Differential effects of diphenyl diselenide $(PhSe)_2$ on mitochondria-related pathways depending on the cellular energy status in Bovine Vascular Endothelial Cells

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Abstract

Cellular energy metabolism varies depending on tissue and cell type, as well as the availability of energy substrates and energy demands. We recently investigated the variations in cellular metabolism and antioxidant responses in primary bovine vascular endothelial cells (BAECs) under different energetic substrate conditions in vitro, specifically glucose or galactose. In this context, pharmacological agents may exert different effects on cells depending on their energy metabolism status. In this study, we aimed to characterize the effects of $(PhSe)_2$, a redox-active molecule known for its prominent cardiovascular effects, on redox-bioenergetic cellular pathways under glycolytic or oxidative conditions in BAECs. Under glucose conditions, $(PhSe)_2$ positively impacted mitochondrial oxidative capacity, as assessed by respirometry, and was associated with changes in mitochondrial cellular dynamics. However, these changes were not observed in cells cultured with galactose. Although $(PhSe)_2$ induced the nuclear translocation of NRF2 in both glucose and galactose media, NRF2 remained in the nuclei of cells cultured in galactose for a longer duration. Additionally, activation of FOXO3a was only detected in galactose media. Notably, $(PhSe)_2$ strongly induced the expression of genes controlling mitochondrial antioxidant capacity and glutathione synthesis and recycling in glucose media, whereas its effects in galactose media were primarily focused on glutathione homeostasis. In conclusion, our findings underscore the critical influence of cellular metabolic status on the antioxidant capacity of redox-active molecules such as $(PhSe)_2$.

Key words: endothelial cell; diphenyl diselenide, glucose; galactose; mitochondria; antioxidants; oxidative metabolism; REDOX; FOXO3; NRF2.

Introduction

Cellular energy metabolism may vary depending on the tissue and cell type as well as on the energy substrates available and the energy demands. Cancer cells, in particular, commonly exhibit a more avid non-oxidative glycolytic metabolism than non-tumor cells [1], although they also need to adapt their metabolism in response to substrate availability, which results in corresponding changes in cell physiology and redox-bioenergetic status [2]. Similarly, pharmacology agents may have different cellular effects, depending on the energy metabolism status of the target cells [3].

The use of different cell culture media conditions provides a suitable model for evaluating cellular metabolic performance *in vitro*. While both glucose and galactose are good sources of cellular energy, galactose enters the glycolytic pathway through the energy-consuming Leloir pathway [4]. When glucose in the culture medium is replaced by galactose, cells are forced to shift to the mitochondrial oxidative metabolism to meet their energy demands. This condition not only activates mitochondrial OXPHOS but fully reprograms mitochondrial function and dynamis, and facilitates the evaluation of mitochondrial susceptibility to various agents revealing sensitivities related to oxidative metabolism that are obscured when cells are cultured in high glucose-containing medium [5][6]. In contrast, glucose catabolism tends to be primarily non-oxidative when the levels of glucose reach a certain threshold, known as the “Crabtree effect”. As a result, cells grown in media with standard of glucose concentrations (25 mM) tend to acquire a highly non-oxidative glycolytic phenotype [7].

The activation of the mitochondrial OXPHOS is controlled by the master transcriptional coactivator PGC-1α and linked to the reprograming of the cellular capacity to regulate its REDOX status [8]. Furthermore, Nrf2 and FoxO, transcription factors that control antioxidant gene expression, and respond to oxidative stress

translocating to the nucleus [9][10], are also well know to be sensitive to the cellular metabolic status [11][12], and are regulated by PGC-1α [13][14]. However, studies that evaluate the redox activity of molecules rarely consider the differential effect of the cellular metabolic status. In a recent study, we observed that, endothelial cells maintained in a galactose-containing medium, not only exhibited higher mitochondrial oxidative capacity and intercellular metabolic coupling than were cultured in galactosa, but also show differences in the nuclear levels of NRF22 and FOXO3 which may be indicative of a differential sensitivity to molecules with redox activity (Galant 2024), highlighting the relevance of redox-metabolic coupling and the need to control for metabolism in cell assys. Accordingly, it is reasonable to expect that pharmacological drug protocols will have different cellular effects depending on the energy metabolism status of the target cells.

