Modulation of mTOR and autophagy in hibernating hamster lung and the application of the potential mechanism to improve the recellularization process of decellularized lung scaffolds

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Abstract

During hibernation, hamsters cycle through periods of deep cooling (torpor) and re-warming (arousal). Reversible lung remodeling was previously observed through hibernation, where smooth muscle actin (SMA) expression was increased during torpor and normalized at arousal, sustaining lung structure throughout torpor. In the present research, the activation of mTOR (mammalian target of rapamycin) which is known to have an important role in remodeling and SMA protein expression was studied in hamster lung. Further, lung serotonin level known to modulate mTOR pathway was investigated. To determine if an increase of serotonin in lung tissue could be affecting SMA expression, cell number and cell matrix adhesion molecules, the effect of serotonin on Human Bronchial Smooth Muscle Cells (HBSMC) was investigated. Further, the potential application of serotonin in recellularization of decellularized rat lungs was studied *in vitro*. In hibernating hamster, mTOR, p-Akt and p-S6 levels decreased during torpor phase but were all upregulated during early arousal. Autophagy increased during torpor and reached maximum value during arousal. Serotonin treatment of HBSMC increased cell number and protein synthesis including SMA expression. It activated mTOR while increasing the expression of adhesion molecules, integrin $\alpha 1$, $\beta 1$ and β -sarcoglycan in cells. Further, serotonin increased cell attachment to decellularized lung matrix, supporting ECM structure. Aberrations in proteostasis have negative effects on cellular growth and metabolism. This research provides new information on regulation of proteostasis in hibernating hamster lung with potential implications on regenerative medicine. It accordingly, further introduces a new approach to more efficient recellularization of lung scaffolds *ex vivo*.

Keywords: Serotonin, adhesion molecules, smooth muscle actin, reactive oxygen species

Introduction

The energy-saving strategies are used by diverse species of mammals to survive stressful environmental conditions. One of these strategies known as hibernation is a unique physiological state characterized by a profound yet reversible sleep-like state. In torpid animals, metabolic rate is decreased to about 2% while the demand for oxygen declines drastically and the heart rate slows, thus eliminating the need for food during the cold winter months. Mammalian hibernation requires an extensive reorganization of metabolism that typically includes a greater than 95% reduction in metabolic rate, selective inhibition of many ATP-consuming metabolic activities and a change in fuel use to a primary dependence on the oxidation of lipid reserves. In an ambient temperature of 5°C, the final body temperature of a hibernating Syrian hamster decreases to around 6-8°C during torpor. In torpor, many physiological processes are substantially suppressed, including a drop in breathing rate from 84±20 to 5-7 times per minute [1]. Typically, the arousal period starts after 3-4 days of torpor and the body temperature rises to the normal value of around 37°C [2]. Investigation of mechanisms of mammalian hibernation and the application of potential findings about this physiological phenomenon to

medical fields has strongly been desired [3].

Hibernating animals have a low rate of protein synthesis and metabolism throughout the torpid phase which in combination with different bio-molecular and structural alterations help them to survive the harsh winter environmental conditions. It was previously demonstrated that structural changes of the bronchial airway through hibernation is similar to what could be observed in lung remodeling as seen in obstructive lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and lung fibrosis [2]. Airway remodeling brings about structural changes in the number, mass, size and/or composition of the airway tissues which are manifested by an increase in collagen and SMA (smooth muscle actin) that could lead to peri-bronchial and peri-bronchoalveolar fibrosis and smooth muscle cell hyperplasia in asthma. The main characteristics of lung remodeling observed during torpor in the Syrian hamster are the increase in collagen and SMA accompanied by significant asthma-like structural changes such as increased matrix deposition in airway wall and increased airway smooth muscle mass leading to wall thickening together with changes in remodeling-associated proteins such as upregulation of TGF- β and ACE but without any significant involvement of immune

system response in the process of remodeling [2,4,5]. The changes observed in hamster lung were mainly located around the bronchioles, veins and the alveoli.

