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Apelin Suppresses Oxidative stress

Apelin Attenuates Oxidative Stress in Human Adipocytes

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Keywords: Apelin, Oxidative stress, Reactive oxygen species, Adipocytokine

Background: Antioxidant effects of apelin on adipocytes are unknown.

Results: Apelin not only suppresses production and release of reactive oxygen species, but also relieves oxidative-stress induced cellular dysfunctions in adipocytes.

Conclusion: Apelin-APJ signaling acts as the negative autocrine feedbacks against oxidative stress in adipocytes.

Significance: Apelin signaling may serve as a potential therapeutic target for metabolic disorders.

SUMMARY

It has been recently recognized that the increased oxidative stress (ROS overproduction) in obese condition is a key contributor to the pathogenesis of obesity-associated metabolic diseases. Apelin is an adipocytokine secreted by adipocytes, and known for its anti-obesity and anti-diabetic properties. In obesity, both oxidative stress and plasma level of apelin are increased. However, the regulatory roles of apelin on oxidative stress in adipocytes remain unknown. In the present study, we provide evidence that apelin, through its interaction with apelin receptor (APJ), suppresses production and release of reactive oxygen species (ROS) in adipocytes. This is further supported by the observations that apelin promotes the expression of anti-oxidant enzymes via MAPK kinase/ERK and AMPK pathways, and suppresses the expression of pro-oxidant enzyme via AMPK pathway. We further demonstrate that apelin is able to relieve oxidative stress -induced dysregulations of the expression of anti- and pro-oxidant enzymes, mitochondrial biogenesis and function, as well as release of pro- and anti-inflammatory adipocytokines. This study, for the first time, reveals the antioxidant properties of apelin in adipocytes, and suggests its potential as a novel therapeutic target for metabolic diseases.

INTRODUCTION

Oxidative stress is a condition in which antioxidant defenses are overwhelmed by reactive oxygen species (ROS). The resulting excess free radicals cause damages to cells and tissues, and are implicated in the pathogenesis of various diseases, such as diabetes, cancer, hypertension and atherosclerosis (1). Oxidative stress is increased in obesity (2, 3). Many studies have demonstrated it is an important link factor between obesity and other metabolic disorders.
(e.g., diabetes) (4, 5). For example, oxidative stress suppresses insulin secretion from pancreatic β cells (6), and also disrupts glucose uptake in muscle and fat tissue (7, 8). And the accompanying inflammation in tissues worsens the problems (e.g., insulin resistance) caused by oxidative stress (9).

It is increasingly recognized that adipose tissue critically regulate metabolic homeostasis via secretion of various adipocytokines and metabolic factors (10). Adipose tissue is also a major source of the plasma ROS (3). Studies have showed that ROS production increases in parallel with fat accumulation in adipocytes (3, 11). On the other hand, excess ROS disrupts adipocyte functions. It, for examples, impairs insulin-induced glucose uptake in adipocytes (12), promotes adipogenesis and lipolysis leading to over-release of free fatty acids (11, 13), and jeopardizes mitochondrial biogenesis and functions which, in turn, further exacerbates the oxidative stress (14, 15). In addition, ROS overproduction in adipose tissue leads to decreased expression of anti-oxidative enzymes, increased expression of anti-oxidative enzymes, increased expression of anti-oxidative enzymes, and dysregulation of adipocytokine expression (e.g., adiponectin and IL-6) (3).

Apelin, a novel peptide hormone and relatively recent addition of adipocytokine family, is abundantly secreted by adipocytes (16, 17). Through interaction with G-protein coupled APJ receptors, apelin plays important roles in regulating energy metabolism, and has been recognized with its anti-obesity and anti-diabetic properties (18). Apelin, for examples, promotes glucose utilization and fatty acid oxidation in muscles of insulin-resistant mice (19, 20), stimulates insulin signaling pathways and glycogen synthesis in hepatocytes and liver tissues of mice (21), and suppresses adipogenesis and lipolysis (22). Interestingly, apelin also shown to alleviate oxidative stress in cardiomyocytes and vascular smooth muscle cells (23, 24). However, its regulations of oxidative stress in adipocytes have never been investigated. Considering that apelin expression in adipocytes is up-regulated in obesity (17) and its plasma level is increased in obese subjects (25, 26), we speculate that apelin may act as the feedback control to limit obesity-associated oxidative stress.

Here, we aim for the first time to reveal the effects of apelin on ROS production and oxidative stress in adipocytes, using human primary adipocyte as the in vitro cell model. Our results show that apelin/APJ suppresses ROS production and release by regulating anti- and pro-oxidant enzyme expressions via MAPK kinase/ERK and AMPK signaling pathways. In addition, we provide evidence that apelin can prevent oxidative-stress-induced dysregulations of ROS-related enzyme expressions, release of anti-inflammatory adipocytokine (adiponectin) and pro-inflammatory adipocytokines (IL-6, TNFα), as well as mitochondrial biogenesis and function. Our experiments also show that oxidative stress stimulates apelin release from adipocytes. This study highlights apelin as a promising therapeutic target for oxidative stress-related metabolic disorders.

