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Advanced Oxidation Protein Products Induce Inflammatory Response and Insulin Resistance in Cultured Adipocytes via Induction of Endoplasmic Reticulum Stress

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Key Words

Advanced oxidation protein products • Adipocyte • Inflammation • Insulin resistance • Endoplasmic reticulum stress • NADPH oxidase

Abstract

Accumulation of advanced oxidation protein products (AOPPs) is prevalent in metabolic syndrome and type 2 diabetes. Adipocyte dysfunction has been recognized as a link between these conditions. To examine the effect of AOPPs on adipocyte perturbation, 3T3-L1 adipocytes were treated with increased levels of AOPPs as seen in these conditions. Exposure of adipocytes to AOPPs induced overexpression of tumor necrosis factor α and interleukin-6. This inflammatory response was completely blocked by nuclear factor-κB inhibitor SN50. AOPPs challenge also impaired insulin signaling, which was partly prevented by SN50. Treatment with AOPPs triggered endoplasmic reticulum (ER) stress, revealed by phosphorylation of PKR-like eukaryotic initiation factor 2α kinase, eukaryotic translational initiation factor 2α, inositol-requiring enzyme 1 and c-jun N-terminal kinase, and by overexpression of glucose regulated protein 78. AOPPs-induced ER stress was mediated by reactive oxygen species (ROS) generated by activation of NADPH oxidase since it was prevented by NADPH oxidase inhibitors or ROS scavenger. Treating the cells with inhibitors of NADPH oxidase or ER stress could completely abolish AOPPs-induced overexpression of adipocytokines and insulin resistance, suggesting that AOPPs induced adipocyte perturbation probably through induction of ROS-dependent ER stress. Our data identified AOPPs as a class of important mediator of adipocyte perturbation. Accumulation of AOPPs might be involved in adipocyte dysfunction as seen in metabolic syndrome and type 2 diabetes.

Introduction

Metabolic syndrome, defined as a cluster of risk factors for type 2 diabetes mellitus, cardiovascular disease and chronic kidney disease, is well-recognized health and economic burdens [1, 2]. The individual risk factors constituting metabolic syndrome include insulin resistance, hypertension, dyslipidemia, and visceral obesity. Recently,

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low-grade inflammation has been recognized as the characteristic feature of the metabolic syndrome since increased level of plasma C-reactive protein, a marker of inflammation, has been demonstrated in this condition [3] and linked to the development of cardiovascular disease [4, 5] and type 2 diabetes mellitus [6] in this patient population.

In addition to the systemic inflammation, local inflammation in fat tissue has been shown in the animal models of obesity [7-9]. Adipocytokines, a number of biologic active compounds secreted by adipocytes, are associated with systemic inflammation in obese subjects [10] and to play an important role in pathogenesis of insulin resistance, type 2 diabetes mellitus and cardiovascular disease [11-15]. However, the factors that may trigger fat inflammation in obesity remain to be determined, and the mechanisms underlying the adipocyte dysfunction have not been completely understood.

Accumulation of advanced oxidation protein products (AOPPs) has been found in subjects with obesity, metabolic syndrome as well as in diabetic patient with or without microvascular complications [16-19]. AOPPs are the dityrosine-containing and cross-linking protein products formed during oxidative stress by reaction of plasma protein with chlorinated oxidants. The plasma concentration of AOPPs closely correlated with the level of dityrosine, a hallmark of oxidized protein, and pentosidine, a marker of protein glycoxidation that is tightly related to oxidative stress. Thus, AOPPs have been considered as the markers of oxidant-mediated protein damage. Our recent studies have found that chronic accumulation of AOPPs promotes inflammation in diabetic and non-diabetic kidney [20, 21] and worsen inflammation and oxidative stress in artery in a hyperlipidemic model [22]. These data suggest that the oxidized proteins, by themselves, may contribute to the persistent oxidative stress as a class of pro-inflammatory mediators in the tissue. However, it is not clear whether AOPPs accumulation affects adipocyte function.

The present study was conducted to test the pathobiological effect of AOPPs on the cellular functions of cultured adipocytes. Our data showed that exposure of adipocyte to AOPPs induced overexpression of TNFα and IL-6 through activation of nuclear factor-κB (NF-κB). AOPPs also induced insulin resistance in adipocytes, which was partly mediated by AOPPs-induced inflammatory responses. AOPPs-induced adipocyte perturbation was associated with endoplasmic reticulum (ER) stress which was induced by NADPH oxidase-dependent generation of reactive oxygen species (ROS). These data provided new information for understanding the molecular basis of adipocyte dysfunction under the conditions of AOPPs accumulation, such as obesity and metabolic syndrome.

Materials and Methods

AOPPs preparation

AOPPs-mouse serum albumin (MSA) was prepared as described previously [23, 24]. Briefly, MSA (100mg/ml, Sigma, St. Louis, MO) was exposed to 200 mmol/L of HOCl (Fluke, Buchs, Switzerland) for 30 min and dialyzed overnight against PBS to remove free HOCl. The ratio of MSA to HOCL has been tested previously and the optimal ratio (MSA to HOCL = 1:134) was selected. The AOPPs preparation was passed through a Detoxi-Gel column (Pierce, Rockford, IL, USA) to remove any contaminated endotoxin. Endotoxin levels in the preparation were determined with the amebocyte lysate assay kit (Sigma) and were found to be below 0.025 EU/ml. AOPPs content in the preparation was determined as described previously. The content of AOPPs was 72.4 ± 9.8 nmol/mg protein in prepared AOPPs-MSA and 0.2 ± 0.02 nmol/mg protein in native MSA.

Culture of adipocytes

Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) at passages 3 to 8 were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (HyClone, Logan, UT), penicillin (200 U/ml), and streptomycin (200 μg/ml) in a humidified atmosphere (95% air and 5% CO_2). Two days after complete confluence, differentiation was initiated by the adipogenic agents (1 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1- methylxanthine) in DMEM containing 10% FBS. After 48 hours, cells were switched to post-differentiation medium and grown in DMEM containing 10 μg/ml of insulin and 10% FBS for another 2 days. Thereafter, the cells were fed with DMEM containing 10% FBS and the media was changed every other day until cells were shown to be maximally morphologically differentiated (above 90% of cells), which commonly occurred at day 8-10 [25]. All Experiments were performed using the differentiated adipocytes. In blocking experiments, the cell were pre-treated with the indicated inhibitors for 1 hour and then incubated with AOPPs in the presence of these inhibitors until the end of the experiments.