Diphenyl diselenide $(PhSe)_2$, is a promising organoselenium compound with antiatherogenic properties [15–17]. The cardiovascular actions of $(PhSe)_2$ have been previously tested in several *in vivo* and *in vitro* models, including hypercholesterolemic mice and rabbits, human LDL, as well as vascular endothelial and macrophage cells [18][19].

$(PhSe)_2$, mechanism of action has been proposed to be related to is antioxidat activity, it has been shown to react with reactive cellular redox-sensitive thiols, such as cysteine and the tripeptide glutathione (GSH), an important antioxidant, functioning as a glutathione peroxidase (GPx) mimic, and facilitating the recycling of reduced glutathione. Additionally, $(PhSe)_2$ has been show to promote the oxidation of critical cysteinyl residues in KEA1, a redox sensing protein that maintains Nrf2 in the cytoplasm, leading to the NRF2 nuclear translocation and activation [20][16][21][22]. Furthermore, $(PhSe)_2$ has been shown to increase the levels of two important mitochondrial antioxidant

enzymes, PRDX3 and MnSOD [21] that are transcriptional targets of FOXO3a [13] and NRF2 [23], although it has to yet been stablished with unclear which transcription factor/s are mediate these effects.

Therefore, aiming to fully characterize the molecular mechanisms involved in the cardiovascular effects of $(PhSe)_2$ and taking into consideration the relevance of metabolic alterations in cardiovascular diseases, we analyzed how $(PhSe)_2$ impacted redox-bioenergetic cellular pathways under glycolytic or oxidative *in vitro* conditions in primary bovine vascular endothelial cells (BAEC).

Materials and Methods

Cell culture and treatments –BAEC were extracted from a fresh bovine thoracic aorta, obtained in an authorized slaughterhouse, as previously described by Peluffo and colleagues [24]. BAEC were cultured in growth medium (DMEM) supplemented with 10% of fetal bovine serum (FBS; Gibco/Invitrogen), containing 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM Hepes, 25 mM glucose, 44 mM $NaHCO_3$ and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells suspensions were seeded in 96, 24 or 6-well plates (100 × 20 mm), at different cell densities, depending on the experimental procedure. Cells were allowed to reach 90% confluence and then submitted to the experimental protocols: i) When BAEC were submitted to the *glycolytic protocol*, culture media was changed to DMEM without FBS. Cells were treated with 1 μM $(PhSe)_2$ or vehicle (DMSO 0.05%) for 3, 6, 12, 24 or 48h. ii) When BAEC were submitted to the *oxidative protocol*, culture media was changed to glucose-free RPMI medium with 5 mM galactose and without FBS. Cells were maintained in this medium for 16 hours and then treated with 1 μM $(PhSe)_2$ or vehicle (DMSO 0.05%) for 3, 6, 12, 24 or 48h.

High-resolution respirometry by Seahorse- To evaluate mitochondrial oxygen consumption in BAEC, cells were plated in XF24 cell culture microplates (24-well plates at a cell density of 5 x 10^3 cells/well), submitted to the glycolytic (glucose) or oxidative (galactose) protocols and then treated with 1 μM $(PhSe)_2$ or vehicle for 3, 12 or 48 h. A calibration cartridge (Seahorse Bioscience) was equilibrated overnight and then loaded with unbuffered DMEM (port A), 0.6 μM oligomycin (port B), 0.3 μM FCCP (port C), and 0.1 μM rotenone plus 0.1 μM antimycin A (port D), all from Sigma-Aldrich. This allowed the determination of basal respiration, maximal respiration, reserve capacity, and non- mitochondrial respiration. In all experiments, the protein concentration in each well was determined at the end of the measurements, using the Pierce BCA protein assay kit (Thermo Scientific) following cell lysis with RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Complete Mini; Roche), and used to calibrate the oxygen consumption data.

MitoSOX Imaging - Mitochondrial superoxide (O_2^-) was evaluated by MitoSOX Red (Molecular Probes, Carlsbad, CA, USA) staining, essentially as described [25]. Briefly, BAEC were grown on coverslips in 24-well culture (1 x 10^5 cells/well) plates, submitted to the glycolytic (glucose) or oxidative (galactose) protocols and treated with 1 μM $(PhSe)_2$ or vehicle (DMSO 0.05%) for 24h. Then, BAEC were exposed to 500 μM H_2O_2 for 4h or 80 μM DMNQ for 2h, and finally incubated with 3 μM MitoSOX Red for 10 min and fixed with paraformaldehyde (2%) and analyzed by fluorescence microscopy (Leica TCS SP5, Buffalo Grove, IL).