**EXPERIMENTAL PROCEDURES**

**Cell culture and differentiation**

Human pre-adipocytes (SP-F-2; Zen-Bio Inc., USA), collected from subcutaneous adipose tissue of non-diabetic male subjects (with body-mass-index 25 - 29.9), were cultured in pre-adipocyte growth medium (DMEM/ Nutrient mixture F-12 medium: 1:1, v/v) containing 15 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin), at 37°C and in a humidified atmosphere containing 5% CO2. Two days after reaching confluence (defined as day 0), the cells were induced to differentiate into mature adipocytes similarly as the previously reported (27, 28). Specifically, the cells were incubated with adipocyte growth medium (serum-free DMEM/Ham’s F-12 medium containing 15 mM HEPES, 2 mM L-glutamine, 33 µM biotin, 17 µM pantothenate, 10 µg/ml transferrin, 0.2 nM triiodothyronine, 100 U/ml penicillin, and 100 mg/ml streptomycin) supplemented with 0.5 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 µM rosiglitazone. On day 2 of differentiation, the cells were treated for 2 days with the growth medium containing insulin and dexamethasone. The medium was thereafter replaced with the growth medium alone, every 2 days in the following 8 – 10 days. Adipocytes differentiation was confirmed by Oil-red-O staining and visual appearance of intracellular lipid droplets.

For knockdown of apelin (APLN) or APJ receptor (APLNR), cells (day 6-7 after induction of differentiation) were transfected with control siRNA or apelin siRNA or APLNR-siRNA (25 nM) (SantaCruz Biotech, USA) using
Lipofectamine-RNAiMAX transfection reagent (Life Technologies, USA) for 2 days. Adipocytes were then maintained in the growth medium for 2 - 3 days before the experiments. Knocking down of apelin or APJ receptor expression was confirmed by EIA or immunoblot analyses (Supplemental Figure 1). All culture media, supplements and sera were purchased from Life Technologies Corp. (Gibco; USA). Unless otherwise stated, all reagents and chemicals were obtained from Sigma-Aldrich Inc. (USA).

Confocal microscopy

Adipocytes grown on the glass-coverslips were placed in an imaging-chamber filled with the growth medium before imaging with a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss GMbH, Germany). For detection of intracellular ROS, the cells were loaded with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carbohy-x-H2DCFDA, 20 µM, Life Technologies, USA) -- a ROS indicator which gives green-fluorescence while being oxidized by ROS. After incubation with the dye for 30 min at 37 °C with 5% CO2 in a humidified incubator, adipocytes were then washed with the growth medium, followed by 200 µM H2O2 treatment for 60 min (or without the treatment as control). Excited at 488 nm, the fluorescence of the dye was monitored with a 505-550 nm filter. For co-localization studies of cellular and mitochondrial ROS, cells were loaded for 30 min with carboxy-H2DCFDA and 0.2 µM MitoTracker Red CM-H2Xros (Life Technologies). The latter, which is a reduced non-fluorescent version of MitoTracker Red, accumulates in mitochondria and fluoresces upon oxidation by ROS generated in mitochondria. To reveal the mitochondrial morphology, the cells were loaded with 0.2 µM MitoTracker Red-FM (Life Technologies). The MitoTracker fluorescence was measured above 580 nm using 543 nm as the excitation wavelength. For detecting superoxide anions (O2•-), the cells were incubated for 30 min with 10 µM dihydroethidium dye (DHE, Sigma-Aldrich) and then washed with the cell medium. DHE oxidation by (O2•-) produces red fluorescent 2-hydroxyethidium (29, 30). In some experiments, the cells were pre-treated with or without pyr-aplein13 (1 µM) and/or TNFα (50 ng/ml) before dye loading and imaging. ROS levels were quantified using a fluorescent plate reader (Victor3, PerkinElmer).

Western blot analyses

The adipocytes were washed twice with ice-cold phosphate-buffer saline (PBS), and scraped in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) containing freshly added protease/phosphatase inhibitor cocktail (Cell Signaling Technology, USA). After centrifugation at 4 °C, the supernatant of the sample was collected and its protein content was determined using a protein assay kit (Bio-Rad Lab, USA) based on Bradford method. Each sample with equal amount of proteins (as the loading control) was mixed with 5x SDS sample buffer, boiled for 5 min, and separated on 12% sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) before transferring the proteins onto a nitrocellulose membrane. The membranes were then blocked at room temperature for 2 - 3 hr with 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween (TBST) (10 mM Tris, 150 mM NaCl and 0.05% Tween-20, pH7.4), followed by subsequent incubation at 4 °C overnight in TBST containing the different primary antibodies (1:200-400 dilution). After washing 3 times (15 min each) with TBST, the membranes were incubated for 6 - 8 hr in TBST containing the horseradish peroxidase-conjugated secondary antibodies (1:2000-3000 dilution), followed by washing again with TBST for 3 times. The protein bands were detected in a G:BOX Chemi XT4 imaging system (Syngene, USA) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA). Antibodies against apelin (sc-33469), APJ receptor (APLNR, sc-33823), superoxide dismutase-1 (SOD-1, sc-8637), catalase (sc-34280), glutathione peroxidase-1/2 (GPx-1/2, sc-30147), p47-phox (sc-14015), PPARγ coactivator-1 (PGC-1α, sc-13067), cytochrome c oxidase subunit I (COX1, sc-23982), succinate dehydrogenase subunit A (SDHA, sc-27992), Akt (sc8312), phospho-Akt (Ser473, sc135651), AMP-activated protein kinase (AMPKα1/2, sc-25792), and phospho-AMPKα1/2 (Thr172, sc-33524) were purchased from SantaCruz Biotechnology. Antibodies against extracellular signal-regulated kinase (ERK1/2, cat.no. 3085-100) and phospho-ERK1/2 (cat.no. 3441-100) were purchased from Biovison Inc. (USA).

