Oxidative stress regulates adipocyte apolipoprotein E and suppresses its expression in obesity

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ABSTRACT

Objective: Endogenous expression of apoE has a significant impact on adipocyte lipid metabolism and is markedly suppressed in obesity. Adipose tissue oxidant stress is emerging as an important mediator of adipocyte dysfunction. These studies were undertaken to evaluate the role of oxidant stress for regulation of adipocyte apoE.

Research Design and Methods: ApoE gene and protein expression in 3T3-L1 adipocytes, or mature adipocytes and adipose tissue from C57/Bl6 mice was evaluated after induction of oxidant stress. The response of adipose tissue and adipocytes from obese compared to lean mice to antioxidants was also assessed.

Results: Oxidant stress in 3T3-L1 cells, or adipocytes and adipose tissue from lean mice significantly reduced apoE mRNA and protein level. Inclusion of an antioxidant eliminated this reduction. Oxidant stress was accompanied by activation of the NF-κB transcription complex and its effect on apoE was eliminated by an NF-κB activation inhibitor. Treatment of freshly isolated adipose tissue or mature adipocytes from obese mice with antioxidant increased apoE expression but had no effect on cells or tissue from lean mice. Incubation of freshly isolated adipocytes from lean mice with stromovascular cells from obese mice significantly suppressed adipocyte apoE compared to incubation with stromovascular cells from lean mice, but this suppression was reversed by inclusion of antioxidant or a neutralizing antibody to TNFα.

Conclusions: Oxidant stress significantly modulates adipose tissue and adipocyte apoE expression. Further, oxidant stress contributes to suppression of adipocyte apoE in obesity. This suppression depends on interaction between adipose tissue stromovascular cells and adipocytes.
Obesity is widely recognized as an increasingly prevalent cause of metabolic and cardiovascular disease (1;2). It has also been recently appreciated that obesity is associated with a chronic inflammatory reaction in adipose tissue and that this inflammation is closely associated with metabolic and cardiovascular risk (1;3;4). Adipose tissue from obese animals or humans is characterized by the influx of inflammatory cells, primarily macrophages, into its stromovascular compartment with increased local production of pro-inflammatory cytokines (5-8). There is also a concomitant increase in the production of reactive oxygen species (ROS) in adipose tissue (9). The localized inflammation with oxidative stress in adipose tissue leads to important changes in adipocyte gene expression with downstream effects on adipocyte lipid metabolism and triglyceride content. Adipose tissue inflammation and oxidant stress also produce a systemic increase in circulating inflammatory cytokines and ROS with adverse effects on systemic insulin action and substrate metabolism (1;9;10).

Mature adipocytes and macrophages express a number of proteins in common and one of these is apolipoprotein E. In macrophages, the endogenous expression of apoE functions primarily to facilitate lipid flux (11;12). However, macrophage-derived apoE in the arterial wall has also been associated with local anti-inflammatory and antioxidant effects (13-15). ApoE is also highly expressed in hepatocytes and steroidogenic cells (16-18). Like macrophages, these two cell types experience high lipid flux related to their differentiated functions of lipoprotein metabolism and steroid hormone secretion respectively. Adipocytes also experience high lipid flux as part of their differentiated function and high level expression of apoE was first noted by Zechner et al (19). More recently, an important role for endogenously expressed adipocyte apoE for modulating adipocyte lipid and lipoprotein metabolism has been established (20).

Comparing freshly isolated or cultured adipocytes from apoE-/- mice to those from wild-type mice demonstrated that adipocytes from the former were smaller, contained less lipid, synthesized less triglyceride and had increased rates of triglyceride hydrolysis (20). Further, these differences were maintained after differentiation of preadipocytes to adipocytes followed by longterm culture in apoE-containing serum. Adipose tissue from apoE-/- mice also accumulated less triglyceride after incubation with apoE-rich VLDL compared to that from wild-type mice, and expressed higher levels of genes involved in fatty acid oxidation. These observations support a role for endogenously expressed apoE in overall adipocyte lipid metabolism. This notion is further supported by physiologically relevant regulation of adipocyte apoE. Adipocyte apoE is increased by the systemic administration of PPARγ agonists and decreased by the systemic administration of the pro-inflammatory peptide angiotensin II (21;22). Furthermore, diet-induced or leptin-deficient obesity leads to marked suppression of adipocyte apoE (23). In addressing potential mechanisms for this suppression in obesity we have previously shown that TNFα, a pro-inflammatory cytokine produced by inflammatory adipose tissue macrophages in obesity, suppresses adipocyte apoE expression (21). Emerging evidence has identified adipose tissue ROS as important effectors producing adipocyte dysfunction in obesity (9). The current studies were undertaken to evaluate a role for oxidative stress in regulating adipocyte apoE expression and its role in mediating the reduced adipocyte apoE expression observed in obesity.
RESEARCH DESIGN AND METHODS