The toxic effect of AOPPs on adipocyte during incubation with AOPPs was assessed by measuring the activity of lactate dehydrogenase in the media using a spectrophotometric assay kit (Sigma, St. Louis, MO).

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the lysed cells using Trizol reagent (Invitrogen). Aliquots of each RNA extraction were reverse-transcribed simultaneously into cDNA using M-MLV reverse transcriptase according to the manufacturer's protocol

(Invitrogen). Each quantitative real-time PCR was performed in a total volume of 25 μl in duplicate by using the SYBR®*Premix Ex Taq*™ kit (TaKaRa, Kyoto, Japan) and the Fast Real-Time PCR system 7300 (Applied Biosystems, CA). The thermal cycling conditions comprised a 30-second step at 95°C, followed by 40 cycles with denaturation at 95°C for 5 seconds, annealing at 60°C (TNF-α, IL-6) or 56°C (18S) for 30 seconds, and extension at 72°C for 60 seconds. The following sets of primers, which were designed using Primer Quest software, were used: TNF-α (122 bp) forward: 5' - AAGCCTGTAGCCCACGTCGTA -3', reverse 5' – GGCACCACTAGTTGGTTGTCTTTG -3'; IL-6 (112 bp) forward: 5' – CCACTTCAC AAGTCGGAGGCTTA-3', reverse 5' – GCAAGTGCATCATCGTTGTTCATAC -3'. 18S (106 bp) forward 5'-GCCGCTAGAGGTGAATT CTT-3, reverse 5'-CGTCTTCGAACCT CCGACT-3'. For normalization of differences in RNA amounts, the 18S ribosomal RNA was co-amplified. Relative quantification of each gene was calculated after normalization to 18S ribosomal RNA by using the comparative Ct method. The results were shown as percentage change in expression with respect to control (unstimulated cells) for all samples.

Adipocytokines measurement with ELISA

Concentrations of TNFα and IL-6 in the supernatants were measured using mouse-specific ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's protocols. Results were normalized by cellular protein levels.

Western Blot analysis

Western blot analyses were carried out as described previously [25]. Briefly, cells were lysed with the radioimmunoprecipitation assay (RIPA) buffer, and the lysates were separated by SDS-PAGE. The separated proteins were then transferred to Polyvinylidene fluoride (PVDF) membranes. The membrane blots were first probed with a primary antibody overnight at 4ºC. After incubation with the appropriate secondary antibody coupled to horseradish peroxidase, the proteins were visualized with the enhanced chemiluminescent system (Pierce, Rockford, IL) and the bands densitometry was analyzed. β-actin was used as internal control. The primary antibodies used included: anti-NF-κBp65, anti-Phospho-NF-κB p65 (Ser536), anti-Akt, anti-phospho-Akt (Ser473), anti-PERK, anti-Phospho-PERK (Thr 981), anti-eIF2α, anti-Phospho-eIF2α (Ser51), anti-JNK1/3, anti-Phospho-JNK (Thr 183/Tyr 185), anti-IRE1α, Anti-phospho-IRE1α (Ser724) (Cell Signaling, Danvers, MA); anti-p47phox, anti-phospho-p47^{phox}, anti-p22^{phox}, anti-Nox4 and anti-GRP 78 (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation analysis

The phosphorylation of p47^{phox} (47 kDa of phagocyte oxidase) and interaction of p47^{phox} with p22^{phox} (22 kDa of phagocyte oxidase) or Nox4 were analyzed by immunoprecipitation [26]. Briefly, equal amounts of cellular protein (100μg) were incubated with anti-p47^{phox}, anti-p22^{phox} or anti-Nox4 antibodies overnight at 4°C, respectively. Protein A sepharose beads (Sigma-Aldrich) were added, followed by incubation for 4 h at 4°C. After washing 3 times with lysis buffer, the immunocomplexes were resolved on SDS-PAGE and then transferred to PVDF membranes. The membranes were then incubated with a HRP-conjugated rabbit anti-phosphoserine antibody (Stressgen Bioreagents Corp. Victoria, BC, Canada) or mouse anti-p47^{phox} monoclonal antibody. To determine total p47^{phox}, p22phox or Nox4, the membranes were washed with an elute buffer, reacted with a mouse anti-p47^{phox} monoclonal antibody, rabbit anti- p22^{phox} or anti-Nox4 polyclonal antibodies. Autoradiograms were prepared as described above.

2-Deoxy-glucose uptake

Glucose uptake was quantified as previously described [27]. Adipocytes deprived of serum overnight were incubated with AOPPs at the indicated concentration for 24 hours or at the 200 μg/ml for the indicated time, and then washed twice with HEPES-buffered saline. Glucose uptake was determined by the addition of 0.5 mM 2-deoxyglucose containing 0.5 μCi of 2-[^{3}H] deoxyglucose (Sigma, St Louis, MO) in HEPES-salts buffer in the presence or absence of insulin (100 nM) at 37°C for 30 min. Non-specific 2-deoxy-D-glucose uptake was determined in the presence of 10 μM cytochalasin B and this value was subtracted from all measurements. The reaction was stopped by aspiration, and extraneous glucose was removed by three washes with ice-cold PBS. Cells were then lysed in 1 N NaOH, and glucose uptake was assessed by scintillation counting. Samples were normalized for protein content by BCA protein determination kit. Triplicate wells for each condition were used in three independent experiments.

Intracellular Production of Reactive Oxygen Species

Intracellular ROS were detected in adipocytes by analyzing the fluorescence intensity of the intracellular fluoroprobe 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFD) (Molecular probe, Carlsbad, CA) [23]. Briefly, adipocytes were incubated with various concentrations of AOPPs for 60 minutes or with 200 μg/ml of AOPPs for the indicated time, followed by incubation with 1 nmol/L of DCFD for 30 minutes. After rinsing with the Krebs–Henseleit buffer (KHB), the fluorescent intensity was measured using a fluorescent microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Untreated cells were used to determine the background fluorescence. The final fluorescent intensity was normalized to the protein content in each group. Data were expressed as percentage of control.