Torpor imposes an increase in intra pulmonary pressure/stress due to very low breathing rates which might threaten to completely deflate the lungs. A similar form of stress on lungs is known to occur under low atmospheric pressure, a condition observed in high altitudes and the consequent lung remodeling [6]. Indeed, remodeling increases pulmonary vascular and bronchial resistance leading to hypertrophy [7]. Lung hypertrophy manifests by an increase in the mass of the cells such as smooth muscle cells, a condition commonly observed during lung remodeling [7]. Thus, mammalian target of rapamycin (mTOR) a kinase known to regulate translational machinery and cell size is very important in regards to tissue remodeling [8]. This kinase responds to multiple extracellular signals (eg., insulin, mitogenic growth factors) that stimulate cell proliferation which could have an essential role in reversible remodeling observed throughout hibernation.

It has been previously found that hamster DDT-1 muscle cells contain serotonin which protects them against hypothermia re-warming induced damage including ROS damage and apoptosis [9]. Serotonin which can work directly as a growth factor is involved in smooth muscle cell mitogenesis and protein synthesis [10]. Serotonin indeed increases cAMP level which is known to be involved in the process of remodeling through serotonin receptors [11]. Further, serotonin increases protein synthesis (anabolic pathways) activating mTOR kinase [9] and thus changes in serotonin concentration in Syrian hamster lung during hibernation might be contributing to reversible lung remodeling.

The aim of this research was to study the changes in mTOR activity and autophagy in relation to serotonin in both hibernating hamster lung and serotonin treated lung smooth muscle cells. Further, as serotonin is implicated in the process of smooth muscle cell growth and SMA expression, the potential effects of serotonin on recellularization of decellularized lung scaffolds was studied *in vitro*.

Methods and materials

Experimental procedures and hibernation model

Twenty (male and female) Syrian golden hamsters (*Mesocricetus auratus*) were subjected to a documented hibernation protocol. In brief, the light:dark-pattern and ambient temperature were respectively lowered after 3 weeks under short-day conditions from 20°C to 5°C and changed to continuous dim light (<1 Lux). Animals were sacrificed by means of an i.p. injection of an overdose of 1.5 ml 6% sodium pentobarbital. Animals were sacrificed on day 1 after entering torpor (torpor early, TE; n=4), on day 3 of torpor (torpor late, TL; n=4), 1.5 hours after onset of arousal (early arousal, EA; n=4) and 8 hours after starting arousal (late arousal, LA; n=4). Summer euthermic animals (EU, n=4) served as controls. Lungs from each phase were flash frozen for analysis. For western blot analyses, frozen

lungs were homogenized (20% w/v) in ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 40 µg/ml PMSF, 100 µg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml aprotinin in PBS) [5].

Cell culture

Human Bronchial Smooth Muscle Cells (HBSMC) were purchased from Promo cells (C-12561) and maintained either in 6 well plates to study serotonin treatment or in 150 cm² culture bottles for cellularization of decellularized lung. All culture plates were kept at 37°C with 5% CO₂ in a low-serum (5% V/V) promoCell Smooth Muscle Cell Growth Medium. Serotonin at a concentration of 150 nM, the same lung serotonin concentration found during arousal phase in hibernation, was used for all the experiments.

To investigate to which extend the potential effects of serotonin on recellularization is due to its ROS inhibiting effects, diphenylene iodonium (DPI) (20 µM) a competitive inhibitor of flavin-containing cofactors which is a potent inhibitor of NOX-dependent ROS production known to also block mitochondria-derived ROS production, was added in a separate experiment to cells and the effects of this ROS production inhibitor on recellularization was investigated.

Measurement of protein synthesis in HBSMC

To show the presence of active protein synthesis in HBSMC and the effect of serotonin treatment on protein synthesis levels, the ratio of protein-to-DNA known as an indice of protein synthesis capacity, was calculated in each sample (non-treated controls and 150 nM serotonin treated). Total genomic DNA was isolated from each sample using the Nucleospin Tissue Kit (Macherey-Nagel, Duren, Germany). The DNA concentration in each sample was measured using a NanoDrop spectrophotometer. Protein concentration in each sample was measured using the Bradford assay.

Assessment of intracellular thiol groups

After 3 days of treatment with serotonin, total levels of cellular sulfhydryl groups (-SH groups) were measured based on the reduction of 5,5 dithiobis-2-nitrobenzoic acid (DTNB, Sigma) and compared to non-exposed control to investigate if serotonin was upregulating this very important intracellular antioxidant which is known to have different effects on cell pathways in relation to cell proliferation and division, beyond its antioxidant activity. Cells were lysed in 10mM Tris-buffer 1% Triton X-100 (Sigma), followed by centrifugation at 7000 x g for 10 minutes. The supernatant was incubated with phosphate buffer containing DTNB at room temperature for 60 minutes. The quantification was conducted at 412 nm using a microplate reader.

