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Generation of reactive oxygen species in adipose-derived stem cells: Friend or Foe?

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Abstract

Introduction—Reactive oxygen species (ROS) participate in cellular apoptosis and are involved in pathophysiological etiology of degenerative diseases. However, recent studies suggest that ROS at low levels may play a pivotal role as second messengers and activate normal cellular processes. Intracellular ROS increase the proliferation, migration, and regenerative potential of ASCs. On the contrary, manipulations that diminish intracellular ROS levels interfere with normal ASC function. ROS generation therefore acts like a double-edged sword.

Areas covered—This review discusses the following research questions: 1) Do ROS stimulate or suppress ASCs? 2) How are ROS generated from ASCs? 3) Which function(s) is/are regulated by intracellular ROS generation? In addition, the antioxidant/antiapoptotic effect of ASCs is briefly introduced.

Expert opinion—Whether ROS is harmful or beneficial is primarily a question of dosage. Low or moderate ROS generation increase the proliferation, migration, and regenerative potential of ASCs. Therefore, it is beneficial to expose ASCs to moderate oxidative stress during manipulation. The addition of a ROS donor in culture can reduce the cost for the expansion of ASCs, and a ROS preconditioning can enhance the regenerative potential of ASCs.

Keywords

adipose-derived stem cells; reactive oxygen species; stimulation; apoptosis; hypoxia

1. Introduction

Oxidative stress, caused by the cellular accumulation of reactive oxygen species (ROS), induces cellular apoptosis or death [1-3]. Although ROS exhibit negative effects on cellular homeostasis, there is conflicting evidence that ROS at low and nontoxic levels can actually promote cell proliferation and survival [4-6]. In addition, ROS are known to serve as a second messenger in the intracellular signal transduction pathway for a variety of cellular processes, including inflammation, cell cycle progression, apoptosis, aging, and cancer [7-9]. Therefore, it becomes necessary to understand the complex role of redox balance in the cellular biology and to investigate the role of ROS in cellular homeostasis (Figure 1). A

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growing body of literature demonstrates that functional characteristics of stem and progenitor cells are under redox control, and physiological levels of intracellular ROS are required to activate the DNA repair pathway for maintaining genomic stability in stem cells and to increase the proliferation/migration of stem cells [10-13]. In addition, stem cells efficiently manage oxidative stress and exhibit a high resistance to ROS-induced death, which correlates with constitutive expression of antioxidant enzymes [14, 15].

Adipose-derived stem cells (ASCs) are regarded as an abundant and reliable source of stem cells for tissue regeneration [16-18]. In addition to their differentiation potential, ASCs secrete growth factors that stimulate recovery of damaged tissue [19-22]. Recently, it has been reported that culturing under hypoxia significantly increases the proliferation and migration *in vitro* [11, 23]. In addition, the secretion of ASCs and the regenerative potential of ASCs were also significantly up-regulated under hypoxia [23, 24]. In the mechanistic explorations, our group found that hypoxia increased intracellular ROS level that in turn triggered a survival signal cascade of ASCs [11]. Platelet-derived growth factor receptor-beta (PDGFR- β) undergoes primary phosphorylation, which is followed by the phosphorylation of Akt and ERK pathways. As described earlier, ROS serve as a second messenger in the intracellular signal transduction pathway of ASCs; however, most studies dealing with the physiological role of ROS focus on its negative aspects, such as apoptosis induction (Table 1). To better address this issue, this review discusses the following fundamental questions: 1) Do ROS stimulate or suppress ASCs? 2) How are ROS generated from ASCs? 3) Which function(s) is/are regulated by ROS generation? In addition, the antioxidant/antiapoptotic effect of ASCs is briefly introduced. Whether ROS is harmful or beneficial is primarily a question of dosage. Under certain conditions, ROS formation appears to exceed protective levels and even becomes harmful. However, this review highlights the positive effects of ROS generation in ASC physiology. Maintenance of ROS at physiological levels increases the proliferation, migration and ASC secretion of growth factor, which may enhance the regenerative potential of ASCs in clinical settings.