To verify the sources of ROS generation, adipocytes were pre-treated for 60 minutes at 37°C with NADPH oxidase inhibitors (diphenylene iodonium, DPI, 10 μmol/L; apocynin 100 μmol/L), nitric oxide synthase inhibitor (N^4-nitro-L-arginine methyl ester, L-NAME, 100 μmol/L), a xanthine oxidase inhibitor (allopurinol, 100 μmol/L), a mitochondria complex I inhibitor (rotenone, 100 μmol/L), a mitochondria complex II inhibitor (TTFA, 10 μmol/L), a mitochondria complex III inhibitor (myxothiazol, 10 μmol/L), a cyclooxygenase inhibitor (indomethacin, 10 μmol/L), a superoxide scavenger that can enter the cells (bovine cytosolic Cu/Zn SOD, 200 U/ml) (all from Sigma). The dose-efficacy of these inhibitors was test and the maximally inhibitory concentrations were used in this study. The experiments were then repeated as described above.

Statistical analysis

All experiments were performed in triplicate. Continuous variables, expressed as mean ± SEM, were compared using one-way ANOVA. Pairwise comparisons were evaluated by the Student-Newman-Keuls procedure or Dunnett's T3 procedure when the assumption of equal variances did not hold. The Dunnett procedure was used for comparisons between reference group and other groups. Two-tailed P value of less than 0.05 was considered statistically significant. Statistical analyses were conducted with SPSS 13.0.

Results

AOPPs induced inflammatory responses in adipocytes via activation of NF-κB

Inflammatory responses such as overexpression of inflammatory adipocytokines have been demonstrated in adipocytes under certain stimulation [28, 29]. We therefore examined the expression of inflammatory adipocytokines in AOPPs-treated adipocytes. The levels of AOPPs used in this study were comparable to the serum concentrations in subjects with obesity and diabetes [16, 18, 19, 23]. As shown in Figure 1, exposure of adipocytes to AOPPs induced overexpression of TNFα and IL-6, at both mRNA (Fig. 1 A & B) and protein levels (Fig. 1 C & D), in a dose- and time- dependent manner. The expression of TNFα and IL-6 were unchanged in cells incubated with native MSA or medium alone (control), suggesting that the overexpression of TNFα and IL-6 was associated with advanced oxidation of MSA. AOPPs at the concentrations used in this study had no toxic effect on adipocyte (data not shown).

Since NF-κB is the major signal mediator which plays a critical role in the regulation of inflammatory cytokine expression [30], we examined whether NF-κB activation was involved in AOPPs-induced inflammatory responses in adipocytes. As presented in Figure 1 (E & F), AOPPs stimulation increased phosphorylation of NF-IκB p65, a key step for the activation of NF-κB, in cultured adipocytes. To further determine the relationship between activation of NF-κB and overexpression of TNFα and IL-6, adipocytes were incubated with AOPPs (200 μg/ml) for 24 hours in the presence or absence of NF-κB SN50 (20 μM), a cell-permeable specific inhibitor of NF-κB translocation [31]. An inactive and mutated form of the nuclear localization sequence was used as the control of SN 50. As shown in Figure 1 (G & H), incubation of adipocytes with NF-κB SN50 completely abolished AOPPs-induced overexpression of the adipocytokines at both mRNA (Fig. 1G) and protein levels (Fig. 1H), suggesting that the induction of adipocytokines was dependent on the activation of NF-κB.

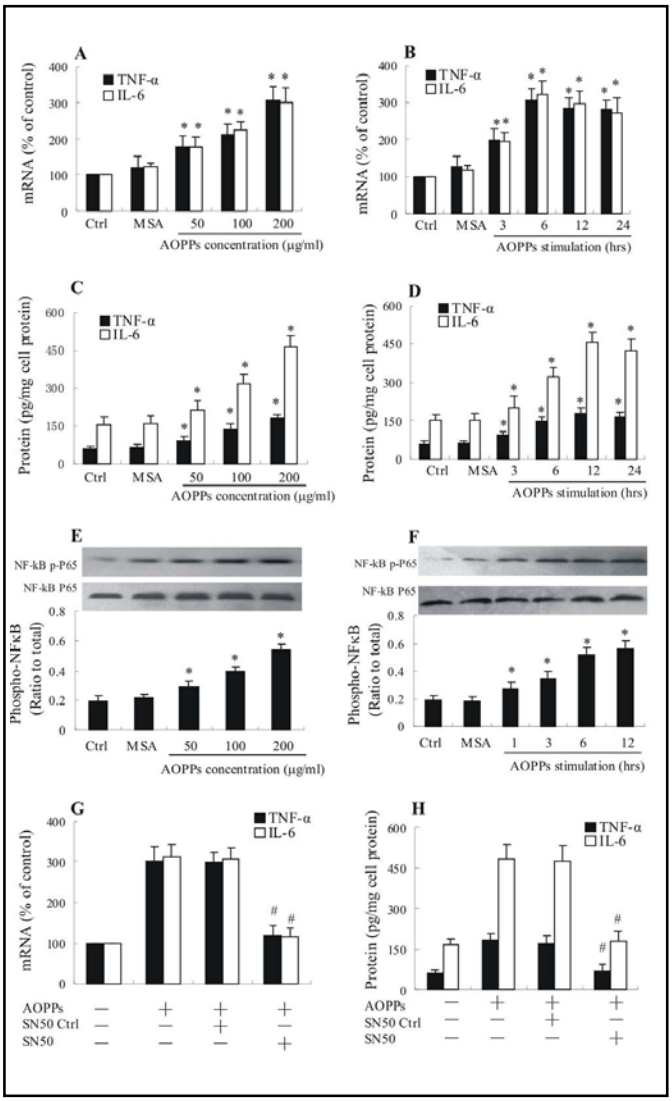
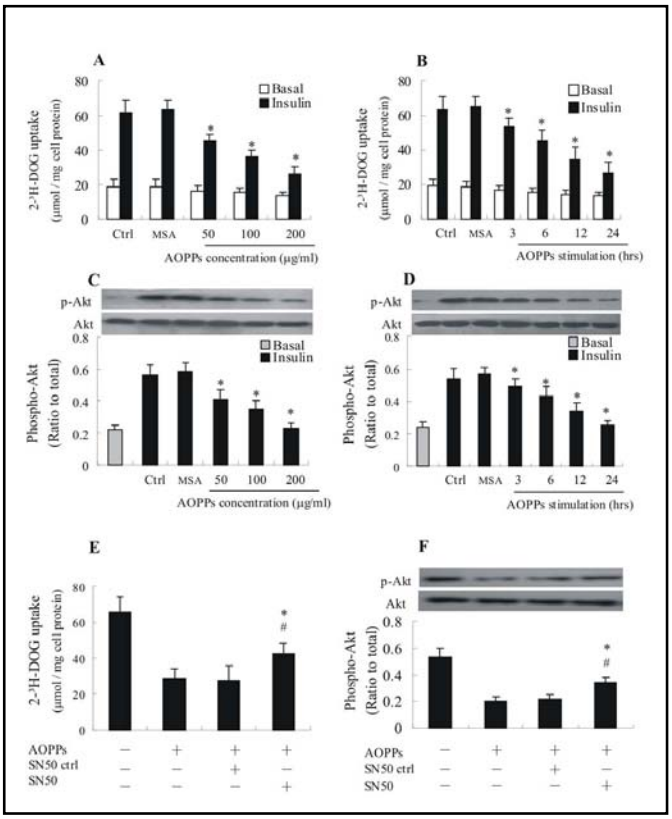