Antibodies

Several antibodies were used for western blot analysis and

immunohistochemistry. The antibodies used were; P-ERK1/2 (cell signaling technology 9101), santacruz Integrin β 1 Antibody (M-106) sc-8978, Integrin α 1 (sc-6584), Integrin β 1 (sc-374429), Sarcoglycan (sc-100956), SMA (MO 851; Dako, Glostrup, Denmark), anti-phospho-mTOR (Millipore 15-105 Pathway Explorer Anti-phospho-mTOR^{ser 2448}), mTOR (Cell Signaling, 7C10), anti-LC3 antibody (Thermoscientific, PA1-16930), EGFR (Santa Cruz SC-03, USA) and TGF- β (Santa Cruz SC-146).

Lung serotonin concentration

Lung samples obtained from each animal were used to measure serotonin concentration. In brief, to extract serotonin and its metabolites from lung, lungs were lysed on dry ice using a lysis buffer without β -mercaptoethanol and the protein concentration in the lysate was determined by the Bradford protein assay. Serotonin was isolated from every 100 μ g protein in each lung lysate. In brief, 100 μ l ethanol was added to every 50 μ l of lung lysate containing 100 μ g protein to precipitate proteins. Ethyl acetate (300 μ l) was added to each supernatant, vortexed for 10 s and centrifuged for 5 min at 2500 rpm. The ethyl acetate layer was transferred to another tube and its content was dried by cold air flow. Ehrlich reagent (50 μ l) was added to each tube containing the dried extract and warmed to 60°C. After 2 hr the intensity of the blue color representing the concentration of serotonin was measured using a 384 well plate with a UV microplate reader at 625 nm. Standard curves were drawn using different concentrations of serotonin to extrapolate the data obtained from lung samples.

Lung immunohistochemistry

For immunohistological evaluation of decellularized and recellularized lungs, lungs were zinc fixed, paraffin embedded, cut in 3 μ m sections, deparaffinized and submitted to staining [12]. Antibody staining was performed by incubating sections with primary antibody against SMA (1:100) with 1% BSA for 1 hr and subsequently washed three times with PBS. Next, sections were incubated with secondary antibody (1:100) with 1% BSA and 1% rat serum for an hour and subsequently washed 3 times with PBS. Dako AEC+High sensitivity substrate chromogen was used to visualize the antibody stain in reddish brown. To demonstrate the localization of the protein in lung sections a Nikon 50i light microscope with a Paxcam camera add on was used to capture the areas around the bronchioles, veins and the alveoli.

To evaluate the percentage of lung area containing SMA after recellularization, a morphometric analysis was performed using Leica Qwin image analysis software (Leica Microsystems).

Decellularization and recellularization of lung

Lungs were decellularized by a detergent perfusion method yielding scaffolds with acellular vasculature, airways and alveoli. In brief; A low-concentration (0.1%) sodium dodecyl sulfate (SDS)-based protocol applying a physiologic perfusion pressure was found to yield acellular whole lung scaffolds.

Histological evaluation revealed no remaining nuclei in lung parenchyma, while the matrix architecture of airways and vasculature was not compromised. To test whether acellular whole lung scaffolds could be repopulated with smooth muscle cells and maintained in culture, serotonin treated and control cells were introduced into the acellular rat lungs using a standard syringe needle and kept in 6 well plates. The reseeded lung constructs were cultured using cell medium at 37°C for up to 5 days. The medium was changed every 6 hours to provide for adequate nutrition and oxygenation of cells inside the scaffolds. Serotonin is known to protect against ROS damage [13]. To evaluate the effect of serotonin in protecting cells in lungs against damage induced by the process, ROS content and Caspase activity were measured. Further, immunohistochemical analysis of SMA expression in lung was performed.

Oxygen radicals present in each sample were determined by a fluorometric method using fluorogenic CM-H2-DCFDA (2,7 dichlorofluorescein diacetate). 10 μ l of each tissue sample in RIPA was loaded into a 96-well FluoroNunc black plate. 150 μ l of 0.1 M phosphate buffer (pH 7.4) and 10 μ l of CM-H2-DCFDA (2,7 dichlorofluorescein diacetate) in methanol was added to each well. The plate was incubated in a water bath (37°C) for 30 min and centrifuged at 12,000 $\times$ g for 8 min. Fluorescence was measured with a Hitachi fluorescence spectrometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Auto fluorescence was subtracted prior to DCF fluorescence [14].