Immunofluorescence (IF)– BAEC were grown on coverslips, 24-well culture (1 x 10^5 cells/well) plates and submitted to the glycolytic (glucose) or oxidative (galactose)

protocols and then treated with 1 μM $(PhSe)_2$ or vehicle for 3, 6, 12, 24 or 48h. At the end of the incubation period, cells were then fixed with 3.7 % formaldehyde, permeabilized with 0.1 % Triton, and incubated consecutively with a primary antibody α-TOMM22 (1:200, Santa Cruz Biotechnology), α-NRF2 (1:200, Cell signaling) or α-FOXO3 (1:100, Cell signaling) and then a secondary antibody (αIgG rabbit ALEXA-488 conjugate, 1:2500). Finally, cells were counterstained with DAPI, mounted and examined under a confocal microscope (Zeiss LSM 700, Obercochen, Germany), as previously described [25].

The total TOMM22 signal was used for the evaluation of the cellular mitochondrial content. Mitochondrial fission was determined as the standard deviation of TOMM22 intensity signal across the cytosol. Mitochondrial subcellular distribution was also evaluated using TOMM22 signal. The perinuclear region signal was compared to the total cytosolic signal, and the asymmetry of the signal in the perinuclear region was also evaluated comparing the max and min signals in opposite nuclear sides. The nuclei were identified by DAPI staining.

Protein extraction and Western blotting - BAEC (5×10^5 cells/well) were grown in 6-well culture plates, submitted to the glycolytic (glucose) or oxidative (galactose) protocols and treated with 1 μM $(PhSe)_2$ or vehicle for 3, 6, 12, 24 or 48 h. The cells were then washed with phosphate-buffered saline (PBS) and lysed in 150 μl lysis buffer containing 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 1% NP40 and 25 mM Tris–HCl pH 7.6, in the presence of protease (Complete, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Cells were harvested by scraping, samples were clarified by centrifugation at 13.000 rpm for 15 min at 4 °C and protein concentration was

determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (40 – 50 μg) from whole extracts were separated by SDS-PAGE electrophoresis, using 10/12% acrylamide/bisacrylamide (29:1) 0.1% SDS gels and transferred to a 0.2 μm PVDF Hybond-P membranes (Amersham/GE healthcare) at 20V for 1h in a semi-dry Trans-Blot SD cell system (Bio-Rad, Hercules, CA). Following the blockade of the membranes using TBS-T (20 mM Tris, 150mM NaCl, 0.1% Tween 20) with 3% BSA, they were incubated with primary antibodies and then secondary antibodies in blocking solution, α-FOXO3 (1:1000, Cell signaling), α-pFOXO3 (1:1000, Cell signaling), α-AKT (1:1000, Cell signaling), α-pAKT (1:1000, Cell signaling), α-MnSOD (1:3000, ADI-SOD-110, Enzo Life Sciences), α-PRDX3 (1:1000; LabFrontier, Daehyun-dong Seodaemun-gu Seoul, Korea), α-GCLC (1:1000; Santa Cruz Biotechnology, CA, USA), α-GCLM (1:1000; Santa Cruz Biotechnology, CA, USA) and β-Tubulin (1:80000; Sigma, St. Louis, M), that was used as loading control. Secondary antibodies α-rabbit and α-mouse IgG were from LI-COR Biosciences (Lincoln, NE). Membranes were then extensively washed with TBS-T and developed using Bio-Rad Clarity Western ECL Substrate. Images were captured using a luminometer scanner and subject to densitometry analysis.

Image analysis - Image J software was used for the analysis of areas, signals in an area and cross section signals from fluorescence and confocal microscopic images for Tomm22, Nrf2, FOXO3 and MitoSOX and also to analyze Western bloting membranes.

Gene expression analysis of genes coding for antioxidant proteins by RT-qPCR - To evaluate mRNA levels, BAEC (5 x 10^5 cells/well) were seeded in 6-well plates and cultivated for 24h. Following cell treatment, total RNA was isolated using TRIzol

Reagent (Invitrogen, Carlsbad, CA) following the manufacturer instructions. 1 μg of total RNA was then reverse transcribed by extension of random primers using M-MLV (Promega, Madison,WI). Relative expression levels were determined by real-time PCR (qPCR) in a 7900 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using the primer sets listed below/in Table 1.

