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ORIGINAL ARTICLE

Aging-related changes in oxidative stress response of human endothelial cells

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Abstract

Background Oxidative stress is strongly associated with aging and age-related diseases and plays a crucial role in endothelial dysfunction development.

Aim To better understand the molecular mechanisms of aging and stress response in humans, we examined changes to young and older human endothelial cells over time (72, 96 and 120 h), before and after H_2O_2-induced stress.

Methods We measured the expression of the deacetylase Sirtuin 1 (Sirt1) and its transcriptional target Forkhead box O3a (Foxo3a); TBARS, a well-known marker of overall oxidative stress, and catalase activity as index of antioxidation. Moreover, we quantified levels of cellular senescence by senescence-associated β galactosidase (SA-βgal) assay.

Results Under oxidative stress induction older cells showed a progressive decrease of Sirt1 and Foxo3a expression, persistently high TBARS levels with high, but ineffective Cat activity to counteract such levels. In addition cellular senescence drastically increased in older cells compared with Young cells both in presence and in the absence of oxidative stress.

Discussion By following the cell behavior during the time course, we can hypothesize that while in young cells an oxidative stress induction stimulated an adequate response through activation of molecular factor crucial to counteract oxidative stress, the older cells are not able to adequately adapt themselves to external stress stimuli.

Conclusions During their life, endothelial cells impair the ability to defend themselves from oxidative stress stimuli. This dysfunction involves the pathway of Sirt1 a critical regulator of oxidative stress response and cellular lifespan, underlining its crucial role in endothelial homeostasis control during aging and age-associated diseases.

Keywords Senescence · Oxidative stress · Sirt1 · Cat · Lipid peroxidation · Endothelial cells

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Introduction

Cardiovascular diseases (CVD) are typical age-associated diseases, in which oxidative stress plays a fundamental role [1–3].

During life, vascular endothelium is constantly exposed to oxidative stress, which occurs when reactive oxygen species (ROS) are produced in excess of the endogenous antioxidants. Oxidative stress is one of the mechanisms responsible of widespread endothelial dysfunction that in turn usually underlies the initial pathologic step of several pathological diseases, including CVD [4]. Indeed, endothelium should not be considered a mere lining between blood and tissues, but rather as an organ involved in crucial physiological functions including transportation of small

and large molecules, vascular tone and blood flow regulation [5, 6].

High ROS concentrations often result in indiscriminate damage to all cellular constituents, including DNA, proteins, and membranes. Sirtuin 1 (Sirt1) is an NAD-dependent deacetylase, which modulates many physiological functions, including oxidative stress response, and has a central role in the inhibition of stress-induced senescence, favoring cellular lifespan extension [7, 8]. Sirt1 controls many transcriptional factors, including Foxo3a, a member of the Forkhead box O family (FOXOs), which, in turn, modulates the expression of some genes, such as the antioxidant enzyme catalase (Cat) [9–11].

Indeed, the role played by Sirt1 in the control of oxidative stress response is now widely recognized. By investigating the molecular impact of exercise training upon aging, previously we found that exercise reduced lipid peroxidation, increased antioxidant molecules expression and induced Sirt1 activity within the heart and adipose tissue of aged rats, suggesting that chronic exercise potentiates Sirt1 activity and exerts an antioxidant effect [12]. Moreover, an increase of Foxo3a nuclear expression and of some Foxo3a transcriptional targets [e.g., manganese superoxide dismutase (MnSOD) and Cat] indicated that chronic exercise improves the efficiency of the cellular protection system [12]. Resveratrol, a well-known Sirt1 activator, is a potential candidate for preventing oxidative stress-induced aging in endothelial cells [13]. Furthermore, Ota et al. [14] demonstrated that Sirt1 inhibition results in premature senescence and sustained vascular growth arrest in human endothelial cells.

The aim of this study was to compare the ability of young and old endothelial cells to react to oxidative stress induction over time. For this outcome we used EA.hy-926 human endothelial cell line, which maintains the characteristics of human umbilical vein endothelial cells (HUVEC) and represents a valid tool to remedy the obstacle of limited lifespan of endothelial cells in cell culture [15].

Materials and methods

Cell culture and oxidative stress induction

This work was performed with the established endothelial cell line EA.hy-926 (American Type Culture Collection, Manassas, VA) that maintains the characteristics of HUVEC [15].