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-6, TNFα, adiponectin, and apelin in the medium was determined using a human IL-6 ELISA kit (RAB0306, Sigma), or a human TNFα ELISA kit (RAB0476, Sigma),
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or a human adiponectin EIA kit (RAB0004, Sigma), or a human apelin EIA kit (RAB0018, Sigma), respectively. Each sample contains the same amount of proteins (determined by Bradford assay as the internal control of the total cell number).

Assays of antioxidant activity, H$_2$O$_2$ release, and ATP production

Total cell antioxidant capacity was measured using the antioxidant assay kit (Sigma) according to the manufacturer’s instructions. In brief, cell lysates with equal amount of proteins were mixed (5 min at room temperature) with myoglobin, hydrogen peroxide and 2,2-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), before measuring the absorbance at 405 nm using a plate reader. The cell antioxidant activity is quantitatively calibrated to the activity of a defined concentration of trolox which is a water-soluble vitamin E analogue.

H$_2$O$_2$ released from adipocytes (total amount of cellular proteins was used as the internal control) was detected using the AmplexRed hydrogen peroxide assay kit (Life Technologies) according to manufacturer’s instructions. The absorbance is measured at ~560 nm and plotted against a standard curve containing serial dilutions of known concentration of H$_2$O$_2$.

The cellular ATP content (total amount of proteins was used as the internal control) was determined using ATP colorimetric/fluorometric assay kit (Biovision) according to the manufacturer’s instructions. Optical density at 570 nm was measured as the reporter.

Statistical Analysis

The variability between data sets was determined by unpaired Student’s $t$ test. All results were expressed as mean ± S.E. based on the measurements from at least three independent experiments. And $p < 0.05$ was considered statistically significant.

RESULTS

Apelin decreases ROS production and release in adipocytes via APJ receptor

In adipocytes, preproapelin (77-amino-acid peptide) is expressed by APLN gene and cleaved into shorter active peptides including pyr-apelin13 (pyroglutamated 13-amino-acid peptide which is the major apelin isoform in human plasma) (31, 32). Apelin’s functional effects are mediated by G protein-coupled APJ receptor which is expressed by APLNR gene (33). We have previously demonstrated that apelin suppresses the adipocyte release of free fatty acids -- one of the main contributors to the development of insulin resistance (22). Since excess reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) and superoxide radical (O$_2^-$) in the condition of oxidative stress also cause insulin resistance (4), we sought to investigate whether apelin has any effects on ROS production and release in adipocytes.

ROS level in live adipocytes was reported by a cell-permeable ROS indicator dye (carboxy-H$_2$DCF-DA) under confocal microscopy (Figure 1A). As shown in Figure 1B and C, the basal ROS level was significantly reduced in the cells pre-treated with apelin (1 μM pyr-apelin13 for 24 hr). In addition, the surge of ROS level after H$_2$O$_2$ stimulation (200 μM for 30 min) was also largely obliterated by apelin. The real-time fluorescence measurement indicates that H$_2$O$_2$ causes acute cellular increase of ROS level (within 5 min) and it can be suppressed by apelin treatment (Figure 1D and E). In order to elucidate whether APJ receptor is involved in the apelin effect on ROS level, APJ receptors were knocked down using APLNR-siRNA. As expected, apelin failed to reduce basal and induced ROS levels in APLNR-gene knockdown adipocytes (Figure 1B and C). Furthermore, as shown in Figure 1F, apelin treatment also suppressed H$_2$O$_2$ release from adipocytes. Taken together, our observations establish that, mediated by APJ receptor, apelin is able to suppress ROS production and release in adipocytes. This suggests the beneficial role of apelin to reduce oxidative stress in adipocytes as well as stress in other tissues stimulated by the released ROS from adipose.