Fig 1. AOPPs induced overexpression of inflammatory adipocytokines in cultured 3T3-L1 adipocyte via activation of NF-κB. A-F: Adipocytes were incubated with the indicated concentration of AOPPs for 24 hours or 200μg/ml of AOPPs for the indicated time. AOPPs treatment increased expression of TNFα and IL-6 at both mRNA (A, B) and protein levels (C, D) in a dose- (A, C) and time- (B, D) dependent manner. Treatment with AOPPs also induced phosphorylation of NF-κB p65 in a dose- and time- dependent manner (E, F). G-H: Adipocytes were treated with AOPP (200μg/ml) for 24 hours in the presence or absence of NF-κB inhibitor SN50 (20 μM). SN50 completely abolished the AOPPs-induced overexpression of the adipocytokines at both mRNA (G) and protein levels (H). Data are expressed as mean ± SD of three independent experiments. ANOVA, $p<0.05$ in A, B, C, D, E&F, *$p<0.05$ *vs* control, #$p<0.05$ *vs* AOPPs treated group.

Fig 2. AOPPs induced insulin resistance in cultured 3T3-L1 adipocyte. A-F: Adipocytes were incubated with the indicated concentration of AOPPs for 24 hours or 200 μg/ml of AOPPs for the indicated time. The dose and time dependent effects of AOPPs on basal and insulin-mediated glucose uptake (A, B) and insulin-stimulated phosphorylation of Akt (C, D) were shown. E-F: Adipocytes were treated with AOPPs (200μg/ml) for 24 hours in the presence or absence of NF-κB inhibitor SN50 (20 μM). SN50 partly improved the AOPPs-induced impairment of insulin-mediated glucose uptake (E) and phosphorylation of Akt (F). Data are expressed as mean ± SD of three independent experiments. ANOVA, $p<0.01$ in A, B, C& D, *$p<0.05$ *vs* control, #$p<0.05$ *vs* AOPP treated group.



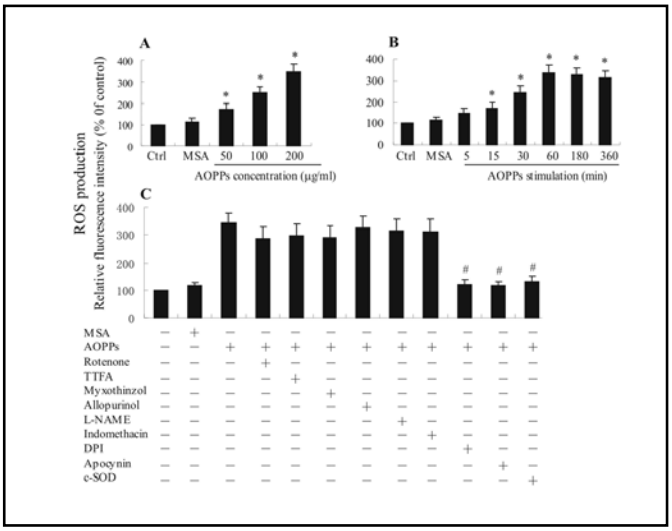


Fig 3. AOPPs induced NADPH oxidase-dependent ROS production in cultured 3T3-L1 adipocyte. The overnight serum deprived adipocytes were treated with indicated concentration of AOPPs for 1 hour or 200 μg/ml of AOPPs for indicated time. The intracellular ROS were determined by measuring the fluorescence of 5 (and 6)-chloromethyl-2',7'-dichlorodro-fluorescein diacetate. AOPPs induced intracellular ROS overproduction in a dose- (A) and time- (B) dependent manner. To verify the source of ROS generation, the experiments were repeated in the presence of rotenone, TTFA, myxothiazol, indomethacin, L-NAME, allopurinol, DPI, apocynin, or c-SOD (C). Data are expressed as mean ± SEM of 3 independent experiments. ANOVA, $p<0.05$ in A and B. *$p<0.05$ *vs* control. #$p<0.05$ *vs* AOPPs group.

AOPPs induced insulin resistance in adipocytes

Insulin resistance is a fundamental feature of metabolic syndrome and has been considered as a consequence of low grade inflammation [1, 32]. To examine whether AOPPs induced insulin resistance, adipocytes were incubated with AOPPs and then stimulated with insulin. The insulin-mediated glucose uptake, a maker of insulin sensitivity, was determined by cell-associated 2-[^{3}H] deoxyglucose. As shown in Figure 2 (A & B), the basal glucose uptake was comparable between AOPPs-treated cells and control cells. Meanwhile, AOPPs treatment induced a significant reduction in insulin-stimulated glucose uptake in a dose- and time- dependent manner.

The activation of Akt is one of the key processes in the insulin-induced signal transduction [33]. To examine the effect of AOPPs on insulin signal transduction, phosphorylation of Akt was measured following stimulation with insulin in cells challenged by AOPPs. As shown in Figure 2 (C-D), AOPPs treatment resulted in decreased phosphorylation of Akt in a dose- and time-dependent manner.

Previous studies have shown that inflammatory cytokines impaired insulin signals [34, 35]. To test whether AOPPs-induced insulin resistance was mediated by the NF-κB activation, the insulin-induced glucose uptake and phosphorylation of Akt were determined in cells treated with AOPPs (200 μg/ml) in the presence or absence of NF-κB SN50. As shown in Figure 2, addition of NF-κB inhibitor attenuated AOPPs-induced reduction in uptake of glucose (E), and phosphorylation of Akt (F), indicating that inhibition of AOPPs-induced inflammatory response may partly improve AOPPs-induced insulin resistance.