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20 % heat-inactivated fetal bovine serum, 100 U penicillin, and 100 µg/ml streptomycin at 37° C in 5 % CO_2.

We evaluated changes occurring during the lifetime of young (PDL 6, "young") and older (PDL 22, "older") EA.hy-926 cells, performing all experiments at 72, 96, and 120 h before and after H_2O_2-induced stress and indicating the endothelial cells (ECs) as "young" and "older" and "Ox young" and "Ox older", respectively.

To induce oxidative stress, the culture medium was aspirated, and cells were exposed to a dose of 500 µM hydrogen peroxide (H_2O_2) for 4 h, as in other previous studies [16, 17]. Then, fresh culture medium was immediately administered to the respective cell cultures.

Western blot

Nuclear cellular extracts were dissolved in $1 \times$ Laemmli buffer, boiled for 5 min, subjected to 10 % SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated overnight with Foxo3a or Sirt1 antibodies (both, 1:1000, Upstate, Lake Placid, NY) according to manufacturer's instructions. After further washing in 0.05 % TTBS, a conjugated goat anti-rabbit polyclonal IgG HRP was used as a secondary antibody. Anti-actin polyclonal antibody (Sigma Aldrich) was used as an internal standard. The blots were visualized with Supersignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and autoradiographed. Protein levels were quantified by scanning densitometry (Quantity One software, Gel Doc-2000, Bio-Rad) and the results are shown as AU. All data are the mean ± SD of three independent experiments.

Lipid peroxidation and catalase activity

Lipid peroxidation was evaluated using an analytical quantitative methodology [18].

It relies upon the formation of a colored adduct produced by the stoichiometric reaction of aldehydes with thiobarbituric acid (TBA). The TBA–TCA solution was prepared by dissolving 0.37 g of TBA in 100 ml of 15 % trichloroacetic acid (TCA) in 0.25 NHCl and butylated hydroxytolouene BHT (7.2 %) was dissolved in ethanol 98 %. The TBARS assay was performed on aliquots (10 µl) of 3×10^5 homogenated cells added to 2 ml of TBA–TCA solution at 100 °C for 30 min. The reaction was stopped by setting the sample in cold water, and after a centrifugation at $15,000 \times g$ for 10 min the chromogen (TBARS) was quantified by spectrophotometric reading at 532 nm. The amount of TBARS was expressed as µM/µg proteins. All data are the mean ± SD of three independent experiments.

Cat activity was determined using the Cayman Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI). Cell lysates were diluted with buffer (1:2) and then incubated

for 20 min in the presence of 3.5 mM of H_2O_2 at room temperature. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The reaction was quenched by the addition of potassium hydroxide. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald; Cayman Chemical, Ann Arbor, MI) as the chromogen.

The absorbance was read at 540 nm using a plate reader. One unit of Cat activity is defined as the amount of enzyme that leads to the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The values were reported as U/μg of protein. All data are the mean ± SD of three independent experiments.

Senescence-associated β-galactosidase activity

Senescence was assessed by β-galactosidase staining. Cells were washed in phosphate-buffered saline (pH 7.4) and fixed with 2 % formaldehyde and 0.2 % glutaraldehyde for 10 min at room temperature. After being washed twice, the cells were incubated at 37 °C for 4 h in a humidified chamber with freshly prepared staining solution (1 mg/ml X-Gal in dimethylformamide, 40 mM citric acid and phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride). At the end of the incubation, the SA-β-gal rate was obtained by counting four random fields per dish and assessing the percentage of SA-β-gal-positive cells from 100 cells per field.

Statistical analysis

All values are reported as mean ± SD. A Shapiro–Wilk's test ($p > 0.5$) [19, 20] and a visual inspection of their histograms, normal QQ plots showed that the levels were normally distributed for all groups. Student's t test was performed on paired data to assess differences within groups. ANOVA analysis was performed to compare the groups and a post hoc Scheffè to identify the differences among groups. A p value <0.05 was considered to be statistically significant. All data were analyzed with the SPSS 21.0 statistical software (SPSS, Chicago, IL).