Apelin increases anti-oxidant enzyme activity and expression, and suppresses pro-oxidant enzyme expression in adipocytes

Previous studies have shown that ROS level in adipocytes was closely related to the activities and expression levels of antioxidant enzymes such as Cu/Zn superoxide dismutase (Cu/Zn SOD or SOD1), catalase, and glutathione peroxidase (GPx) (3, 34), which are essential to prevent high ROS level caused oxidative stress and cellular damage (35). As shown in Figure 1G, antioxidant activity in adipocytes was greatly augmented by apelin treatment in dose-dependent manner. Consistently, the expression of antioxidant enzymes (SOD1, catalase, and GPx) was markedly increased in apelin-treated adipocytes (Figure 2A-D). Contrarily, apelin significantly suppressed the expression of p47phox which is a cytosolic subunit of the...
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superoxide-producing enzyme complex -- nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (36) (Figure 2E). On the other hand, siRNA knockdown of apelin or APJ expression in adipocytes produced opposite effects, confirming the autocrine regulation of apelin on the expression of ROS-related enzymes in adipocytes (Figure 2F-J, Supplemental Figure 1). Therefore, it may be concluded that apelin-APJ can refrain ROS level, at least in part, by regulating the expression and activities of both anti- and pro-oxidant enzymes.

**TNFα–induced ROS production and TNFα–impaired antioxidant enzyme expression are relieved by apelin**

It has been increasingly recognized that ROS overproduction critically contributes to the development of insulin resistance that characterizes type 2 diabetes (4, 8). It has been shown that TNFα can decrease insulin sensitivity in adipocytes by inducing ROS overproduction (37, 38), and apelin is able to improve TNFα–induced insulin resistance in adipocytes (39). It was also recently discovered that apelin acts oppositely to TNFα in regulating the trafficking of insulin receptors in adipocytes (40). We thus hypothesize that apelin may suppress TNFα–induced ROS generation in adipocytes. To test this hypothesis, adipocytes were simultaneously loaded with the carboxy-H₂DCF-DA and MitoTracker Red CM-H₂XRos, which are fluorescent reporters for intracellular ROS and mitochondrial ROS levels, respectively (Figure 3A). As shown in Figure 3B - F, we observed that apelin not only reduces intracellular ROS level, but also decreases ROS generation in mitochondria (an important source of cellular ROS). In agreement with the previous findings (38, 41), TNFα stimulated both the intracellular and mitochondrial ROS levels. As we anticipated, apelin treatment suppressed the TNFα–induced ROS overproduction (Figure 3B - F).

Since the primary ROS generated by mitochondria is superoxide anion (O₂⁻) (42), we also investigated the intracellular level of O₂⁻ which can be detected by dihydroethidium (DHE) - a widely used superoxide fluorescent indicator (29, 43). Similarly, apelin treatment resulted in decreased O₂⁻ level, and also abolished the TNFα-stimulated O₂⁻ generation in adipocytes (Figure 3D). These results indicate that apelin is able to ameliorate the TNFα-induced ROS overproduction (hence oxidative stress and associated insulin resistance in adipocytes).

It is known that oxidative stress in adipose tissue is accompanied by increase of mRNA expression of NADPH oxidase subunits, and decrease of mRNA expression of antioxidant enzymes (3, 44). Consistently as shown in Figure 3G-K, TNFα-treated adipocytes exhibited a significant decrease in antioxidant enzyme contents (SOD1, catalase, GPxs); and decreased expression of the antioxidant enzymes was also observed after exposure to excessive ROS (200 µM H₂O₂) which did not apparently affect cell viability (Supplemental Figure 2). The decrease of antioxidant enzyme expression was essentially rectified by apelin pre-treatment (Figure 3G-K). On the other hand, TNFα or H₂O₂ treatment caused increase of intracellular NADPH oxidase subunit (p47phox) which can be attenuated by apelin. These results further support the assertion that apelin is protective against the deleterious effects of oxidative stress by regulating expression of anti- and pro-oxidant enzymes.

**The mitochondrial biogenesis and function impaired TNFα or H₂O₂ are ameliorated by apelin**

It is well established that enhanced mitochondrial biogenesis and function can improve insulin sensitivity while impaired mitochondrial biogenesis and function are observed in adipose tissue of obese and diabetic subjects (45, 46). Studies also demonstrated that mitochondrial morphology is abnormal in adipocytes from diabetic and obese mice (47, 48). In addition, factors that lead to ROS overproduction (such as FFA, TNFα, etc.) is usually accompanied by reduced mitochondrial biogenesis and function, and increased insulin resistance in adipocytes (38, 49, 50). Mitochondrial dysfunction, in turn, leads to produce more ROS (15). Here, we investigated the apelin’s effects on ROS impaired mitochondrial morphology, biogenesis and function.

Mitochondrial morphology in live adipocytes can be resolved by confocal microscopy using the specific mitochondria-staining dye (MitoTracker) (51-53). As shown in Figure 4, mitochondria are short and densely packed in mature adipocytes; and apelin treatment increased the mitochondria density (Figure 4A). In comparison, mitochondria in TNFα-treated adipocytes assume tubular morphology while those in H₂O₂ treated cells appear swollen. Interestingly, the aberrant morphologies observed in the pro-inflammatory factor or ROS...
treated cells were largely remedied by apelin (Figure 4A).