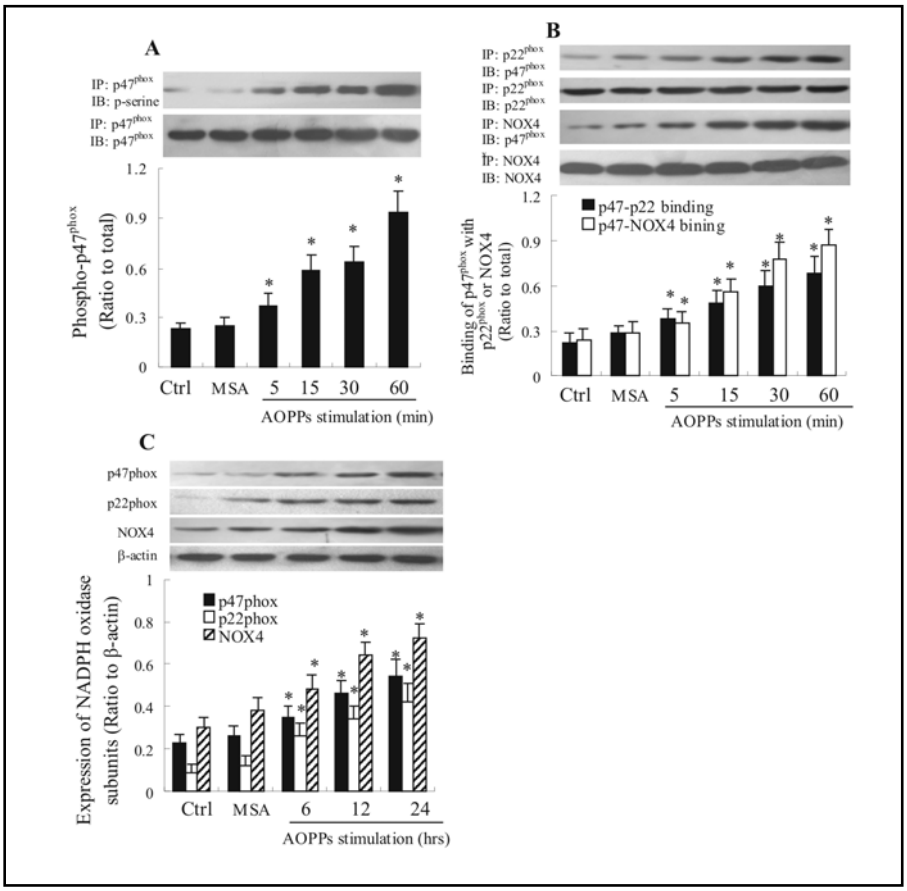


Fig 4. AOPPs activated NADPH oxidase in cultured adipocyte. The overnight serum deprived cells were treated with 200 μg/ml of AOPPs for indicated time. AOPPs induced phosphorylation of $p47^{phox}$ (A) and enhanced interaction of $p47^{phox}$ with $p22^{phox}$ and Nox4 (B). Exposure of cells with AOPPs for the indicated time upregulated expression of $p47^{phox}$, $p22^{phox}$ and Nox4 (C). Data were expressed as mean ± SEM of 3 independent experiments. ANOVA, $p<0.05$ in A, B, and C. *$p<0.05$ *vs* control.

AOPPs induced NADPH oxidase-dependent ROS production in adipocytes

It has been demonstrated that ROS play an important role in the promotion of inflammatory signaling and insulin resistance [36-39]. To examine the effect of AOPPs on intracellular ROS production, the fluorescence intensity of the intracellular fluoroprobe (DCFD) was evaluated. ROS generation was significantly increased in cells treated with AOPPs as compared with unstimulated cells or cells treated with native MSA (Fig. 3 A&B). AOPPs increased ROS generation in a dose- (Fig. 3 A) and time- (Fig. 3 B) dependent manner. To verify the enzymatic source of ROS generation, the response of cells to AOPPs was examined in the presence of inhibitors of various enzymatic systems involved in ROS production (Fig. 3C). AOPPs-induced ROS production was completely suppressed by the NADPH oxidase inhibitor DPI or apocynin, but not by the nitric oxide synthase inhibitor, the mitochondria complex inhibitors, the cycloxygenase inhibitor or the xanthine oxidase inhibitor. These inhibitors alone, at the concentration used in the experiments, had no effect on ROS production compared with cells cultured in medium alone. These data indicated that NADPH oxidase played a central role in AOPPs-induced ROS production.

To further examine the mechanism underlying ROS production, we investigated NADPH oxidase activity in AOPPs-challenged adipocytes. As shown in Fig. 4A, AOPPs treatment induced rapid phosphorylation of $p47^{phox}$. Native MSA had no effect. To examine the interaction of activated $p47^{phox}$ with membrane subunits $p22^{phox}$ and Nox4, we immunoprecipitated $p22^{phox}$ and Nox4 with specific antibodies and probed for the co-existence of $p47^{phox}$. As shown in Fig. 4B, the binding of $p47^{phox}$ with $p22^{phox}$ and Nox4 rapidly increased in AOPPs-stimulated cells. Thus, AOPPs challenge promoted the interaction of $p47^{phox}$-$p22^{phox}$ complex with Nox4, a key step for ROS generation in nonphagocytic cells [40].

Increased expression of NADPH oxidase subunits might be necessary for its sustained activation. To examine the effect of AOPPs on expression of the enzyme subunits, adipocytes were incubated with AOPPs for 6~24 hours. Compared with unstimulated cells, the expression of $p47^{phox}$, $p22^{phox}$ and Nox4, the essential subunits of NADPH oxidase in adipocytes [41], were all significantly increased after 6-hour exposure to AOPPs (Fig. 4C).