Results

We evaluated changes occurring during serial passaging of young (PDL 6) and older (PDL 22) EA.hy-926 cells (ECs), performing all experiments at 72, 96, and 120 h, before ("young" and "older") and after ("Ox young" and "Ox older") H_2O_2-induced stress.

Sirt1 and Foxo3a expression

The expression of Sirt1 and Foxo3a in young and older ECs was measured before and after the induction of oxidative stress (Fig. 1, panel A, B).

The increase in Sirt1 (panel A) correlated to rise in Foxo3a expression in both the young and older ECs (panel B). Under stress condition, the Ox Young ECs enhanced the expression of both Sirt1 and Foxo3a over time (all comparisons $p < 0.000$). On the contrary, in the Ox older
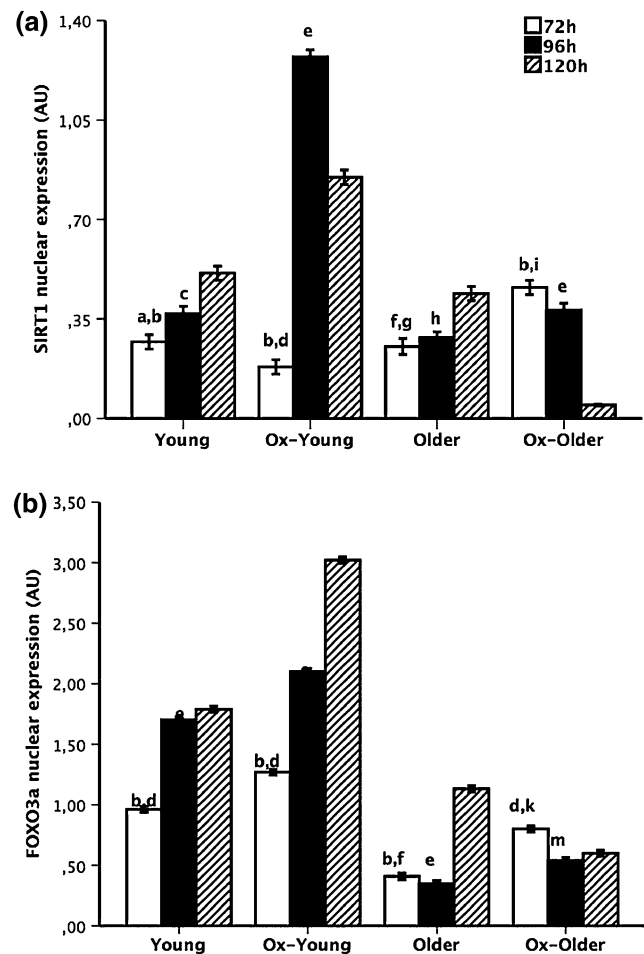

Fig. 1 Sirt1 and FOXO3a nuclear expression in the ECs groups over time. The increase in Sirt1 (*panel* **A**) correlated to rise in Foxo3a expression in both the young and older ECs (*panel* **B**). Under stress condition, the Ox young ECs enhanced the expression of both Sirt1 and Foxo3a over time. On the contrary, in the Ox older ECs both the expression of Sirt1 and Foxo3a strongly decreased over time (*panel* **A**, **B**). In Ox young, Sirt1 expression levels at 120 h remained higher when compared with the levels recorded in the other cellular groups (*panel* **A**). The results showed the mean values ± SD of three independent experiments. *a* 72 vs 96 h $p = 0.013$, *b* 72 vs 120 h $p < 0.000$, *c* 96 vs 120 h $p = 0.007$, *d* 72 vs 96 h $p < 0.000$, *e* 96 vs 120 h $p < 0.000$, *f* 72 vs 96 h $p = 0.034$, *g* 72 vs 120 h $p = 0.004$, *h* 96 vs 120 h $p = 0.003$, *i* 72 vs 96 h $p = 0.020$, *k* 72 vs 120 h $p = 0.003$, *m* 96 vs 120 h $p = 0.036$

ECs both the expression of Sirt1 and Foxo3a strongly decreased over time (Fig. 1, panel A, B). In Ox Young, Sirt1 expression drastically increased between 72 and 96 h (+100 %) and then decreased between 96 and 120 h (−20.7 %) remaining higher than value measured at 72 h. In addition, this expression level was higher when compared with the levels recorded in the other cellular groups (all comparisons $p < 0.000$) (Fig. 1, panel A). In Table 1 the intergroup significant p values are reported.