To measure Caspase 3/7 activity, tissue samples were lysed in 50 mM potassium phosphate buffer (pH 6.9, 5%, w/v), sodium orthovanadate 10 mM and protease inhibitor. Bradford assay was conducted and 500 μ g of protein was deposited in each well of a black Immuno plate (Nunc) and diluted to 50 μ l with the lysis buffer. Fifty μ l of Caspase substrate (Promega Apo-ONE[®]) was added to each well. The plates were left on a shaker for 3 h and fluorescence was read at an excitation of 499 nm and emission maximum at 521 nm.

Statistical analysis

The statistical data analyses, based on the analysis of ten slices each from four animals, were performed using the One-way ANOVA with Tukey's test or unpaired t-test with Welch's correction. Statistical significance difference was accepted at $P < 0.05$ (GraphPad Prism version 5).

Results

MTOR activity, autophagy, p-ERK levels and serotonin concentration in hamster lung

MTOR directly regulates protein synthesis in response to different stimuli. To investigate the changes in mTOR activity in relation to autophagy and extracellular signal-regulated kinases (ERK) which regulate various aspects of cell physiology including proliferation and importantly have an established role in remodeling process, these proteins were studied in

hamster lung. Further, serotonin concentration known to affect the activity and amount of these proteins was measured in lung tissues. It was found that there was a decrease in mTOR activity (**Figure 1C**) during early torpor most probably due to inhibition of phosphorylation of mTOR protein (**Figure 1A**) rather than the expression of mTOR (**Figure 1B**). The activity of

mTOR was increased during arousal in hamster lung (**Figure 1C**). Autophagy was highly increased during late torpor and early arousal (**Figure 2C**), which is most probably due to an increase in LC3-II (**Figure 2B**) compared to LC3-I (**Figure 2A**).

ERK phosphorylation was decreased during torpor and increased during early arousal (**Figure 3A**).

Figure 1. MTOR activity in hamster lung throughout hibernation phases. (A) phosphorylation of mTOR protein (B) expression of mTOR (C) mTOR activity. Data are Means $\pm$ SD (n $\geq$ 3 per group) * difference to euthermia, # difference to torpor or torpor early, P<0.05. Western blot expression is normalized to β -actin; lanes of western blot insets are in the same order as in the X-axis.

Figure 2. Autophagy levels in hamster lung throughout hibernation phases. (A) LC3-I expression, (B) LC3-II expression, (C) LC3 II/LC3-I showing autophagy levels. Data are Means $\pm$ SD (n $\geq$ 3 per group) * difference euthermia, # difference to torpor, P<0.05. Western blot expression is normalized to β -actin; lanes of western blot insets are in the same order as in the X-axis.

The change in lung serotonin concentration was investigated at different phases of hamster hibernation. Concentration of lung serotonin was slightly increased during torpor early, decreased during the torpor late phase and was increased during arousal phase (Figure 3B).

Analysis of the effects of serotonin treatment on multiple parameters in HBSMC

To investigate the effect of serotonin on protein synthesis,

autophagy and the expression of several adhesion molecules, serotonin was added to cultured HBSMC *in vitro* and western blot analysis was performed. Serotonin increased mTOR activity (Figures 4A and 4B) and protein synthesis (Figure 4C) while it inhibited autophagy (Figure 4D and 4E). Serotonin also increased cell number compared to non-treated control cells (Figure 4F). Further serotonin increased the expression of cell_ECM adhesion molecules Sarcoglycan β (Figure 4G) and Integrins $\alpha 1$ and $\beta 1$ (Figure 4H). It also increased SMA levels in treated cells compared to control (Figure 4I). It was surprisingly noted that the addition of a ROS blocker (DPI) to the cell medium did not only slightly but significantly inhibit normal cell proliferation but it also inhibited serotonin induced cell proliferation (Figure 4F), and finally it decreased SMA production by these cells (Figure 4I).