To investigate the effects of apelin on mitochondrial biogenesis, we examined the expression of PGC1α (a master regulator of mitochondrial biogenesis) (54) and the mitochondrial proteins including cytochrome c oxidase subunit I (COX1) and succinate dehydrogenase complex subunit A (SDHA). As shown in Figure 4B-E, apelin is able to enhance the basal expression of these markers for mitochondrial biogenesis and partially recover the expression impaired by TNFα or H2O2. On the contrary, PGC1α expression was largely decreased in the apelin- or APLNR- gene knockdown adipocytes (Figure 4G, Supplemental Figure 1). This further ratifies the autocrine stimulation of apelin on the expression of PGC1α. Cellular ATP production is an indicator of mitochondrial function (45, 46). We further demonstrate that apelin can abrogate TNFα or H2O2 impairment on ATP production (Figure 4F). Taken together, we provide evidence that the impaired mitochondrial biogenesis and function in oxidative stress can be remedied by apelin.

Signaling pathways underlying the apelin–mediated expression of the proteins related to mitochondrial biogenesis and ROS generation

Mitochondrial biogenesis depends on at least two separate pathways: AMP-activated protein kinase (AMPK) and phosphatidylinositol 3 kinase/Akt (PI3K/Akt) (55). In agreement with the previous report (39, 56) that apelin increased activation (phosphorylation) of AMPK Akt, and ERK1/2 in adipocytes in a dose-dependent manner (Figure 4A-C, Supplemental Figure 3A-C). As shown in Figure 4D and F, inhibiting AMPK phosphorylation by dorsomorphin (a potent and selective AMPK inhibitor) (57) (Supplemental Figure 3D) abolished the apelin-stimulated expression of PGC1α (a nodal regulator of mitochondrial biogenesis). Dorsomorphin treatment also effectively blocked the apelin-induced expression of the mitochondrial proteins (COX1 and SDHA). Blocking the PI3K/Akt pathway by LY294002 (a potent and selective PI3K inhibitor) (58) (Supplemental Figure 3E) similarly abolished the apelin-induced expression of COX1 and SDHA (Figure 4G and H). In contrast, blocking the MAPK kinase by its specific inhibitor (PD98059) (59) (Supplemental Figure 3F) had no effect on the expression of PGC1α, COX1, and SDHA. These observations suggest that apelin stimulates mitochondrial biogenesis through two distinct routes: 1) activating the AMPK signaling which leads to increase of PGC1α expression; and 2) activating the PI3K/Akt signaling which enhances expression of mitochondrial proteins.

Studies have shown that AMPK signaling (likely via PGC1α) stimulates the expression of anti-oxidant enzymes (60, 61) whereas PI3K/Akt signaling impairs ROS scavenging (62, 63). Consistently, we observed that blocking the AMPK pathway largely eliminated the apelin-stimulated expression of anti-oxidant enzymes (catalase, GPx) and apelin-suppressed expression of pro-oxidant enzyme (P47phox subunit of NADPH oxidase) and inhibiting PI3K/Akt led to up-regulation of anti-oxidant enzymes (SOD1 and catalase) (Figure 4D, I-K). In addition, apelin stimulation on antioxidant enzyme expression is also dependent on MAPK kinase/ERK pathway, since PD98059 treatment effectively eliminated the apelin-induced expression of SOD1, catalase, and GPx. Taken together, our results show that apelin-mediated expression of anti- or pro-oxidant enzymes are, at least in part, dependent on AMPK and MAPK kinase/ERK signaling pathways.

Apelin attenuates the ROS-stimulated release of pro-inflammatory adipocytokines

ROS overproduction (oxidative stress) is closely associated with inflammation in adipose tissue (9). Specifically, pro-inflammatory adipocytokines secreted from adipocytes increase ROS generation in adipocytes; ROS overproduction, on the other hand, stimulates adipocytic release of pro-inflammatory factors (38, 44). Thus, oxidative stress and inflammation in adipose form a vicious positive cycle. Figure 3 and 4 demonstrate that apelin is able to suppress TNFα-induced ROS production. Here, we further sought to investigate the apelin effects on ROS-stimulated release of pro-inflammatory (IL6 and TNFα) and anti-inflammatory (adiponectin) adipocytokines.

As shown in Figure 6A-C, ROS (H2O2) stimulated adipocytic release of pro-inflammatory factors (IL6 and TNFα) and reduced release of anti-inflammatory factor (adiponectin), indicating that ROS can cause or worsen local inflammation in adipose tissue. Interestingly, apelin treatment largely rectified these effects (Figure 6A-C), suggesting its ability to attenuate the ROS-induced inflammation in adipose tissue. ROS also suppressed the expression of APJ receptor, while
apelin treatment induced it (Figure 6D-E). On the other hand, it was observed that H$_2$O$_2$ enhances apelin release from adipocytes (Figure 6F). This is consistent with the previous report that phorbol myristate acetate (PMA, a potent ROS inducer) can acutely stimulate adipocytic release of apelin (16). Therefore, it is likely that the excess release of apelin from adipocytes (instead of increased APJ receptor expression) acts as a negative autocrine control to the oxidative stress and inflammation in adipose.