Ox older cells showed persistently high TBARS levels, with high, but ineffective, Cat activity

With regard to lipid peroxidation (measured by TBARS), young and older ECs showed a similar behavior, on the contrary, the Ox young cells showed a decrease of TBARS over time, while the Ox older cells showed a decrease between 72 and 96 h (−43.13 %) followed by an increase of TBARS between 96 and 120 h (+25 %). Of note, at the end of the time course, Ox young raised a level of TBARS lower when compared with the level of young ECs ($p < 0.000$).

Importantly, even if the TBARS levels decreased also in Ox older over time, they remained very higher compared with those recorded in all other groups (vs young $p < 0.000$; vs Ox young $p < 0.000$; vs older $p = 0.001$) (Fig. 2, panel A).

Both young and older ECs showed an increase of Cat activity from 72 to 120 h, but the Older ECs raised a larger augment of the enzyme activity at 120 h (older vs young, $p < 0.000$). After oxidative stress induction, the Ox young ECs demonstrated a drastic increase from 72 to 96 h followed by a decrease from 96 to 120 h of Cat activity. On the other hand, the Ox older ECs showed a progressive increase of Cat activity over time, reaching a very high level at the end of time course (Fig. 2, panel B). In Table 1 the intergroup significant p values are reported.

Endothelial cell senescence

Both in the absence and in the presence of stress, young and older ECs displayed a similar behavior showing a progressive β-gal activity increase over time. Of note, the senescence levels recorded in Ox older were much higher than in Ox young ($p = 0.001$) (Fig. 3). In Table 1 the intergroup significant p values are reported.

Discussion

The aim of the present study was to investigate the differences between young and older endothelial cells during serial passaging and to evaluate the ability of these cells to defend themselves from oxidative stress induction.

Table 1 The statistical p values by ANOVA and Scheffè post hoc intergroup analysis

	Scheffè post hoc		
	72 h	96 h	120 h
Sirt1 nuclear expression			
Young			
Ox young	<0.0001	<0.0001	<0.0001
Older	0.377	<0.0001	<0.0001
Ox older	<0.0001	0.566	<0.0001
Ox young			
Older	<0.0001	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Older			
Ox older	<0.0001	<0.0001	<0.0001
ANOVA	<0.0001	<0.0001	<0.0001
Foxo3a nuclear expression			
Young			
Ox young	<0.0001	<0.0001	<0.0001
Older	<0.0001	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Ox young			
Older	<0.0001	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Older			
Ox older	<0.0001	<0.0001	<0.0001
ANOVA	<0.0001	<0.0001	<0.0001
TBARS levels			
Young			
Ox young	<0.0001	<0.0001	<0.0001
Older	0.007	0.067	0.002
Ox older	<0.0001	0.001	<0.0001
Ox young			
Older	<0.0001	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Older			
Ox older	<0.0001	0.067	<0.0001
ANOVA	<0.0001	<0.0001	<0.0001
Cat activity			
Young			
Ox young	0.057	<0.0001	0.693
Older	0.055	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Ox young			
Older	0.98	0.6	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Older			
Ox older	<0.0001	<0.0001	<0.0001
ANOVA	<0.0001	<0.0001	<0.0001
Beta-galactosidase levels			
Young			

Table 1 continued

	Scheffè post hoc		
	72 h	96 h	120 h
Ox young	<0.0001	<0.0001	<0.0001
Older	<0.0001	0.019	<0.0001
Ox older	<0.0001	0.002	<0.0001
Ox young			
Older	0.98	<0.0001	<0.0001
Ox older	<0.0001	<0.0001	<0.0001
Older			
Ox older	<0.0001	0.287	<0.0001
ANOVA	<0.0001	<0.0001	<0.0001

Oxidants (such as hydrogen peroxide and reactive lipid species) act as cell signaling molecules, but they can be inductors of pathological mechanisms in dependence of their dose and the cellular context [21, 22].

Physiologically there is a balance between oxidant and antioxidant species, but this equilibrium is compromised during aging and diseases leading to a condition, commonly called "oxidative stress", that is often associated with endothelial dysfunction [23, 24].