Lung recellularization and cellular damage analysis

To investigate if the effects of serotonin or a ROS blocker (DPI) on lung cells could facilitate lung recellularization, lungs were decellularized and scaffolds were recellularized using serotonin and DPI treated and untreated HBSMC (Figure 5A). The general outer morphology in serotonin treated lungs (Figure 5B) was very similar to a normal lung while bullous lesion formations and indentations could be observed on untreated recellularized control lungs (Figure 5C). This could probably point to the structural instability of recellularized lung scaffold. Decellularized lungs maintained ECM structure (Figure 5E) while no SMA was detected compared to control (Figure 5D). After 5 days of isolated organ culturing, the cells were fully dispersed and engrafted throughout the scaffold. In recellularized lungs the presence of SMA was mostly observed around the bronchi and veins although in serotonin treated lungs the dispersion was more structured with higher cell-ECM binding and SMA expression (Figure 5F) compared to control (Figure 5G). To show to what level the presence of serotonin had improved recellularization of lung and to which degree its ROS inhibiting properties were involved in the process photos were made from cross sections of the recellularized lungs and the area covered by SMA was measured in each sample. The area covered by SMA was higher in serotonin treatment compared to control (Figure 5I) but DPI a ROS production blocker (Figure 5H) did not improve recellularization compared to untreated lung. ROS formation and Caspase activity were significantly lower in serotonin treated samples compared to control (Figure 5J and 5K). ROS blocker, DPI, did not facilitate lung recellularization (Figure 5H). Finally, serotonin treatment also upregulated EGFR and TGF- β , known to become up-regulated in hamster lung during early arousal (Figure 5L and 5M).

Discussion

Lowering body temperature during torpor enables hibernators to survive through cold winter months when food sources are limited. Hibernating animals protect their organs through

Figure 4. The effect of serotonin on lung smooth muscle cells, (A) mTOR and p-mTOR expression, (B) mTOR activity as per p-mTOR/mTOR ratio, (C) Protein synthesis levels, (D) LC3-I and LC3-II expression (E) Autophagy levels as per LC3-II/LC3-I ration, (F) Cell number, (G) Sarcoglycan β , (H) Integrin α 1 and integrin β 1 expression as cell-ECM adhesion molecules, (I) SMA α expression. Data are Means $\pm$ SD (n $\geq$ 3 per group) * difference to non-treated cells, # difference to serotonin treated cells P<0.05. Western blot expression is normalized to β -actin; lanes of western blot insets are in the same order as in the X-axis.

Figure 5. Lung decellularization and lung scaffold recellularization using serotonin treated and untreated HBSMC. (A-C) Lungs were decellularized and recellularized using HBSMC, (D) Control lung, (E) Decellularized lungs maintained ECM structure but no SMA was detected compared to control, (F) After 5 days, serotonin treated cells were fully dispersed and engrafted throughout the recellularized scaffold, (G) Scaffold containing untreated cells control, (H) Scaffolds containing DPI treated cells, show similar or lower recellularization levels compared to scaffolds containing untreated cells (I) Area covered by SMA, (J,K) Caspase activity and Ros formation were significantly lower in serotonin treated samples compared to untreated control, (L) Expression of EGFR in serotonin treated lungs, (M) Expression of TGF-β in serotonin treated lungs. Data are Means ± SD (n ≥ 4 per group) * difference to control lung, # difference to untreated recellularized lung, + difference to serotonin treated lung P < 0.05.

different mechanisms. For example the metabolic rate is highly decreased during the torpor phase and the damage sensitive tissues such as brain and lung go through changes which are similar to tissue remodeling [2]. The very low breathing rates during torpor should normally impose a very high level of pressure on lungs which would damage pulmonary structure due to lung deflation and collapse. As hibernating hamsters do not show any sign of lung damage

during arousal, remodeling might be protecting the structure of lung throughout the torpor phase [2]. This remodeling was found to be reversible during arousal phase without significant tissue damage. Due to the importance of protein synthesis in remodeling, mTOR, a kinase known to control protein synthesis and anabolism was studied. It was found that mTOR was significantly less active during torpor while it was highly activated during arousal. Autophagy, a basic

catabolic mechanism, was upregulated during the late phase of torpor and remained high in early arousal. The simultaneous activation of anabolic and catabolic pathways during early arousal might have an important role on the reversibility of lung remodeling and prevention of significant lung damage.