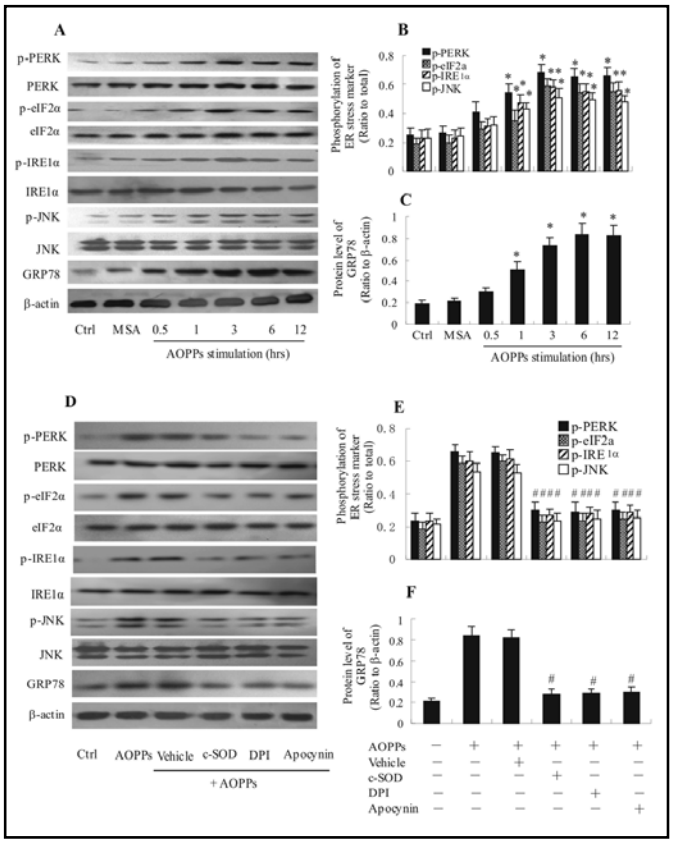


Fig 5. AOPPs induced ER stress through activation of NADPH oxidase-dependent ROS generation in cultured 3T3-L1 adipocyte. A: Representative product of western blot for expression of ER stress markers in adipocytes treated with 200 μg/ml of AOPPs for the indicated time. Incubation with AOPPs induced phosphorylation of PERK, eIF2α, IREα and JNK (B) and increased expression of GRP78 (C). In a separate experiment, adipocytes were incubated with AOPPs (200 μg/ml) combined with NADPH oxidase inhibitors (DPI or apocynin) or scavenger of ROS (c-SOD) for 6 hours. The representative products of western blot for expression of ER stress markers were shown (D). AOPPs-induced phosphorylation of PERK, eIF2α, IREα and JNK (E) as well as overexpression of GRP78 (F) were blocked by c-SOD, DPI or apocynin. Data are expressed as mean ± SD of three independent experiments. *p<0.05 *vs* control. #p<0.05 *vs* AOPPs-treated group.

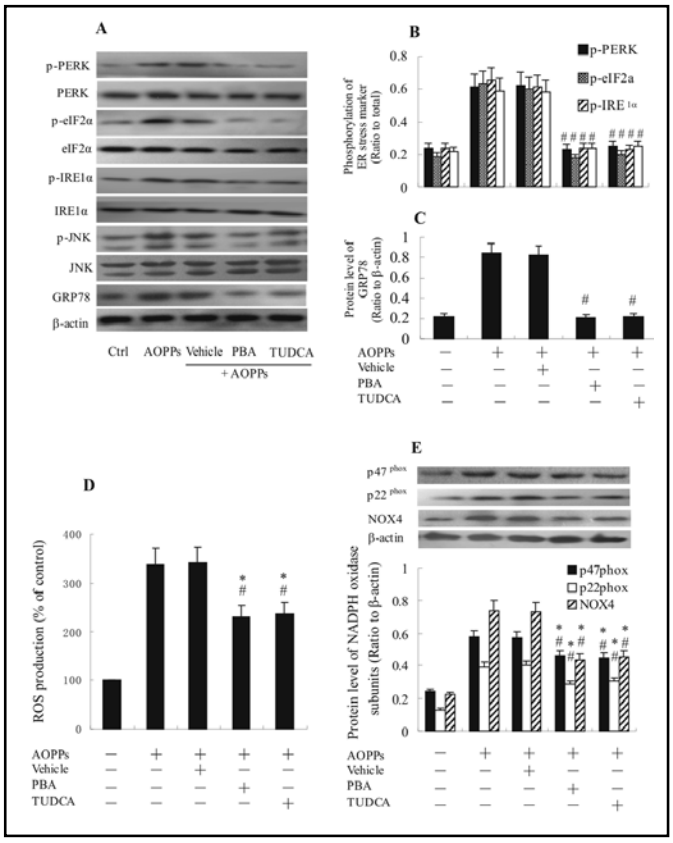


Fig 6. ER stress was involved in AOPPs-induced ROS generation and NADPH oxidase expression. Adipocytes were treated with AOPPs (200 μg/ml) in the presence of ER stress inhibitor (PBA or TUDCA) for 24 hours and subjected to analysis of intracellular ROS generation and NADPH oxidase expression. Both PBA and TUDCA completely inhibited AOPPs-induced ER stress (A, B, C). ER stress inhibitor partly suppressed AOPPs-induced ROS generation (D) and NADPH oxidase expression (E). Data are expressed as mean ± SEM of 3 independent experiments. *p<0.05 *vs* control; #p<0.05 *vs* AOPPs-treated group.

AOPPs triggered ER stress in adipocytes via activation of NADPH oxidase-dependent ROS generation

Increasing evidence shows that ER stress is linked to adipocyte dysfunction [42]. To explore the involvement of ER stress in AOPPs-induced adipocyte perturbation, we firstly examined whether ER stress was triggered by AOPPs in adipocytes. The cells were incubated with AOPPs (200μg/ml) for the indicated time. The ER stress markers [42], phosphorylation of PKR-like eukaryotic initiation factor 2α kinase (PERK), eukaryotic translational initiation factor 2α (eIF2α), inositol-requiring enzyme 1 (IRE1α) and c-jun N-terminal kinase (JNK), and expression of glucose regulated protein 78 (GRP78) were determined by western blot. As shown in Figure 5 (A, B & C), these ER stress markers were significantly increased in AOPPs-treated adipocytes compared with cells in medium alone. The ER stress markers was

increased with the time of AOPPs exposure ($p<0.05$) and reached a plateau after 3 hours, suggesting that ER stress was induced in AOPPs-treated cells.

Several studies have shown that ROS is able to activate ER stress [42-44]. To determine the role of AOPPs-induced ROS production in ER stress, the ER stress markers were determined in the presence or absence of NADPH oxidase inhibitor (DPI or apocynin) or a cell-permeable scavenger of ROS (c-Cu/Zn SOD). As shown in Fig. 5 (D, E & F), AOPPs-induced phosphorylation of PERK, eIF2α, IRE1α and JNK, as well as overexpression of GRP78 were completely abolished by the NADPH oxidase inhibitor or the ROS scavenger, suggesting that AOPPs-induced ER stress was dependent on ROS generation which was mediated by activation of NADPH oxidase.