Materials. Cell culture media, fetal bovine serum (FBS) and antibiotics were purchased from Invitrogen (Carlsbad, CA). Goat-derived apoE antiserum was from International Immunology (Murrieta, CA). Phospho-Ikappa Bα and Ikappa Bα antibodies were from Cell Signaling Tech (Danvers, MA). TNFα neutralizing antibody was from Biovision (Mountain View, CA). The NF-κB activation inhibitor, 6-amino-4-(4a-phenoxyphenylethylamino) quinazoline (QNZ) was purchased from Calbiochem. Liberase Blendzyme 3 was from Roche. Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), hydrogen peroxide, N-acetyl-L-cysteine (NAC), xanthine oxidase, hypoxanthine, glucose oxidase and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO).

Cell culture and isolation of primary adipocytes. 3T3-L1 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in 10% FBS supplemented Dulbecco’s modified Eagle’s medium (DMEM) with penicillin and streptomycin in a 5% CO2 incubator at 37°C. Two days post confluence, cells were differentiated by incubating in differentiation medium containing 0.5 mM IBMX, 0.2 μM dexamethasone and 10μg/ml insulin. Three days after addition of this differentiation cocktail, cells were placed in DMEM containing 10μg/ml insulin and 10% FBS. All experiments were performed 10 days post differentiation.

Male C57Bl/6J (10-12 weeks old) mice, male ob/ob (10-12 weeks old) mice, or their lean littermates were from Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Illinois, Chicago. Intraabdominal fat pads were harvested, washed with sterile PBS, and separated into two parts. One part was used for tissue incubation and the other part was used for isolation of mature adipocytes and the stromovascular fraction. For tissue incubation, fat pads were minced into 1-2 mm pieces under sterile conditions and incubated in DMEM containing 0.5% BSA and treated as described in the figure legends. For isolation of mature adipocytes and the stromovascular fraction, fat pads were cut into 1-2 mm pieces and digested using 0.5 mg/ml Liberase Blendzyme 3 in DMEM for 1h at 37°C in a shaking water bath. After digestion, cells were centrifuged at 300g for 5 min and the suspended mature adipocytes were separated from the pelleted stromovascular fraction for experiments. For some experiments, mature adipocytes from lean mice were incubated in serum free DMEM containing 0.5% BSA with 1.0 X 10^6 stromovascular cells from either lean or ob/ob mice. After 4h, stromovascular cells were pelleted and floating adipocytes were collected for measurement of apoE mRNA. Twenty mM NAC, 4 μg/ml anti- TNF antibody or both were added during some incubations as indicated in the figure legends. For some experiments, the stromovascular fraction from adipose tissue harvested from the subcutaneous space of ob/ob mice was isolated as described above.

ApoE mRNA quantitation. Total RNA was isolated from adipose tissue, floating adipocytes, stromovascular cells or 3T3-L1 adipocytes using RNeasy mini kit (Qiagen, Valencia, CA). First strand cDNA was synthesized from 1 μg of total RNA using Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Real time PCR was performed on each sample using the Mx3000p Quantitative PCR system (Stratagene, La Jolla, CA) using iTaq SYBR® Green Supermix with ROX. The relative quantity of apoE mRNA was calculated after correction for β-actin mRNA abundance and was expressed for each experiment as fold change compared to the experimental control.
Oxidative stress and adipocyte apoE gene expression was evaluated in 3T3-L1 cells. Oxidative stress was modeled by exogenous addition of hydrogen peroxide. Incubation of 3T3-L1 cells with this agent for only 10 minutes reduced apoE mRNA level by approximately 50% in cells harvested 4 and 18 hr after treatment (Fig. 1A). Endogenous cellular production of ROS was induced by incubating cells for 18 hr with glucose oxidase or xanthine oxidase (26;27). These incubations increased the production of endogenous cellular ROS to 21.5 ± 1.9 and 14.9 ± 1.5 μM/well respectively, compared to 6.0 ± 0.8 μM/well in control cells (p < 0.05 for control compared to glucose oxidase or xanthine oxidase). Incubation with either agent also significantly reduced apoE mRNA level in adipocytes (Fig. 1B and C). NAC is an antioxidant compound previously shown to mitigate the effect of ROS on adipocyte gene expression in vitro and in vivo (9,28). Treatment of cells with NAC alone had no