All metabolic reactions can be divided into either catabolic or anabolic reactions. Autophagy is the transportation and degradation of damaged, denatured or aged proteins. It is a common cellular physiological process which can maintain cell homeostasis, specially the breakdown of cellular components can ensure cellular survival during starvation by maintaining cellular energy levels. Indeed hibernating animals lose body fat during torpor while the muscle mass is protected from catabolism despite long periods of inactivity and lack of food intake [15]. Interestingly it was even found previously that SMA was increased in hamster lung during the late torpor phase, potentially indicating that protein synthesis is to some degree active throughout this phase [2]. According to the present data obtained from recently conducted experiments and the previously published work, it could be deduced that autophagy does not affect muscular tissue during torpor but might have an effect on collagen content as collagen levels are decreased while torpor phase progresses. Early arousal though brings about a reduction in SMA content which could be due to higher autophagy levels during this phase and a reduction in a still not recognized muscle protecting substance which might only be present during torpor. Researchers have shown that a muscle maintaining protein, SKG1 (serum/glucocorticoid-induced kinase 1) is activated during torpor [16] which could be protecting muscle mass. Autophagy has been suggested to simultaneously contribute to cell metabolism and initiate a pathway to sensitize cells to apoptotic cell death [17]. Thus, although to exclude toxic, damaged proteins, the higher levels of autophagy might be necessary during early arousal, hamsters probably have developed a counter mechanism to prevent unnecessary tissue brake down by activating protein synthesis pathways to repair any potential damage and prevent healthy tissue breakdown. MTOR is an essential kinase in protein synthesis and is known to be activated by different growth hormones and amino acids. As it was previously shown that serotonin, a potential growth factor, is present in hamster cells and protects different non-hamster cells against hypothermia induced damage [9], serotonin and its metabolites were measured in hamster lung lysates. Serotonin was found to be higher in lung during early torpor and arousal. Serotonin is a well known neurotransmitter, whose actions have hitherto been attributed mainly to result from stimulation of 5-HT receptors. It is already known that serotonin plays an important role in hibernation. Previously, serotonin concentrations were measured in small intestines of hamsters showing an increase of serotonin concentrations just days before the start of torpor and the early torpor which was reduced by the end of torpor where the reserves of bioactive substances was severely decreased.

The concentrations were found to be high in the tissues of spontaneously awakened animals. It has also been found that the serotonin "utilization" index shows a continuous and active catabolism of serotonin during torpor, thus, proving that this biogenic amine performs an important function in hibernation [18]. Its also known that serotonin increases the levels of cAMP (cyclic adenosine monophosphate) in cells which has a very important role in cell proliferation and division [19]. Amongst the different functions of cAMP is its potential to increase the phosphorylation of ERK (extracellular-signal-regulated kinases) phosphorylation. ERK1/2, when active is implicated in cell proliferation and survival pathways [20]. In the present experiments although ERK phosphorylation was lowered, it was certainly maintained throughout torpor and was increased during arousal. It has previously been reported that the activity of ERK, is markedly enhanced after ischemia *in vivo* and chemical anoxia *in vitro* probably as a survival promoting measure against cellular damage [21]. Thus, the present observation on ERK phosphorylation during arousal might be a protective measure against cellular damage through a periodical short term increase in serotonin concentrations.

Although the increase of serotonin and phosphorylation of ERK accompanied by the previously found increase in hydrogen sulfide levels [5] are potentially sustaining the survival of the cells in tissues through different survival pathways, the increase in SMA expression and remodeling might be playing an even more essential role in preventing lung damage due to pressure induced lung collapse during torpor phase [2]. Indeed, the reversibility of lung remodeling could only be dependent on the mechanisms that potentially break down excess structural molecules which had sustained lung structure during torpor phase while maintaining or repairing the parts necessary for normal lung function. This specially could point to the importance of the simultaneous activation of catabolic and anabolic pathways in the process of reversible lung remodeling.