It has also been shown that ROS play a role in both downstream and upstream of ER stress and expression of NADPH oxidase can be induced during ER stress [45, 46]. To investigate whether ER stress was involved in AOPPs-induced ROS generation and NADPH oxidase expression, the cells were treated with AOPPs in the presence of ER stress inhibitor, 4-Phenylbutyrate, PBA, or Tauroursodeoxycholic acid, TUDCA, [47], for 24 hours and then subjected to analysis. As shown in Fig. 6, PBA or TUDCA completely inhibited AOPPs-induced ER stress (A, B, C). These inhibitors significantly, but not completely, suppressed ROS generation (D) and NADPH oxidase expression (E). This data suggest ER stress can positively modulate AOPPs-induced ROS generation and NADPH oxidase expression by feedback.

AOPPs-induced adipocytes perturbation was dependent on activation of NADPH oxidase and ER stress

To determine the role of NADPH oxidase-ER stress pathway in AOPPs-induced inflammatory response and insulin resistance in adipocytes, the phosphorylation of NF-κB, expression of TNFα and IL-6, insulin-mediated uptake of glucose and phosphorylation of Akt were evaluated in the presence or absence of NADPH oxidase inhibitors (DPI or apocynin) or ER stress inhibitors (PBA or TUDCA). Treatment with either NADPH oxidase inhibitors or ER stress inhibitors completely blocked AOPPs-induced phosphorylation of NF-κB (Fig. 7 A) and overexpression of TNFα and IL-6 at both mRNA (Fig. 7B) and protein levels (Fig. 7C). Addition of these inhibitors also prevented the cells from AOPPs-induced reduction in insulin mediated- uptake of glucose (Fig. 7D) and phosphorylation of Akt (Fig. 7E). Consistent with this information, AOPPs-induced inflammation and insulin resistance was also inhibited by c-Cu/Zn SOD (data not shown). These data suggested that AOPPs-induced inflammatory response and insulin resistance might be mediated by the activation of NADPH oxidase and ER stress.

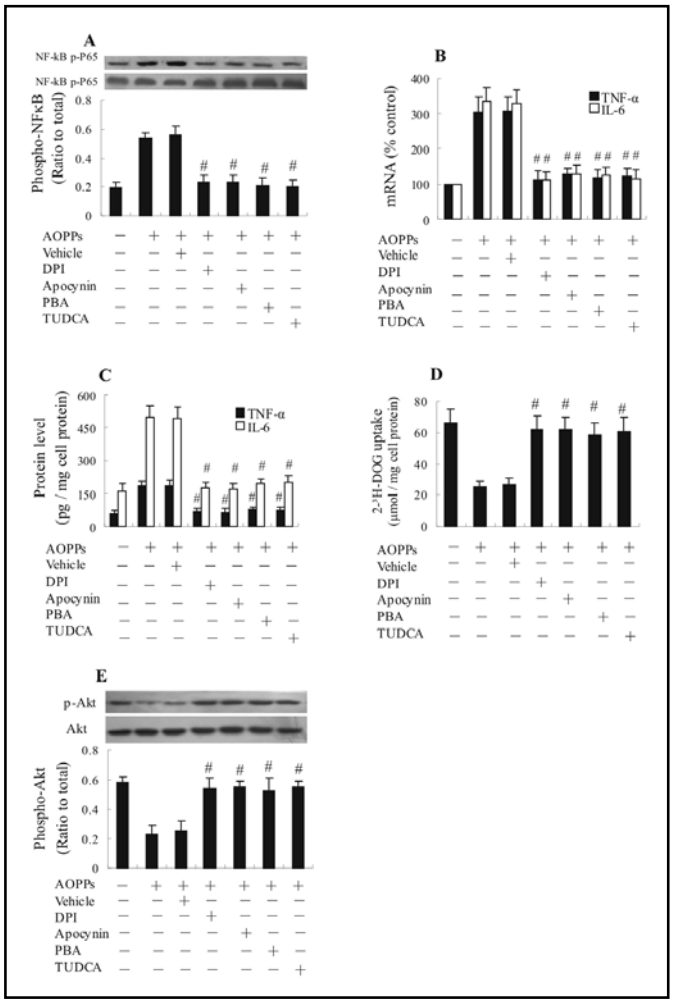


Fig 7. AOPPs-induced adipocyte perturbation was dependent on activation of NADPH oxidase and ER stress. Adipocytes were incubated with AOPPs (200 μg/ml) in the presence of NADPH oxidase inhibitors (DPI or apocynin) or ER stress inhibitors (PBA or TUDCA) for 24 hours. Treatment with DPI, apocynin, PBA or TUDCA completely inhibited AOPPs-induced phosphorylation of NF-κB (A), overexpression of TNFα and IL-6 at both mRNA (B) and protein levels (C). Blocking the activation of NADPH oxidase or ER stress also inhibited AOPPs-induced impairment of insulin-mediated glucose uptake (D) and phosphorylation of Akt (E). Data are expressed as mean ± SD of three independent experiments. #$p<0.05$ *vs* AOPPs-treated group.

Discussion

Increased recognition of adipocyte dysfunction as a link between obesity, insulin resistance, and type 2 diabetes mellitus has highlighted the importance of determining mechanisms underlying the patho-physiological abnormalities in adipocytes [12, 13]. Here we demonstrated *in vitro* that increased level of AOPPs, as seen in obesity and diabetes, induced inflammatory response and insulin resistance in cultured adipocytes. Native MSA had no effect on adipocyte, suggesting that the observed effects were due to the protein modification, not a property of MSA or other contaminations. Our data also showed that AOPPs-triggered adipocyte perturbation was associated with ER stress which was induced by NADPH oxidase-dependent ROS generation.

Recent studies have revealed that the plasma levels of pro-inflammatory cytokines, such as TNFα and IL-6, are increased in subjects with obesity, metabolic syndrome and type 2 diabetes mellitus. Inflammation has been recognized as the link between obesity, insulin resistance and type 2 diabetes mellitus. Accumulation of AOPPs has been observed in these conditions. One important finding of the present study was that increased level of AOPPs might be not only a marker of oxidative stress, but also a contributor to adipocyte inflammation. AOPPs treatment triggered overexpression of the inflammatory adipocytokines in adipocytes, which was mediated by activation of NF-κB, since NF-κB inhibitor completely blocked the response. Exposure of adipocytes to increased level of AOPPs also induced insulin resistance, revealed by decrease in insulin-mediated uptake of glucose and phosphorylation of Akt. Blocking the inflammatory response with NF-κB inhibitor significantly improved AOPPs-induced insulin resistance, suggesting that the inflammation might be partly involved in the pathogenesis of AOPPs-induced insulin resistance. Consistent with our finding, previous studies report that pro-inflammatory cytokines such as IL-6 and monocyte chemoattractant protein 1 impair insulin sensitivity [34, 48]. In addition, insulin resistance occurs in several models of oxidative stress [36, 38, 39] and inflammation plays an important role in the pathogenesis of insulin resistance in the animal model of obesity [7, 8, 32]. Collectively, AOPPs contributed to adipocyte perturbation by upregulation of inflammatory adipocytokines and impairment of insulin signaling.