Target	Forward	Reverse
GCLC	5'-gcacggcatcctccagttcct-3'	5'-tcggatggttggggtttgtcc-3'
GCLM	5'-gtgatgccaccagatttgact-3'	5'-cccactcgtgcgcttgaat-3'
GPx1	5'-ctgctcattgagaacgtagca-3'	5'tctcgccattcacctcgca-3'
MnSOD	5'-ggaacaacaggtcttatccccct-3'	5'-ttacttgctgcaagccgtgtatc-3'
Prx3	5'-ttcgggcttcgctcatccga-3'	5'-atggtatgaggaactggtgct-3'

Table 1 Primer pairs used in RT-qPCR to quantify gene expression changes

Statistical analysis and graphics. Numerical raw data was compiled and processed in Excell files (Microsoft) and then final data was incorporated into GraphPad PRISM® software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA), that was used for statistical analysis and preparation of graphs. Normal (Gaussian) distribution was evaluated with the Shapiro-Wilk normality test. Significance of differences among data sets was evaluated using unpaired test *t* or two-way of variance analysis (ANOVA), using Bonferroni's correction for multiple comparisons. Results are expressed as mean ± SEM. $p < 0.05$ was considered statistically significant. $n \geq 4$ in all experiments. The number of independent experiments is indicated in the corresponding figure legends.

Results

Effect of $(PhSe)_2$ on mitochondrial respiration in BAEC

We first tested the differential effect of $(PhSe)_2$ on mitochondrial respiration in BAEC cultured in standard glucose containing media or in galatose-containing medium to force

the cells to rely on OXPHOS for ATP production. We found that 3h treatment with $(PhSe)_2$ decreased maximum respiration in both glucose- and galactose-containing medium (Fig. 1A, C and D) but the oxygen consumption rate (OCR) recovered after 48h of treatment with $(PhSe)_2$ in both glucose- and galactose-containing medium (Fig. 1B, C and D). Furthermore, when the maximum OCR was normalized for the mitochondrial volume, estimated by immunofluorescence analysis of the mitochondrial protein Tomm22, we found that this ratio was significantly increased following 48h of treatment with $(PhSe)_2$ in glucose-containing medium, suggesting a higher respiratory capacity per mitochondria in this context (Fig. 1E). However, in galactose-containing medium the treatment with $(PhSe)_2$ did not significantly change the maximum respiration/mitochondrial volume unit ratio (Fig. 1F).