Western blotting: Total protein was extracted from cells or tissue using RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X, 20 mM Tris base, 150 mM NaCl and 5 mM EDTA), supplemented with protease inhibitor cocktail. Samples were centrifuged for 5 min, the top fat layer was discarded and the middle clear layer of solubilized protein was collected. Total protein concentration was analyzed using Biorad DC protein assay. Fifty micrograms of protein for each sample was subjected to SDS-PAGE analysis, transferred to nitrocellulose and probed with antibodies for apoE, IκBα phosphorylated IκBα, or β-actin. Western blot images were quantitated using ImageQuant TL software (GE Healthcare, Piscataway, NJ) using β-actin as an internal loading control.

NF-κB pathway involvement. NF-κB pathway activation was first assessed by detecting the level of IκBα phosphorylation. Following treatment of 3T3-L1 adipocytes with 1 mM H2O2 for 0, 1, 5 or 10 min, cells were lysed in the presence of phosphatase inhibitor cocktail. Fifty micrograms of total cell extract was subjected to western blot analysis using an antibody specific for IκBα phosphorylated at serine 32/36. Blots were stripped and re-probed with an antibody to total IκBα protein. The involvement of the NF-κB pathway was also assessed by adding QNZ (an inhibitor of NF-κB complex activation) to cells in the presence or absence of 1 mM H2O2.

Measurement of cellular H2O2. Cellular ROS was measured using fluorescent dye CM-H2DCFDA[5(-and-6)-chloromethyl-2′7′-dichlorohydrofluorescein deacetate acetyl ester] (Molecular Probes) as previously described with minor modifications (24). After treatment with glucose oxidase or xanthine oxidase, differentiated adipocytes were washed with phenol free DMEM and incubated with 2 μM CM-H2DCFDA for 45 min at 37°C. Fluorescence was analyzed using BIOTEK Synergy H Fluorescent plate reader at an excitation wavelength of 485 nm and emission at 530 nm. Cellular ROS concentration was quantitated using a H2O2 standard curve.

Statistical analysis. Statistical differences between experimental groups were evaluated using Student’s two-sample t-test. P values of < 0.05 were considered significant. All data are expressed as mean ± SD of the sample replicate number indicated in the figure legends.

RESULTS

In the first series of experiments, we evaluated the impact of oxidative stress on adipocyte apoE gene expression in 3T3-L1 cells. Oxidative stress was first modeled by the exogenous addition of hydrogen peroxide (9;25). Incubation of 3T3-L1 cells with this agent for only 10 minutes reduced apoE mRNA level by approximately 50% in cells harvested 4 and 18 hr after treatment (Fig. 1A). Endogenous cellular production of ROS was induced by incubating cells for 18 hr with glucose oxidase or xanthine oxidase (26;27). These incubations increased the production of endogenous cellular ROS to 21.5 ± 1.9 and 14.9 ± 1.5 μM/well respectively, compared to 6.0 ± 0.8 μM/well in control cells (p < 0.05 for control compared to glucose oxidase or xanthine oxidase). Incubation with either agent also significantly reduced apoE mRNA level in adipocytes (Fig. 1B and C). NAC is an antioxidant compound previously shown to mitigate the effect of ROS on adipocyte gene expression in vitro and in vivo (9,28). Treatment of cells with NAC alone had no
effect on apoE mRNA or protein level in 3T3-L1 cells (Fig. 2A-C). However, inclusion of NAC with either hydrogen peroxide or glucose oxidase eliminated significant down-regulation of apoE expression.