2. ROS generation

ROS are generated from a number of sources including NADPH oxidase, the mitochondrial electron-transport system, xanthine oxidase, cytochrome p450, uncoupled nitric oxide synthase, and myeloperoxidase [25, 26, 28]. Among the different subcellular sources, mitochondria are considered to be the main source of ROS, and the respiratory chain reaction in mitochondrial wall contributes superoxide anion [27]. During respiration, a small but significant proportion of O₂ molecules are converted to superoxide anion radicals (O₂^{•-}) by complex I and complex III of the respiratory chain [28]. The flux of superoxide anion is related to the concentration of potential electron donors and the local oxygen concentration. Chemicals such as antimycin and rotenone inhibit complex I and III and generate ROS by the respiratory chain reaction, which has diverse effects on the proliferation and angiogenic potential of ASCs [27, 29, 44].

In addition to the passive production by the mitochondria, intracellular ROS can be directly generated by the NADPH oxidase enzyme (Nox) [11, 30, 32]. This enzyme was originally characterized in the phagocytes, which utilize a Nox-generated burst of superoxide anion as a mechanism of defense against pathogens. Moreover, generation of ROS through Nox activation was reported in some nonphagocytotic cells, which is an essential component of cellular signaling and survival [31-34]. In particular, Nox is reportedly one of the major sources of ROS in stem/progenitor cells and is activated by growth factors and hypoxia [11, 32, 35]. ROS generated from Nox play an important role in redox signaling involved in angiogenesis, as well as stem cell mobilization and proliferation. Therefore, the Nox-induced ROS activates cell survival pathways such as MAPK and PI3K/Akt

pathways [34]. Nox isoforms have been identified in a number of different tissues. Several homologues of gp91phox (also known as Nox2), Nox1, Nox3, Nox4, and Nox5, as well as the dual oxidases (Duox) 1 and 2, have been identified [36-38]. Nox1, Nox2, Nox4, and Nox5 are reportedly expressed in endothelial cells; Nox2 and Nox4 are also found expressed in mesenchymal stem cells [12, 39, 40]. Our preliminary study found that the expression of Nox4 and Nox5 in ASCs was relatively high, while that of Nox1Nox2 and Nox3 was not. Our group first demonstrated the Nox family-mediated ROS generation in ASCs by showing that ROS level was reduced by diphenyleneiodonium, a potent NADPH oxidase inhibitor [11]. Although free radicals generated under hypoxic conditions have still not been identified, the expression of Nox4 and its involvement in ROS generation suggests that H₂O₂ is the most likely generated free radical under hypoxia.

Low oxygen tension (2-8% O₂ concentration) is an important characteristic of the stem cell niche and hypoxia provides a conducive environment for the maintenance of stem cell properties [58, 59]. In addition, mesenchymal stem cell culture under hypoxic conditions *in vitro*, enhance their proliferative and self-renewal capacities [60]. Suga et al. [59], established surgically induced ischemia models by disrupting blood supply, which induced cell death including adipocytes, vascular endothelial cells, and blood-derived cells. However, hypoxia induced the proliferation of ASCs, which indicates that hypoxia also act as stimulator *in vivo*. Despite the fact that hypoxia is considered to be beneficial, ASCs are routinely cultured in normoxia (20% O₂ concentration).