We here investigated the role of Sirt1, now largely recognized as a crucial factor in the control of the cellular redox state homeostasis, proposing that this protein could act as redox stress sensor addressing the cells forward an adequate response.

In a previous research, we studied the effects of exercise training on heart and adipose tissue in aged rats and found that exercise was a natural inductor of Sirt1. We recorded an increase in the expression of Foxo3a and antioxidant scavengers such as MnSOD and Cat, while a reduction of lipid peroxidation occurred, suggesting that Sirt1, by control of Foxo3a, promoted the antioxidant effects of the training. Noteworthy, Cat expression but not MnSOD expression significantly decreased in the heart of aged rats [12].

Moreover, in other studies we demonstrated that exercise training induced antioxidant and anti-senescent effects in human endothelial cells via modulation of Sirt1 and Cat activities [16, 17, 25, 26].

The present investigation emphasized the role of Sirt1 in the determination of aged-associated changes in the oxidative stress response of endothelial cells.

Sirt1 and Foxo3a expression progressively increased over time both in young and older endothelial cells. Of note, in Ox young cells a progressive increase of Sirt 1 expression corresponded to an increase of Foxo3a, while in the Ox older cells a drastic decrease in both Sirt1 and Foxo3a expression occurred.
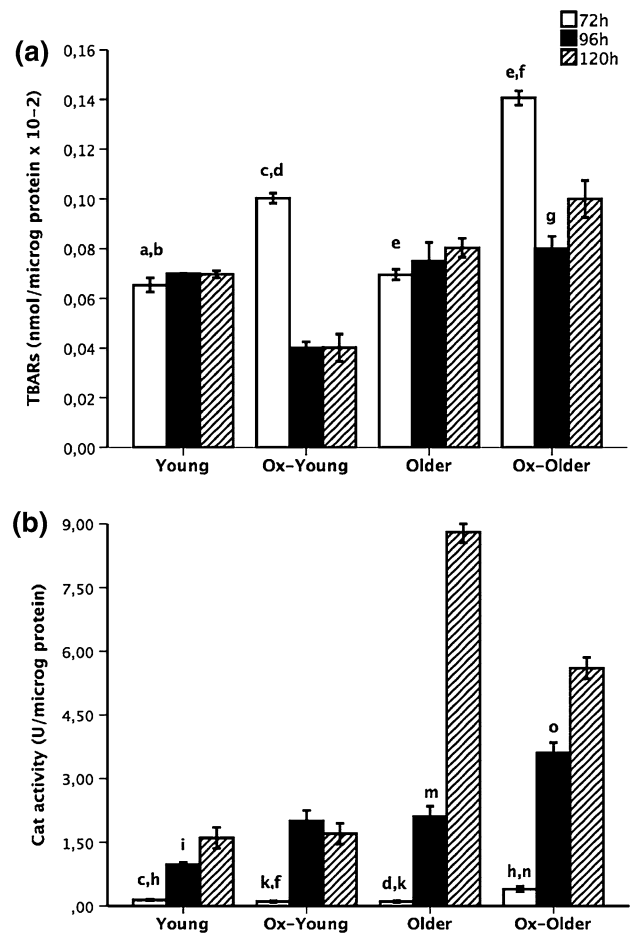
Fig. 2 TBARS and catalase activity in the ECs groups over time. The TBARS levels decreased in Ox older over time, but remained very higher compared with all other groups (*panel* **A**). Both young and older ECs showed an increase of Cat activity from 72 to 120 h, but the Older ECs raised a larger augment of the enzyme activity at 120 h. After oxidative stress induction, the Ox young ECs demonstrated an increase from 72 to 96 h followed by a decrease from 96 to 120 h of Cat activity. On the other hand, the Ox older ECs showed a progressive increase of Cat activity over time, reaching a very high level at the end of time course (*panel* **B**). The results showed the mean values ± SD of three independent experiments. *a* 72 vs 96 h $p = 0.020$, *b* 72 vs 120 h $p = 0.039$, *c* 72 vs 96 h $p < 0.000$, *d* 72 vs 120 h $p < 0.000$, *e* 72 vs 120 h $p = 0.010$, *f* 72 vs 120 h $p = 0.001$, *g* 96 vs 120 h $p = 0.001$, *h* 72 vs 120 h $p = 0.002$, *i* 96 vs 120 h $p = 0.010$, *k* 72 vs 96 h $p = 0.001$, *m* 96 vs 120 h $p < 0.000$, *n* 72 vs 96 h $p = 0.012$, *o* 96 vs 120 h $p = 0.003$

Moreover, in young endothelial cells, a transient oxidative stress induced a longer term increase in Cat activity with reduction in TBARS; by contrast, the older cells showed persistent high TBARS levels associated with high, but ineffective Cat activity to counteract such stress.