The NF-κB pathway has been identified as an important mediator of oxidant stress on adipocyte apoE gene expression. We next evaluated the participation of this pathway for the effect of oxidant stress on the adipocyte apoE gene using two complementary approaches. Activation of the NF-κB transcription complex requires phosphorylation of the inhibitory IκBα subunit at ser 32/36. This phosphorylation triggers IκBα proteosomal degradation with subsequent activation of the NF-κB transcription complex. Treatment of 3T3-L1 cells with hydrogen peroxide for 5 min increased the cellular level of phosphorylated IκBα (designated IκBα-P in the upper panel of Fig. 3A). After 10 min, there was a significant decrease in total cellular IκBα consistent with its increased degradation. These results indicate that treatment of 3T3-L1 adipocytes with hydrogen peroxide at the dose and time utilized for our experiments was sufficient for activation of the NF-κB transcription complex. To further evaluate the importance of the NF-κB pathway for the apoE gene response, we evaluated the effect of QNZ, an inhibitor of NF-κB activation (29). Addition of QNZ alone had no effect on apoE mRNA level in 3T3-L1 adipocytes (Fig. 3B). Inclusion of QNZ with hydrogen peroxide, however, completely eliminated the suppression of apoE gene expression produced by the latter agent.

We next extended our observations to examine the impact of oxidative stress on apoE expression in freshly isolated adipose tissue and mature adipocytes from C57Bl/6J mice (Fig. 4A). Measurement of apoE mRNA level in whole murine adipose tissue, mature adipocytes, and the stromovascular fraction showed that mature adipocytes accounted for the preponderance of apoE message level in adipose tissue, as we have previously reported (20). Treatment of these fractions with hydrogen peroxide for 10 min produced significant reduction of apoE mRNA level in whole adipose tissue and mature adipocytes, completely consistent with results obtained in 3T3-L1 cells. ApoE expression in the stromovascular cell fraction was also reduced by treatment with hydrogen peroxide; however, its overall contribution to adipose tissue apoE mRNA level, and its level of expression compared to mature adipocyte expression was small. We next evaluated the effect of oxidant stress on apoE protein expression in freshly isolated adipose tissue. Adipose tissue was incubated with or without H2O2 for 10 min, and then harvested 18h later for Western blot. H2O2 treatment reduced apoE protein level by over 70% (Fig. 4B).

Obesity leads to increased production of ROS in adipose tissue and we have previously shown that adipose tissue and mature adipocyte apoE expression are reduced in obese mice compared to lean mice. We, therefore, next evaluated a potential role for ROS in contributing to reduced adipose tissue and adipocyte apoE expression in obesity. We approached this question by comparing the impact of treating freshly isolated adipose tissue or mature adipocytes from obese and lean mice with the antioxidant NAC (Fig. 5). As we have previously reported, apoE expression is lower in adipose tissue and adipocytes isolated from ob/ob mice compared to lean littermate controls (23). Treatment of adipose tissue or adipocytes isolated from lean mice with NAC did not significantly impact apoE expression level. However, treatment of adipose tissue or mature adipocytes isolated from obese mice with NAC increased adipose tissue mRNA level by 5-fold and 4-fold respectively. These results indicate that adipose tissue oxidative stress is an important factor contributing to the suppression of adipose tissue and adipocyte apoE in obesity.
Obesity is characterized by an influx of inflammatory cells into the stromovascular compartment of adipose tissue. Signaling between inflammatory cells in the stromovascular compartment and adipocytes has been identified as an important contributor to adipocyte dysfunction in obesity (5-7,30). We next addressed the question of whether the adipose tissue stromovascular fraction from obese mice could modulate the expression of apoE in adipocytes obtained from lean mice (Fig. 6A). Freshly isolated mature adipocytes from lean mice were incubated alone, or with the stromovascular cell fraction from lean mice or from obese mice. After 4h of incubation, apoE mRNA levels were suppressed in the adipocytes incubated with the stromovascular cell fraction from obese mice by over 80%. We next evaluated whether the suppressive effect of the stromovascular fraction was different for stromovascular fractions isolated from visceral or subcutaneous adipose depot of obese mice (Fig. 6B). Addition of the obese stromovascular fraction from either fat depot significantly suppressed adipocyte apoE mRNA level, but the suppression produced by the visceral stromovascular fraction was significantly greater than that produced by the subcutaneous stromovascular fraction.