Hypoxic culturing (2% O₂ concentration) induces ROS generation by the Nox-family in cytosolic membrane and mitochondria (Figure 2). Acute increase in cellular ROS levels activates receptor-type or non-receptor-type tyrosine kinases in ASCs. Of them, PDGFR- β is first to get phosphorylated, followed by the phosphorylation of PI3K/Akt/mammalian targets of the rapamycin (mTOR) and ERK1/2 signaling pathways [11]. Then, activation of these signaling pathways inhibits the degradation of hypoxia-inducible factor-1 alpha (HIF-1 α) by propyl-hydroxylation of the von Hippel Lindau tumor suppressor protein and increases cytosolic HIF-1 α levels in ASCs [23]. The accumulated HIF-1 α translocates to the nucleus where it modulates the transcription of its target genes after binding to the hypoxia-responsive element (HRE) in the nucleus. Among these target genes, expression of the VEGF gene is significantly up-regulated resulting in increased VEGF secretion [23, 42-43].

3. ASC stimulation by ROS

Despite the accumulating evidence that regards intracellular ROS as the signaling molecules of ASC stimulation, there are only few reports demonstrating the stimulatory effect of ROS [11, 27]. However, recent observations of our group indicate that low or intermediate levels of ROS generation exhibit positive effects on ASCs.

3.1 Proliferation

The effects of ROS generation on the proliferation of ASCs were previously examined in 3T3 preadipocytes and human ASCs, but the results are quite different [8, 11, 23, 44, 45]. Carriere et al. [44] investigated the effects of ROS on the proliferation of 3T3 preadipocytes and found that rotenone and oligomycin, inhibitors of complex I and of ATP synthase respectively, increased mitochondrial ROS levels and inhibited cell growth in preadipocytes. Jee et al. reported that hypoxia and the anti-oxidant 4-(3,4-dihydroxy-phenyl)-derivative could also induce ASC proliferation [8]. Recently, Song et al. modulated Thioredoxin1 (TRX1) and Thioredoxin2 (TRX2) expressions and found that the overexpression of TRX1 and TRX2 increased the proliferation of ASCs by decreasing ROS production, and it inhibited oxidant-induced apoptosis [45]. However, our group investigated the proliferative effect of hypoxia (2% O₂ concentration) in human ASCs [14, 15], and found that ROS

generation by hypoxia significantly increased the proliferation and survival of human ASCs [11, 23]. In that study, Akt and ERK1/2 signaling pathways were phosphorylated by ROS generation. Our group also found that antimycin treatment (0.01–10 μ M concentration) significantly increased the proliferation of ASCs (Figure 3A). These opposite results can be explained by the difference in intracellular ROS concentration of the ASCs. Therefore, a precise control of intracellular ROS levels is a key determining factor for ASC proliferation.

3.2 Expression of early undifferentiation markers

Recently, 4 transcription factors (Oct4, Sox2, Klf4, and c-Myc) have been shown to reprogram fibroblasts and generate induced-pluripotent stem cells [46–48]. Although they are not as abundant as embryonic stem cells, ASCs express early undifferentiation markers such as Oct4, Sox2, Nanog, Rex1, and c-Myc [8, 49, 50]. ASCs may undergo an increase in developmental potential and multipotency via the up-regulation of these transcription factors. Like proliferation, the intracellular ROS level has a different effect on the early undifferentiation gene expression of ASCs and plays a pivotal role in the maintenance of ASC multipotency. Jee et al. reported that a combination of low oxygen tension and 4-(3,4-dihydroxy-phenyl)-derivative induced ASCs to de-differentiate into more primitive stem cells and significantly increased the mRNA expression of Rex1 and Oct4 [8]. They also reported that selenium improved Rex1, Nanog and Oct4 expression in ASCs and induced DNA demethylation on the promoter regions of these genes via the inhibition of ROS generation [49]. Recently, George et al., generated a mouse mutant with a mutated inner mitochondrial membrane peptidase 2-like gene and examined the effects of excessive mitochondrial ROS generation on stem cell self-renewal. ASCs from mutant mice showed impaired ability to proliferate, and formed significantly few and smaller colonies in colony formation assays, although they retained adipogenic differentiation capability *in vitro* [61]. However, an experiment by our group showed that ROS generation under hypoxia (2% O₂ concentration) increased the mRNA expression of Oct4 and Rex1 in ASCs, and a low concentration of antimycin (1–100 nM) increased mRNA expression of Oct4, Sox2, and Rex1 in ASCs (Figure 3-C). Although it depends on concentration, low-level ROS generation increases the expression of early undifferentiation markers.