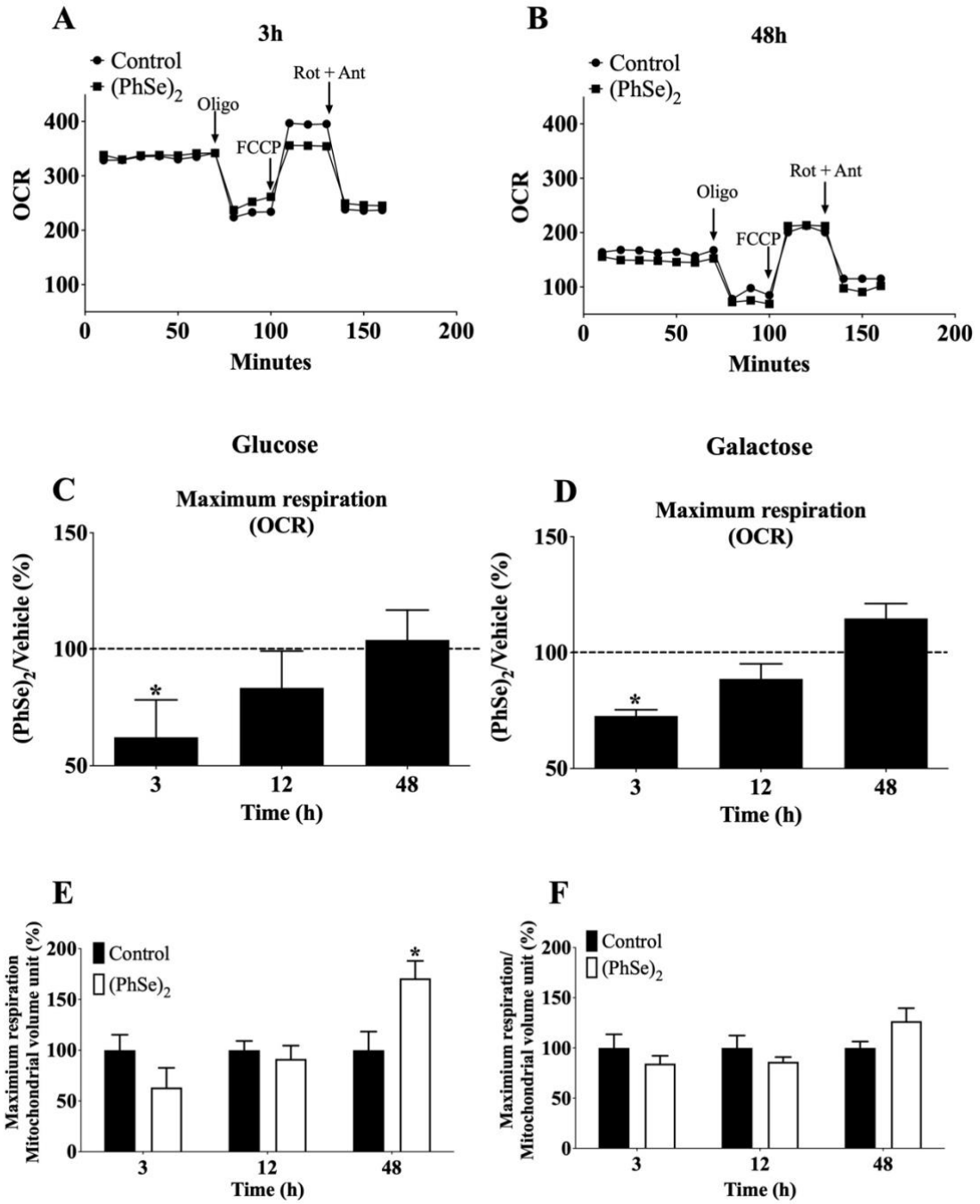

Fig. 1 Effect of $(PhSe)_2$ on mitochondrial respiration in BAEC conditioned in glucose- or galactose-containing medium. (A) Representative respirometry assay of BAEC conditioned in glucose-containing medium for 3h and (B) 48h, data corrected for protein amount. (C) Maximum respiration in BAEC conditioned in glucose-containing medium. (D) Maximum respiration in BAEC conditioned in galactose-containing medium. (E) Ratio of maximum respiration per mitochondrial volume unit in BAEC treated with $(PhSe)_2$ for 3, 12 or 48h in glucose-containing medium and (F) galactose-containing medium. (A and B) Plots of O_2 consumption (OCR) quantification was corrected using total μg of protein and expressed as the percentage of treatment with $(PhSe)_2$ (black bars) with respect to vehicle group (dashed line). (C and D) Average points recorded in samples

with $(PhSe)_2$ after addition of FCCP/average points recorded in samples with vehicle after addition of FCCP x100. (E and F) The mitochondrial volume unit was analyzed by Tomm22 immunofluorescence of 4 independent experiments (Figure 2). The average of the samples after FCCP by Seahorse/average of Tomm22 was analyzed by immunofluorescence. Data were represented as mean ± SEM (n=4). $*p < 0.05$, indicate statistical difference from vehicle group by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

Effect of $(PhSe)_2$ on mitochondrial dynamic in BAEC conditioned in glucose or galactose medium

To understand the changes elicited in the mitochondria by $(PhSe)_2$ we monitored mitochondrial dynamics by immunofluorescence analysis of Tomm22 using confocal microscopy. Total mitochondrial volume, fusion and subcellular distribution were analyzed in BAEC treated with $(PhSe)_2$ for 3, 12 and 48h in glucose- or galactose-containing medium. We found that in glucose-containing medium (Fig 2A-E), although the treatment with $(PhSe)_2$ for 48h decreased total mitochondrial content (Fig. 2B), it increased mitochondrial fussion (Fig. 2C), reduced the mitochondrial content in the perinuclear region (Fig. 2D), consistent with the observed increase in oxidative capacity per mitochondria. Furthermore, it increased the asimetrical distribution of mitochondria within the cells, that could be related to intercellular coupling/organization of the epithelium polarity (Fig. 2E). In contrast, cells maintained in galactose-containing medium (Fig. 2F-J) did not change the total mitochondrial content (Fig. 2G), fission (Fig. 2H), or mitochondrial localization in the perinuclear region (Fig. 2I) in response to the treatment with $(PhSe)_2$. However, a transient increase in the the asimetrical distribution of mitochondria was observed at 12 h, that reverted after 48h of treatment (Fig. 2J).