Another new finding of the study was that AOPPs-induced adipocyte perturbation might be mediated by induction of ER stress. Exposure of adipocytes to AOPPs activated the key processes of ER stress such as phosphorylation of PERK, eIF2α, IRE1α and JNK, as well as upregulation of GRP78. Moreover, inhibition of ER stress by TUDCA or PBA, the chemical chaperones of ER stress inhibitor, completely blocked AOPPs-induced inflammatory response and insulin resistance. Thus, ER stress may present a directly critical mechanism underlying AOPPs-induced inflammatory response and insulin resistance. AOPPs exposure induced activation of JNK through a ROS-dependent pathway. It has been shown that ER stress can induce IRE1alpha-mediated JNK activation, resulting in insulin resistance in the liver and adipose tissues [49]. Also, inflammatory cytokines and oxidative stress can all lead to JNK activation [11]. This information suggests that JNK may be an important linker between oxidative stress, ER stress and insulin resistance. Consistent with our finding, evidence of ER stress in adipose tissue has been shown in subject with obesity and diabetes [50, 51], which is linked to the activation of inflammatory network and induction of insulin resistance *in vivo* and *in vitro* [49, 52-54].

How AOPPs challenge induced ER stress in adipocytes remains to be studied. Our data provided several lines of evidence suggesting that NADPH oxidase-dependent ROS generation played a central role in AOPPs-induced ER stress. First, Exposure of adipocytes to AOPPs increased ROS production. AOPPs-induced ROS production was completely prevented by NADPH oxidase inhibitor DPI or apocynin, but not by

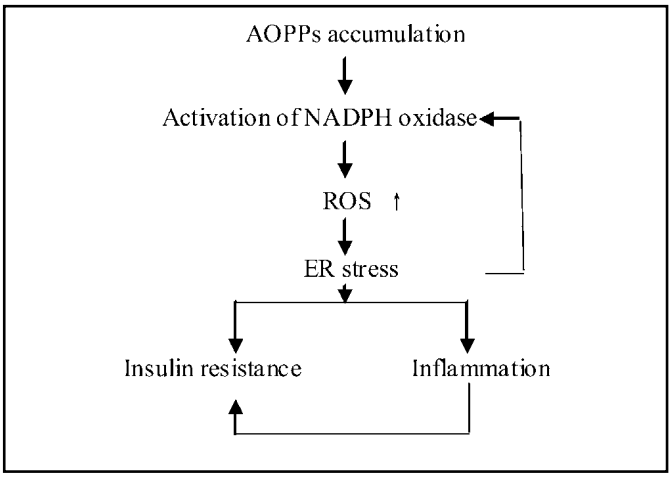


Fig 8. Schematic illustration depicting signal pathway linked with AOPPs induced- perturbation in cultured adipocytes. AOPPs induce overproduction of ROS through activation of NADPH oxidase, which results in ER stress. Induction of ER stress lead to insulin resistance and inflammation, as well as enhancing ROS production via a positive feedback. Inflammation can be partially involved in insulin resistance.

the inhibitors of nitric oxide synthase, xanthine oxidase, mitochondria complex I, mitochondria complex II, mitochondria complex III or cyclooxygenase, which are enzymatic systems involved in ROS generation. Second, AOPPs challenge activated NADPH oxidase, revealed by enhanced phosphorylation of cytosolic subunit p47phox and increased binding of p47phox with the membrane subunit p22phox, a key process for activation of NADPH oxidase. Moreover, Expression of the key regular subunits of the NADPH oxidase, p47phox, p22phox and Nox4, was significantly upregulated after several hours' incubation of adipocytes with AOPPs. Increased expression of NADPH oxidase subunits is necessary for sustained enzymatic activity. Finally, inhibition of NADPH oxidase with its inhibitor or sequestration of ROS by a cell-permeable Cu/Zn SOD completely prevented adipocytes from ER stress, suggesting that ROS produced by activation of NADPH oxidase might be the critical mediator of AOPPs-induced ER stress. ER stress inhibitor partly suppressed AOPPs-induced ROS generation and expression of NADPH oxidase, suggesting ER stress might contribute to ROS generation and activation of NADPH oxidase by feedback. This data is consistent with the emerging evidence that ROS generation can be upstream and downstream of ER stress [46]. Consistent with our results, several studies have shown that ROS is able to induce ER stress in neural and tumor cells [43, 44] . In addition, increased activity of NADPH oxidase in adipose tissue has been found in animal model of obesity [36, 38].

The detailed structure feature of advanced oxidation protein products such as advanced oxidation albumin need to be further studied. AOPPs prepared by incubation of albumin with hypochlorite have been shown to exert similar effect patterns on cell biology as do AOPPs extracted from patients with diabetes and end stage renal disease in several *in vitro* studies [23, 24, 55-57]. The signal pathways triggered by AOPPs in adipocytes remain to be further dissected. AOPPs have been demonstrated to disrupt the cell functions via a receptor for advanced glycation end products (RAGE) in vascular endothelial cells [23, 55] and CD36 [58] in renal tubular epithelial cells. Whether these or other receptors are involved in AOPPs-induced adipocyte signaling needs to be further studied.

In summary, we have identified AOPPs as a new class and potentially important mediators for adipocytes perturbation. Increased level of AOPPs triggered inflammatory response and insulin resistance in cultured adipocytes, probably through induction of ROS-dependent ER stress (Fig. 8). Accumulation of AOPPs is prevalent in subjects with obesity and metabolic syndrome as well as in patients with type 2 diabetes mellitus. Therefore, understanding the pathobiological effect of AOPPs on adipocytes might be an important step toward development of new intervention for inflammation and insulin resistance in metabolic syndrome and type 2 diabetes mellitus.

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