We performed experiments to determine if the effect of the stromovascular fraction from obese mice was related to the induction of oxidant stress in adipocytes (Fig. 7). Adipocytes from lean mice were incubated alone or with the visceral stromovascular fraction from obese mice plus the additions indicated in the figure. Consistent with the results in Fig. 6A, the obese stromovascular fraction alone produced a greater than 80% suppression of apoE mRNA level. Inclusion of antioxidant NAC during the incubation significantly attenuated the effect of the obese stromovascular fraction on adipocyte apoE expression. Because we have previously demonstrated an important role for TNFα in regulating adipocyte apoE (21;31), we also evaluated the effect of including a neutralizing antibody to TNFα during these incubations. The inclusion of this antibody also opposed the suppression of adipocyte apoE by the obese stromovascular fraction. The inclusion of both the antioxidant and neutralizing antibody restored adipocyte apoE to control level. In separate experiments, we evaluated the effect of the NF-κB inhibitor QNZ on suppression of adipocyte apoE by the obese stromovascular fraction. QNZ alone had no effect on adipocyte apoE mRNA level (1.1 ± 0.2 compared to control). Addition of the obese stromovascular fraction reduced apoE mRNA level to 0.4 ± 0.1 compared to control (p<0.01). Addition of QNZ with the obese stromovascular fraction prevented down-regulation of apoE mRNA level (1.01 ± 0.1). The above results indicate that cross talk between adipocytes and stromovascular cells enhances oxidant stress-related reduction of adipocyte apoE in obesity.

DISCUSSION

The current studies were undertaken to evaluate the importance of oxidative stress for modulating adipocyte apoE expression. Using 3T3-L1 cells, we show that ROS reduce apoE mRNA and protein expression, and that this reduction can be prevented by the general antioxidant NAC (Figs. 1 and 2). Analogous to other adipocyte genes regulated by oxidative stress, these effects on the apoE adipocyte gene are mediated by the NF-κB pathway (Fig. 3). We extend our observations from 3T3-L1 cells to primary cells by showing that ROS also suppress apoE mRNA and protein level in freshly isolated murine adipose tissue and adipocytes (Fig. 4). Using freshly isolated adipose tissue and adipocytes from ob/ob mice and lean littermate controls, we show that the suppression of apoE level that we have previously reported in ob/ob mice could be partially reversed by NAC (Fig. 5). We further show that interaction between the ob/ob stromovascular fraction...
and adipocytes is important for producing the suppression of adipocyte apoE, and that this suppression is reversed by incubation with NAC and a neutralizing antibody to TNFα (Fig. 6). In aggregate, our results establish that oxidant stress regulates adipocyte apoE gene expression, that oxidative stress contributes to the suppression of adipocyte apoE in obesity, and that this suppression in obesity depends on interaction between the adipose tissue stromovascular fraction and adipocytes. Both ROS and TNFα play an important role in mediating the impact of the obese stromovascular fraction on adipocyte apoE, and both appear to utilize NF-κB activation as a final common pathway for suppressing its expression. Both ROS and TNFα (21;31) act directly on adipocytes to regulate apoE gene expression. This is consistent with the interpretation that NAC and TNFα-neutralizing antibodies also act directly on adipocytes during co-incubation with the obese stromovascular fraction to prevent reduction of apoE expression (Fig. 7). However, our experiments do not rule out an additional effect of NAC or TNFα-neutralizing antibodies on stromovascular fraction cells during the co-incubation. Our observations also establish that the stromovascular fraction from the visceral fat depot of obese mice more effectively reduces adipocyte apoE expression compared to that from the subcutaneous depot. This is consistent with observations that in obesity, visceral fat may be the primary contributor to metabolic derangements (1;3).