**DISCUSSION**

Adipocytes (fat cells), the major depots for excess energy, are also active endocrine cells producing a variety of secretory factors (adipocytokines) that play critical roles in regulating metabolic homeostasis (10, 64, 65). Obesity (excess fat accumulation) is closely associated with various metabolic disorders (66). It has been recently recognized that the increased oxidative stress (ROS overproduction) in obese condition is a key contributor to the pathogenesis of obesity-associated metabolic diseases, particularly, insulin resistance (2, 5, 67). Adipose tissue is a major source of plasma ROS level (3). Oxidative stress in adipose tissue, in turn, impairs adipocyte functions and accelerates ROS overproduction (68, 69). In addition, the local inflammation induced by excess ROS further exacerbates the oxidative stress (9). Such vicious positive cycles in adipose tissue lead to systematic oxidative stress and inflammation which causes development of insulin resistance in peripheral tissues as well as other diseases (e.g., atherosclerosis and hypertension) (1, 4).

In this study, we provide evidences that apelin (secreted by adipocytes as an adipocytokine, and other cells) is able to halt the abovementioned adverse chain reactions. Based on our experiments, the following scenario may be proposed (Figure 7). As shown, excess ROS inhibits release of anti-inflammatory factors (adiponectin) and promotes release of pro-inflammatory factors (TNFα and IL6) from adipocytes (Figure 6A-C). Such inflammatory responses, in turn, further stimulate the production of ROS, because, for example, the autocrine interaction between the release TNFα and its receptor on adipocyte membrane inhibits the expression of anti-oxidant enzymes (SOD, GPx, and catalase) and increases the expression of pro-oxidant enzymes (e.g., NADPH oxidase) (Figure 3). In addition, excess ROS impairs the mitochondrial biogenesis and function (Figure 4). It has been shown that mitochondrial malfunction leads to ROS overproduction (15, 68). Excess ROS together with the triggered vicious positive feedbacks promote the development of insulin resistance. For example, it is known that oxidative stress impairs the function of insulin receptor substrate-1 (IRS1) whereby the translocation of glucose transporter (GLUT4) and thus glucose uptake are reduced (8).

On the other hand, excess ROS enhances the release of apelin from adipocyte to initiate the negative controls to the oxidative stress (Figure 1 and 6F). Specifically, the autocrine interaction between the released apelin and APJ receptors on adipocyte membrane stimulates the expression of anti-oxidant enzymes via AMPK and MAPK kinase/ERK signaling pathways, and inhibits the expression of pro-oxidant enzymes via AMPK signaling (Figure 1 and 5). The resulting suppression of ROS rectifies the positive echo between oxidative stress and inflammation (Figure 3 and 6A-C). Furthermore, the apelin-APJ activation also remedies ROS-impaired mitochondrial biogenesis and function by, at least in part, stimulating the expression of PGC1α (a nodal transcription factor of mitochondrial biogenesis) through AMPK pathway (Figure 4 and 5). The notion that apelin signaling is a beneficial physiological response to oxidative stress in adipose is supported by the previous observation that the plasma apelin level is elevated in obesity (25, 26). It also explains the previous observations in mice that apelin treatment leads to a significant decrease in plasma lipid hydroperoxide (an index of systemic oxidative stress) (23), and improves glucose uptake in adipose (18). Dray et al has demonstrated that intravenous injection of apelin has a powerful effect to enhance glucose utilization in skeletal muscle and adipose tissue in high-fat fed obese and insulin-resistant mice (20). As reported in this study, endothelial NO synthase (eNOS) activation is essential to apelin’s effect on skeletal muscle (20). It is conceivable that eNOS activation may be partly attributed to apelin inhibition on ROS level (70, 71).

Oxidative stress can increase fat tissue mass since ROS facilitates adipogenesis via accelerating mitotic clonal expansion (11, 72). It is also known that mitochondrial oxidative stress triggered by ROS inhibits aconitase in the tricarboxylic acid cycle (TCA cycle) leading to increased biosynthesis of fatty acids (73, 74).
Moreover, ROS stimulates lipolysis by recruiting the hormone sensitive lipase to the lipid droplets (13, 75), resulting in increased release of free fatty acids which, in turn, leads to increase of ROS generation and insulin resistance (76, 77). We have previously shown that apelin suppresses adipogenesis and lipolysis (22). Our current study implies that the antioxidant effects of apelin can attenuate ROS-induced adipogenesis, lipogenesis, lipolysis, and release of free fatty acids. Lipoic acid (an antioxidant) is a commercial dietary supplement which has been shown to be beneficial for reducing body adiposity and insulin resistance (78, 79). This study and our previous study suggest that apelin-APJ signaling pathways may serve as the novel therapeutic targets for obesity and obesity-associated metabolic diseases.
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FOOTNOTE

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The abbreviations used are: Akt, protein kinase B; AMPK, 5’ adenosine monophosphate-activated protein kinase; APJ, apelin receptor; carboxy-H2DCFDA, 6-carboxy-2’,7’-dichlorodihydro fluorescein diacetate; COX1, cytochrome c oxidase subunit I; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; GPx, glutathione peroxidase; IL6, interleukin 6; MAPK, mitogen-activated protein kinase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3K, phosphatidylinositol-3 kinase; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SDHA, succinate dehydrogenase subunit A; SOD-1, superoxide dismutase-1; TNFα, tumor necrosis factor alpha.