Interestingly, while in young, older and also in Ox older we observed an increase of Cat activity over time, in Ox young Cat activity first increased and then decreased at the end of the time course, as Sirt1 expression. It is conceivable that in young cells the Sirt1 pathway is more effective
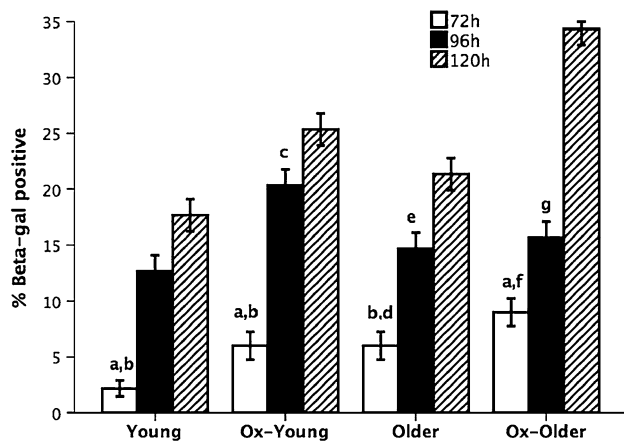
Fig. 3 Endothelial cell senescence in the ECs groups over time. Both in the absence and in the presence of stress, young and older ECs displayed a similar behavior showing a progressive β-galactosidase activity increase over time, with the senescence levels recorded in Ox older much higher than in Ox young. The results showed the mean values ± SD of three independent experiments. a 72 vs 96 h $p = 0.001$, b 72 vs 120 h $p = 0.000$, c 96 vs 120 h $p = 0.013$, d 72 vs 96 h $p = 0.000$, e 96 vs 120 h $p = 0.002$, f 72 vs 120 h $p = 0.001$, g 96 vs 120 h $p = 0.001$

and, consequentially, young cells are more able to defend themselves from oxidative stressors when compared with older cells. The drastic increase in Cat activity of older cells at 120 h could be difficult to explain and this represents a limitation of the study. Indeed, apart from H_2O_2 detoxification, Cat has a role in the protection against NO/peroxynitrite [27] and it is implicated in the regulation of angiogenesis and neovascularization [28] and apoptosis [29]. Therefore, it is possible that the high level of Cat activity in older cells could be linked to the involvement of this enzyme in processes not investigated here.

Many authors assigned to Sirt1 a key role in aging and cellular senescence [30]. Recently, Hwang et al. [31] demonstrated that oxidative stress could lead to reduction of Sirt1 and its control on target proteins including Foxo3a, thereby enhancing the senescence as well as endothelial dysfunction.

We showed that during time course, the cellular senescence progressively increased in young and in older cells and, actually, the Sirt1 pathway was activated in both cell groups. It seems that an accelerating aging occurred during the cells culture. However, the significant differences found between groups cannot be influenced by this event because all the cells culture had the same start condition, and were plated and settled in the same way. Of note, after stress induction Ox older raised a level of senescence much higher when compared to Ox young cells.

Taken together, these findings suggest that older endothelial cells suffered by a severe impairment in the activation of oxidative stress response. By following the cell behavior during the time course, we can hypothesize that while in young cells an oxidative stress induction stimulated an adequate response through activation of molecular factor crucial to counter oxidative stress, the older cells are not able to adequately adapt themselves to external stress stimuli.

Conclusions

During their life, endothelial cells impair the ability to defend themselves from oxidative stress stimuli. This dysfunction involves the pathway of Sirt1, a critical regulator of oxidative stress response and cellular lifespan, underlining the crucial role of Sirt1 in endothelial homeostasis control during aging and age-associated diseases.

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