3.3 Migration

The effects of ROS on the migration of stem cells have been studied extensively in hematopoietic stem cells and endothelial progenitor cells. These stem cells circulate in the vascular system and are involved in the regeneration of a variety of tissues, and their migration can be enhanced by ROS [13, 51]. ASCs localize in subcutaneous and visceral regions, and regenerate skin and adipose tissue through migration and self-renewal mechanisms. When local inflammation or injury occurs, ROS and chemokines are secreted from damaged cells and induce ASC migration. The effect of ROS on ASC migration was directly measured *in vitro*, which showed that hypoxia significantly increased the migration of human ASCs, whereas neutralizing ROS generation by NAC or DPI treatment decreased ASC migration [11]. We further investigated the mechanism of action and found that activities of matrix metalloproteinases 2 and 9 were increased by ROS generation in a preliminary study. In addition, low-dose treatment with antimycin (0.1–10 μ M) significantly increased the migration of ASCs (Figure 3B). It appears reasonable to believe that local hypoxia or inflammation induces the migration of ASCs through generation of ROS.

3.4 Differentiation

ASCs have multi-differentiation potentials, which is major mechanism of action underlying tissue regeneration by ASCs. Adipogenic, angiogenic, osteogenic and chondrogenic differentiation has been largely reported [17, 27, 52–54]. Differentiation of ASCs is regulated by a multitude of factors, and ROS are shown to regulate ASC differentiation.

Carriere et al. (2004) tested the implication of mitochondrial ROS in adipogenic differentiation of ASCs [52]. Mitochondrial ROS generation demonstrated a very strong negative correlation with adipogenic differentiation of 3T3 preadipocytes. Moreover, mitochondrial ROS positively controlled the expression of the adipogenic repressor CHOP-10/GADD153. However, there are contradicting reports that ROS generation induces adipogenesis. Kanda et al., recently reported the ROS involvement in adipocyte differentiation of mesenchymal stem cells (MSCs) and found that the increase in the intracellular ROS level via Nox4 mediates adipocyte differentiation in MSCs [62]. Mouche et al., showed that Nox4 is expressed at high levels both in white and brown preadipocytes and that differentiation into adipocytes results in a decrease in their Nox4 mRNA content [53]. These were confirmed *in vivo* by demonstration of localization of Nox4 expressing cells chiefly within the preadipocyte-containing stromal or vascular fraction, rather than in the intact adipose tissue with mature adipocytes.

Although controversial, ASCs are reported to differentiate into endothelial cells as well [63, 64]. Carriere et al. investigated the pro-angiogenic differentiation of human ASCs by mitochondrial ROS generation [27]. Transient stimulation of mitochondrial ROS generation in ASCs did not affect their ability to differentiate into endothelial cells *in vitro*, but strongly improved revascularization and the number of ASC-derived CD31-positive cells *in vivo*. Moreover, ASC preconditioning by mitochondrial ROS effectively protected ASCs against apoptosis. Recently, the age- and ROS-dependent pro-angiogenic potential of ASCs was examined by Effimenco et al. (2011) who found that aged ASCs exhibited impaired angiogenic stimulation which was mitigated by hypoxia preconditioning [54]. In general, ROS generation stimulates angiogenic differentiation of ASCs.

Instead of the direct effect of ROS, there are many articles that investigated the effect of hypoxia on the chondrogenic and osteogenic differentiation. Regulation of chondrogenic and osteogenic differentiation by hypoxia is quite contrastive. While it induces chondrogenic differentiation in one hand, hypoxia inhibits the osteogenic lineage [65-67]. Therefore, preconditioning with hypoxia is recommended for promoting chondrogenic differentiation of ASCs.