FIGURE LEGENDS

**FIGURE 1** Apelin suppresses intracellular ROS production and release, and increases the antioxidant activity in adipocytes. [A] Bright-field image of a human adipocyte and its confocal image of intracellular ROS level (reported by fluorescent carboxy-H2DCF-DA). Scale bars = 10 µm. [B] Representative confocal images of intracellular ROS in adipocytes, without (control) or with exposure to pyr-apelin13 (1 µM, 24 hr), before (upper lane) or after (lower lane) 30-min stimulation with 200
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μM H₂O₂. Some adipocytes were transfected with APLNR-siRNA to knock-down expression of APJ receptors. Scale bars = 50 μm. [C] Intracellular ROS levels indicated by the fluorescence intensity of carboxy-H₂DCF-DA. The values are normalized to the intensity in control cells (mean ± S.E., n = 4). [D - E] Real time-responses of ROS level after H₂O₂ exposure. The statistics (mean ± S.E.) in E is obtained from 11 cells (3 independent experiments). [F] H₂O₂ release from adipocytes without (control) or with exposure to 1 μM pyr-apelin13 for 24 hr (mean ± S.E., n = 4). [G] Antioxidant activities in adipocytes after 24 hr treatment with various doses of pyr-apelin13 (0 - 5 μM). The results are expressed in terms of Trolox-equivalent antioxidant-capacity (mean ± S.E., n = 5). Student’s t test was used: *p < 0.05; **p < 0.01 vs. control; #p < 0.05; ##p < 0.01 between indicated pairs.

FIGURE 2 Apelin modulates the expression of enzymes related to ROS in adipocytes. [A – E] Western blot analyses of the expression of SOD1 (~23 kDa), catalase (~64 kDa), GPx (~23 kDa), p47 phox (~47 kDa) and actin (~43 kDa) in adipocytes treated with various doses of pyr-apelin (0 nM, 100 nM, 1 µM, 5 µM, 24 hr). [F – J] Western blot analyses of the expression of SOD1, catalase, GPx, p47 phox and actin in adipocytes transfected with control-siRNA, apelin-siRNA, or APLNR-siRNA. Representative immunoblots are shown in A and F, respectively. The statistics of the blot intensity in B – E and G - J (mean ± S.E., n = 3) is normalized to that of actin (as the internal control). Each sample contains the same amount of total proteins (as the loading control). Student’s t test was used: *, p < 0.05; **, p < 0.01 vs. control (0 nM apelin, or control-siRNA).

FIGURE 3 [A – F] Apelin suppresses TNFα-induced ROS production. [A] Confocal images of intracellular ROS (detected by carboxy-H₂DCF-DA, upper-left) and mitochondrial ROS (detected by MitoTracker Red CM-H₂XRos, lower-left). The merged fluorescence image (upper-right) and bright-field image (lower-right) are also shown. Scale bars = 10 μm. [B - D] Representative confocal images of intracellular ROS, mitochondrial ROS, and intracellular superoxide levels (detected by DHE), without (control) or with exposure to pyr-apelin13 (1 μM) and/or TNFα (10 ng/ml) for 24 hr. Scale bars = 50 μm. [E - F] The statistics (mean ± S.E., n = 4) of intracellular (E) and mitochondrial (F) ROS level based on the fluorescence intensity normalized to that in control cells. [G - K] Apelin remedies TNFα- or ROS- impaired expression of ROS-relevant enzymes. Cells were pre-incubated without (-) (control) or with (+) 1 μM pyr-apelin13 for 24 hr, followed by 12 hr exposure to 10 ng/ml TNFα or 200 μM H₂O₂. The representative immunoblots of SOD1, Catalase, GPx, p47 phox, and actin are shown in G. The statistics of the blot intensity in H - K (mean ± S.E., n = 4 - 5) is normalized to that of actin (as the internal control). Each sample contains the same amount of total proteins. Student’s t test: *, p < 0.05; **, p < 0.01 vs. control; #p < 0.05; ##p < 0.01 between indicated pairs.