While high-level expression of apoE in human and murine adipose tissue has been known for some time, its physiologic function has only recently been explored (19;20). Freshly isolated adipocytes from apoE-/- mice are smaller and contain less triglyceride compared to those from wild-type mice despite in vivo exposure to a hyperlipidemic environment. In the apoE-/- mouse model, reduced triglyceride in freshly isolated adipocytes could be due to the absent expression of apoE in adipocytes or, alternatively, to lack of apoE on the surface of circulating lipoproteins. An important role for endogenous adipocyte apoE for modulating adipocyte triglyceride content and adipocyte lipoprotein metabolism is supported by the following observations; a) the difference in lipid content between apoE-/- adipocytes and wild-type adipocytes is maintained after longterm culture in apoE-containing serum, b) in apoE-/- adipocytes incubated with apoE-containing serum the adenoiral-mediated expression of apoE significantly increases adipocyte triglyceride mass, c) freshly isolated adipose tissue from apoE-/- mice accumulates significantly less triglyceride after incubation with apoE-rich VLDL compared to that from wild-type mice (20).

The above observations indicate that endogenous apoE expression is important for adipocyte acquisition of triglyceride from extracellular triglyceride-rich lipoproteins (TGRL). In this way, the level of apoE expression in adipocytes could influence the partitioning of lipid in circulating TGRL between adipocytes and other tissues. For example, reduced TGRL lipid deposition in adipose tissue resulting from reduced adipocyte apoE expression (such as that observed in obesity) could favor lipid delivery to liver and muscle, where deposition of this lipid has been implicated in producing tissue-specific insulin resistance (32-35). We have previously shown that the lipogenic response to PPARγ agonists is defective in apoE-/- adipocytes even when incubated in the presence of apoE-containing serum. Increased adipocyte apoE expression could also, therefore, participate in the expansion of adipose tissue that is observed with administration of PPARγ agonists (36-38). These issues will require further study.

The above considerations underline the importance of integrating adipocyte apoE into an overall model of adipocyte lipid metabolism. As noted above, systemic
administration or treatment of isolated cells with PPARγ agonists increases adipocyte apoE expression (21). Conversely, treatment with the pro-inflammatory peptide angiotensin II reduces adipocyte apoE (22). Diet-induced or leptin-deficient obesity leads to reduced apoE expression and we have previously demonstrated that TNFα reduces adipocyte apoE expression (21;23). The observations in the current manuscript indicate that ROS and oxidant stress present in obesity are an important pathway for suppressing adipocyte apoE expression. This pathway has high pathophysiologic importance given the emerging evidence that oxidative stress in adipose tissue is increased in both obesity and diabetes (9;28); two diseases becoming increasingly prevalent worldwide.

Our observations also provide evidence for an important interaction between adipocytes and adipose tissue stromovascular cells for inducing oxidative stress with reduced apoE expression in adipocytes. This pro-oxidant and pro-inflammatory interaction suggests that interventions that reduce adipose tissue inflammation and oxidant stress will increase adipocyte apoE expression. PPARγ agonists suppress a pro-inflammatory phenotype in macrophages, and produce apoptosis of adipose tissue macrophages (39). In addition, a specific anti-inflammatory effect of PPARγ agonists in adipose tissue has been demonstrated (40;41). PPARγ agonists could therefore increase adipocyte apoE by both a direct effect on the adipocyte apoE gene (21), and by suppressing adipose tissue inflammation.

