each individual lot of oxLDL was assessed for potency. Cell cultures were serum-starved with DMEM:Ham’s F-12 + 0.1% fetal bovine serum for 48 hours and then treated with 10-100 μg/ml LDL or oxLDL Intracel Resources (Fredrick, MD) in the same medium. After 24 hours of exposure, the health of the cells was visually ascertained by shape, adherence, and granularity. The concentration that caused morphological change, without significant cell or protein loss and induced a ≥ 70% reduction in CREB protein at 24 hours was used during all experiments treated with each characterized lot of oxLDL. LDL treatment was always with 100 μg/ml LDL.

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