FIGURE 4 Apelin improves TNFα- or ROS -impaired mitochondrial biogenesis and function in adipocytes. Cells were incubated without (-) (control) or with (+) 1 μM pyr-apelin13 for 24 hr, followed by 12 hr exposure to without or with 10 ng/ml TNFα / 200 μM H₂O₂. [A] Confocal images of mitochondria (detected by MitoTracker) in adipocytes, and their corresponding bright-field images and 3x magnification images are shown in upper-right and lower-right, respectively. Scale bars = 10 μm. [B] ATP content in adipocytes, as indicated by various treatment, was determined by using ATP colorimetric assay kit (mean ± S.E., n = 4). [C - F] Western blot analyses of the expression of PGC-1α (~ 90 kDa), COX1 (~ 57 kDa), SDHA (~ 70 kDa), and actin in adipocytes as indicated by various treatment. The representative immunoblots are shown in C; the statistics of the blot intensity in D – F (mean ± S.E., n = 4 - 5) is normalized to that of actin (as the internal control). [G] Western blot analyses of the expression of PGC-1α in adipocytes transfected with control-siRNA, apelin-siRNA, or APLNR-siRNA. The upper panel shows the representative immunoblots, and the lower panel shows the statistics of the blot intensity normalized to that of actin (mean ± S.E., n = 3). Each sample
contains the same amount of total proteins. Student’s t test: *, $p < 0.05$; **, $p < 0.01$ vs. control; #, $p < 0.05$ between indicated pairs.

**FIGURE 5** Signalling pathways underlying the apelin-mediated expression of the proteins related to mitochondrial biogenesis and ROS level. [A – C] Western blot analyses of the phosphorylation and total protein levels of [A] AMPK (~62 kDa), [B] Akt (~60 kDa) and [C] ERK1/2 (~42 - 44 kDa) in adipocytes (15 min, 30 min, 2 hr), without (-) (control) or with (+) exposure to 1 µM pyr-apelin13. The top panels show the representative immunoblots. The lower panels show the statistics (mean ± SEM, n = 3) of the optical density ratio between the blots: pAMPK and AMPK, or pAkt and Akt, or pERK1/2 and ERK1/2. [D – K] Western blot analyses of the expression of p47phox, PGC-1α, COX1, SDHA, SOD1, Catalase, GPx, and actin in adipocytes, after 24 hr incubated without (-) (control) or with (+) 1 µM pyr-apelin13 in the absence (-) or presence (+; added 1 hr before apelin treatment) of 50 µM PD98059, 10 µM LY294002, or 1 µM dorsomorphin. The representative immunoblots are shown in D. The statistics of the blot intensity in E - K (mean ± S.E., n = 4) is normalized to that of actin. Each sample contains the same amount of total proteins. Student’s t test: *, $p < 0.05$; **, $p < 0.01$ vs. control; #, $p < 0.05$, ##, $p < 0.01$ between indicated pairs.

**FIGURE 6** [A - C] Apelin modulates ROS-induced dysregulation of IL-6, TNFα, and adiponectin release from adipocytes. After 24 hr exposure to without (-) or with (+) 1 µM pyr-apelin13, the media were changed, and adipocytes were treated without (-) (control) or with (+) pyr-apelin and/or 200 µM H2O2 for 12 hr. IL6, TNFα, and adiponectin release from adipocytes were measured with ELIZA or EIA [A – C]. [D - E]. Expression of APJ (~40 kDa) and actin in adipocytes were analyzed by western blot. Representative immunoblots are shown in D; the statistics of the blot intensity in E is normalized to that of actin (as the internal control). [F] ROS stimulate apelin secretion from adipocytes. Apelin release from adipocytes (2hr, 12 hr), without (-) (control) or with exposure to (+) 200 µM H2O2, were measured with EIA. The results are shown as mean ± S.E. (n = 4 - 6) of 3 - 4 independent samples (Each sample contains the same amount of total proteins). Student’s t test: *, $p < 0.05$; **, $p < 0.01$ vs. control; #, $p < 0.05$ between indicated pairs.

**FIGURE 7** The apelin-APJ signaling pathways on regulating oxidative stress.
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FIGURE 1

A

B

C

D

E

F

G

Fluorescence intensity (a.u.)

% increase of fluorescence intensity after H₂O₂ stimulation

H₂O₂ release (nmol/mg protein)

Antioxidant Activity (mM)

FIGURE 2

A

B

C

D

E

F

G

H

I

J

Relative density

Control Apelin APLNR siRNA - APLNR siRNA

Control Apelin APLNR siRNA - APLNR siRNA

Control Apelin APLNR siRNA - APLNR siRNA

Control Apelin APLNR siRNA - APLNR siRNA

Control Apelin APLNR siRNA - APLNR siRNA

Control Apelin APLNR siRNA - APLNR siRNA
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FIGURE 3
FIGURE 4

Apelin Suppresses Oxidative stress
FIGURE 6

Apelin Suppresses Oxidative stress
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**FIGURE 7**

[Diagram showing the interaction between Apelin, AMPK, ERK, PGC1α, and TNFα with their respective effects on ROS, anti-oxidant enzyme (SOD, CAT, GPx), pro-oxidant enzyme (NADPH oxidase), pro-inflammatory factor release (TNFα, IL6), insulin resistance, and mitochondrial biogenesis and function.]