3.5 Paracrine effect

The transplanted ASCs act as “building blocks” in the human body. In addition, ASCs exhibit a paracrine effect through the secretion of growth factors. The functional improvement and attenuation of tissue injury following ASC transplantation can be reproduced in part by treatment with a cell-free conditioned medium of ASCs, which supports the paracrine mechanism of ASCs [19, 21, 22]. Secretion of these paracrine factors is reportedly regulated by ROS generation [11, 23, 27, 43]. Rehman et al. first demonstrated an increased secretion of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) under hypoxia, that have been shown to be responsible for the enhanced regenerative potential of ASCs in ischemia models [43]. Direct evidence for the effect of ROS on the secretion of ASCs was reported by Carriere et al. (2009) who found that mitochondrial ROS generated by antimycin and rotenone increased HGF and VEGF production in human ASCs [27]. Our group also found that hypoxia-induced ROS generation increased the VEGF and bFGF expression in ASCs, which was associated with an accelerated wound-healing in animal models [23].

4. ROS-induced apoptosis

Accumulation of ROS secondary to excessive production has the potential to damage cells and is the underlying basis of aging and disease. Overflow of intracellular ROS lead to excess mitochondrial Ca^{2+} entry that eventually triggers cellular apoptosis and necrosis.

Severe cellular stress induces apoptosis of ASCs via ROS generation [8, 41, 49, 55, 56]. For example, exposure of human ASCs to high glucose concentrations significantly increases ROS production and reduces the viability of ASCs [55]. Ceramide induced ROS generation and disruption of mitochondrial membrane potential, and evoked cellular death through both caspase-dependent and caspase-independent mechanisms [41]. Selenium, a powerful ROS scavenger, improved the early undifferentiation markers and survival of ASC [49]. Song et al. modulated antioxidant gene expressions and found that an overexpression of TRX1 and TRX2 increased the proliferation of ASCs by decreasing ROS production [45]. In high intracellular ROS concentration, ROS might damage ASCs and antioxidants could rescue ASCs from apoptosis.

5. Antioxidant and antiapoptotic effect of ASCs

Stem cells efficiently manage oxidative stress [10, 11, 13, 15]. A set of stress response genes is highly expressed in stem cells and they are a high resistance to oxidative stress [14, 15]. Like other stem cells, ASCs are resistant to oxidative stress and play a key role in the protection of neighboring mature cells from oxidative damage. Our group demonstrated their antiapoptotic and antioxidant effects through dermatological application of ASCs and their soluble factors [17, 19, 22, 57]. A conditioned medium of ASCs (ASC-CM) was used to evaluate the antioxidant and antiapoptotic effects of ASCs because these functions are mediated by soluble factors of ASCs [17, 19]. We analyzed the ASC-CM and detected several antioxidant and antiapoptotic proteins such as insulin-like growth factor binding proteins, granulocyte-colony stimulating factor, platelet-derived growth factor, super oxide dismutase, and HGF [19]. The antioxidant and antiapoptotic effect of ASCs was also investigated in dermal fibroblasts and epidermal keratinocytes after inducing oxidative stress by tert-butyl hydroperoxide or UVB [17, 19, 22-]. A cell survival assay revealed that incubation with ASC-CM protected these cells from free radical attack. In a cell-cycle analysis, ASC-CM treatment was reported to reduce apoptosis as evidenced by reduction in caspase-3 activity. In addition, ASC-CM enhanced the superoxide dismutase and glutathione peroxidase activities in fibroblasts and keratinocytes. Collectively, these results indicate that ASCs exhibit an antioxidant/antiapoptotic function, which plays a key role in protecting the neighboring cells from oxidative stress.