Serotonin activates mTOR and has mitogenic activity which could be important for cell proliferation and differentiation [22]. Serotonin is also involved in the process of remodeling through serotonin receptors [11]. Thus, to investigate if the proliferation and healing induced through serotonin could affect cells in lung recellularization, the effects of serotonin were directly studied on human bronchial smooth muscle cells. Serotonin increased HBSMC number and SMA expression while upregulating free thiol antioxidant groups in cells. Further it increased cell-ECM adhesion molecules such as integrin $\alpha 1$ and $\beta 1$ and increased the area covered by muscle cells in recellularized lung scaffolds. Integrins are transmembrane receptor proteins which are made of glycoproteins and normally anchor the cell to the basement membrane by its cytoskeleton [23]. For example rat aortic smooth muscle cells attach to surfaces coated with collagen types I and IV and cell attachment to these substrates appears to be mediated by members of the beta 1 integrin family of extracellular matrix

receptors [24]. Many functions of smooth muscle cells, such as adhesion, migration, proliferation, contraction, differentiation and apoptosis are determined by surface adhesion receptors involved in cell–cell binding and interactions between cells and extracellular matrix proteins. Further, serotonin increased cell-ECM adhesion molecules. Dystrophin–glycoprotein complex (DGPC), links actin filaments of muscle cells to ECM [25] and an increase in beta sarcoglycan, a subcomplex of DGPC through serotonin treatment could be a contributing factor in attachment of muscle cells to the ECM. Interestingly muscle cells did not attach non-specifically, meaning that the SMA staining showed the presence of muscle cells mainly around the ECM spaces belonging to veins and bronchi, pointing to the presence of cell specific receptors on extracellular matrix. Thus, the reintroduction of serotonin treated cells into long scaffolds increased cell-ECM attachment, resulting into stronger restructured lungs in a shorter period of time.

Serotonin has direct and indirect antioxidant properties. Its indirect antioxidant properties are due to an increase in intracellular thiol (-SH) content [9]. Indeed increasing intracellular -SH groups through the use of certain substances such as ketanserine has shown beneficial effects in facilitating the wound healing process [26]. Also the use of free thiol group inducing substances such as serotonin and NaHS which releases H₂S could limit the side effects of organ preservation [27]. Further, the use of NaHS was shown to inhibit cellular senescence in Werner progeria syndrome fibroblasts [28]. Regardless, it could be possible that in the present case the effects observed through serotonin treatment are beyond its antioxidant activity or the increase in free -SH groups in cells. Interestingly, here it was shown that blocking ROS through DPI, a ROS production inhibitor, inhibited cell growth and SMA expression. Indeed, it has been previously shown by researchers that probably DPI and surprisingly NAC (N-acetyl-L-cysteine) might be inhibiting cell proliferation through different mechanism affecting cell cycle [29]. Indeed, it should not be neglected that there is literature pointing to the fact that active thiol chemistry is very important for cell growth and viability, affecting many signaling pathways [30,31]. Although the direct effects of -SH groups on recellularization is beyond the scope of this paper, the presence of higher free -SH groups through serotonin treatment might play a part in the positive outcome observed in serotonin treated recellularized lung.

The results show that the adhesion of cells to the extracellular matrix and the rate of cell growth are playing important roles in serotonin treated lung recellularization. It was further shown that serotonin has the potential to upregulate TGF- β as also previously suggested [32] and EGFR as it is shown in the present experiments, very similar to what is observed during hibernation where these proteins are down regulated through torpor and become rapidly upregulated during arousal. Indeed serotonin was previously shown to induce pulmonary muscle cell proliferation through growth factor

receptors [33]. These changes could probably keep the integrity and structure of lung tissue during the potentially strenuous period of late torpor into early arousal. The down regulation of SMA during early arousal compared to torpor is most probably due to other mechanisms such as autophagy pathways which clean the organ of dead tissue contributing to normalization of pulmonary structure and function during arousal. This is strengthened by the fact that no potential side effects of this whole process such as breathing distress are observed in animals during arousal. Serotonin, due to its demonstrated effects, could be preventing the break down of lung matrix through upregulation of adhesion molecules inside the lung. It could be perceived that a very delicate balance between protein synthesis and autophagy is brought about through growth factors, especially serotonin, which could be preventing lung damage throughout hibernation.

Conclusion

Autophagy and mTOR pathways which regulate proteostasis seem to be carefully regulated and balanced throughout hibernation, especially during arousal when both pathways seem to be simultaneously active. Its known that aberrations in proteostasis have negative effects on cellular growth and metabolism [34]. Serotonin might play an important role in regulating proteostasis and the regenerative process observed in hamster lung. Further, the use of serotonin seems to be a plausible approach to achieve more efficient recellularization of decellularized lung scaffolds in tissue engineering.

Competing interests

The author declares that she has no competing interests.

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