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REFERENCES


Oxidative stress and adipocyte apoE


FIGURE LEGENDS

Figure 1. Oxidative stress reduces apoE mRNA level in adipocytes. A. 3T3-L1 adipocytes were preincubated for 6h in serum free medium and then treated with 0, 0.5 or 1 mM H2O2 for 10 min. Cells were then incubated in serum free medium for an additional 4h or 18h before being harvested for measurement of apoE mRNA. B. Adipocytes were treated with or without 25mU/ml of glucose oxidase, or C. 10Um/ml xanthine oxidase with 0.6mM hypoxanthine for 18h in serum-free medium. Total RNA was extracted and apoE mRNA level was measured using RT-PCR. Each experiment was performed using triplicate samples and was repeated 3 times with similar results. Results shown are from a representative experiment as mean ± SD.*P<0.001 for the difference compared to untreated control.
Figure 2. NAC abrogates the effect oxidative stress on apoE expression in adipocytes. 3T3-L1 adipocytes in serum free medium were incubated with 20mM NAC for 1h prior to incubation with the indicated oxidative stress agents. A) Cells were treated with 1mM H₂O₂ for 10 min in the presence or absence of NAC and then harvested 4h later for measurement of apoE mRNA. B) Cells were treated with 25mU/ml glucose oxidase (GO) for 18h in the presence or absence of NAC, and then harvested for measurement of apoE mRNA. C) Western blot showing apoE protein expression level in adipocytes treated with 1mM H₂O₂ for 10 minutes in the presence or absence of NAC. Cells were then harvested 18h after treatment with H₂O₂. Signal quantitation is shown in parentheses. Experiments were performed using triplicate samples and repeated 3 times with similar results. Results shown as mean ± SD, *p< 0.002, †p<0.02 for the difference compared to untreated control.
Figure 3. NF-κB pathway mediates the effect of oxidative stress on apoE expression in adipocytes. A) 3T3-L1 adipocytes in serum free medium for 6h were treated with 1mM H₂O₂ for 0, 1, 5 and 10 min. Cells were then immediately lysed in the presence of phosphatase inhibitor cocktail. Fifty micrograms of protein were resolved on SDS-PAGE and probed with phospho-IκBα (Ser 32/36) antibody. The nitrocellulose membrane was stripped and reprobed with anti-IκBα antibody. The blots shown are representative of three separate experiments. B) 3T3-L1 adipocytes were placed in serum free medium for 6h and the preincubated with 100nM NF-κB activation inhibitor QNZ for 1h before treatment with 1mM H₂O₂ for 10 min. Cells were washed and incubated for an additional 4h without additions. The results shown are from a representative experiment performed using triplicate samples and repeated 2 times with similar results. Results shown as mean ± SD , * p< 0.001 for the difference compared to untreated control.
Figure 4. Effect of oxidative stress on apoE mRNA level in freshly isolated adipose tissue, mature adipocytes and stromovascular cells from C57Bl/6J mice. A. Intraabdominal fat pads were isolated and adipose tissue, mature adipocytes and the stromovascular fraction (SVF) were separated and treated with 1 mM H$_2$O$_2$ for 10 minutes. Cells and tissue were washed and incubated in serum free medium for an additional 4h before harvest. All results are expressed as fold change compared to apoE mRNA level in untreated adipose tissue. Results shown are mean ± SD of cells and adipose tissue from 6 mice, *p< 0.005 for the difference compared to untreated control. B. Western blot for the effect of 1mM H$_2$O$_2$ (10 min) on apoE protein expression in isolated adipose tissue harvested 18h after treatment with H$_2$O$_2$. Signal quantitation is shown in parentheses. †p<0.02 for the difference compared to control.
Figure 5. NAC increases apoE mRNA level in freshly isolated adipose tissue and mature adipocytes from ob/ob mice. A. Freshly isolated adipose tissue or B. freshly isolated mature adipocytes from fat pads of ob/ob mice or lean littermate controls were isolated as described in Methods and incubated alone or with 20mM NAC for 4h. Total RNA was extracted and apoE mRNA was measured. Results are expressed as fold change compared to apoE level in untreated lean and are mean ± SD of cells and adipose tissue from 6 mice, *p<0.0005 for comparison of untreated vs. NAC treated. NS = Non Significant.
Figure 6. Importance of stromovascular-adipocyte cross talk for regulating adipocyte apoE expression in obesity.  A. Mature adipocytes from lean mice were incubated alone or with \( 1.0 \times 10^6 \) cells from the stromovascular fraction (SVF) of lean mice or ob/ob mice for 4h as described in Methods. Adipocytes were harvested for measurement of apoE mRNA. B. Mature adipocytes from lean mice were incubated alone or with \( 1 \times 10^6 \) cells isolated from the stromovascular fraction of visceral or subcutaneous fat from ob/ob mice. After 4h, adipocytes were harvested for measurement of apoE mRNA. Results are mean +/- SD from 5 lean mice and are representative of 2 experiments with similar results. *\( p<0.01 \), **\( p<0.001 \).
Figure 7. Role of ROS and TNFα in suppression of adipocyte apoE by the obese stromovascular fraction. Adipocytes from lean mice were incubated alone or with $1.0 \times 10^6$ SVF cells from ob/ob mice alone or with the indicated additions. After 4h, adipocytes were harvested for measurement of apoE mRNA. Results are mean ± SD of adipocytes from 5 lean mice and are representative of 2 experiments with similar results. *$p<0.01$. 

Figure 7