6. Conclusion

While excess ROS are involved in apoptosis, low and moderate intracellular ROS levels act as a signal transducer that activates ASCs. PDGFR- β , Akt and ERK pathways are primarily phosphorylated, and related gene expressions are up-regulated. ROS generation serves as a second messenger in the intracellular signal transduction pathway of ASCs. Therefore, a precise control over ROS generation is essential to keep them at appropriate levels that stimulate ASCs and has a positive effect in ASC physiology.

7. Expert opinion

Since generation of ROS stimulates the proliferation, migration, and regenerative potential of ASCs under well controlled conditions, it could be beneficial to expose ASCs to moderate oxidative stress during cell manipulation. The addition of a ROS donor may reduce the costs for the expansion of ASCs in culture, while ROS preconditioning may potentially enhance the regenerative capacity of ASCs in clinical application. However, future studies are needed to address the efficiency of ROS donors and optimal concentrations for ASC stimulation. In addition, future research should be directed to develop a direct approach for monitoring the intracellular ROS concentration in ASCs and for measuring the critical concentration of ROS that determines the fate of ASCs.

Acknowledgments

Declaration of Interest:

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highlights

- ROS at low levels may play a pivotal role as second messengers and activate normal cellular processes of ASCs.
- PDGFR- β , Akt and ERK pathways are phosphorylated, and related gene expressions are up-regulated by ROS generation.
- Intracellular ROS increase the proliferation, migration, and regenerative potential of ASCs.
- The addition of a ROS donor may reduce the costs for the expansion of ASCs in culture, while ROS preconditioning may enhance the regenerative potential of ASCs in clinical use.

Figure 1.
A positive or negative aspect of ROS generation

Figure 2.
Signaling events induced by ROS generation under hypoxia

Figure 3.

Stimulatory effect of antimycin on ASCs. Note that antimycin significantly increased the proliferation (A) and migration (B) of ASCs (n=3, **P<0.01). In addition, mRNA expression of Oct4, Sox2 and Rex1 (C) was increased with antimycin treatment.

Table 1

ROS-induced functional alteration of ASCs.

ROS donor/scavenger	Cell type	Functional alteration	Reference
Hypoxia	Human ASC	Hypoxia generates ROS, which increases the proliferation, migration and growth factor secretion of hASCs	[11]
Antimycin/Rotenone	Human ASC	Preconditioning by antimycin or rotenone (mitochondrial ROS generation) improves the pro-angiogenic potential of ASC-based therapy	[27]
Antimycin/Rotenone	3T3 preadipocyte	Mitochondrial ROS control CHOP-10/GADD153 expression, and trigger hypoxia-dependent inhibition of adipocyte differentiation.	[52]
Rotenone/Oligomycin	3T3 preadipocyte	Inhibition of preadipocyte proliferation by mitochondrial ROS	[44]
Ceramide	Human ASC	Ceramide induces cell death through both caspase-dependent and caspase-independent mechanisms mediated by ROS generation in hASCs	[41]
High glucose level	Human ASC	ASCs isolated from diabetic rats and exposed at high concentration of glucose have an impaired pro-angiogenic action and the functional impairment is partly due to ROS generated by chronic exposure to high glucose concentrations	[56]
NOX4 expression	Human ASC, 3T3 preadipocyte	NOX4 is expressed at high levels both in white and brown preadipocytes and that differentiation into adipocytes results in a decrease in their NOX4 mRNA content.	[53]
Hypoxia and 4-(3,4-dihydroxy-phenyl)-derivative (DHP-d)	Human ASC	Hypoxia and a novel anti-oxidant, DHP-d, directly induce hASCs to de-differentiate into more primitive stem cells and increased migration activity.	[8]
Selenium	Human ASC	Selenium induces improvement in stem cell behavior of hASCs and increases proliferation, migration and telomerase activity	[49]
Mutated inner mitochondrial membrane peptidase 2-like (Immp2l) gene	Mice ASC	Mutant mice showed impaired capability for proliferation, formed significantly few and smaller colonies in colony formation assays, although they retained adipogenic differentiation capability <i>in vitro</i>	[61]