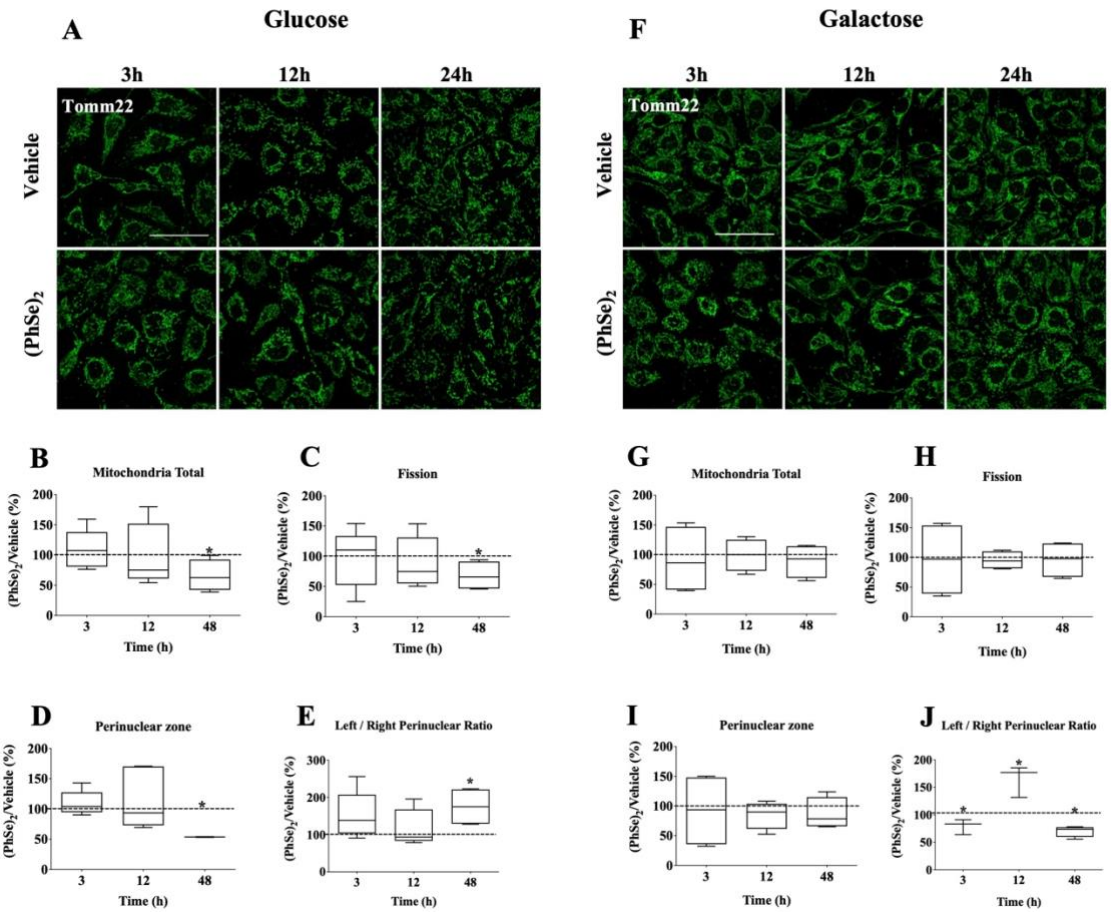

Fig. 2 Effect of $(PhSe)_2$ on mitochondrial dynamics in BAEC conditioned in glucose- or galactose-containing medium. BAEC were treated with $(PhSe)_2$ (1 μM) or vehicle (DMSO) for 3, 12, or 48h in medium containing glucose (A-E) or galactose (F-J) and the mitochondrial parameters were determined by immunofluorescence of Tomm22 antibody. (A and F) Representative image of mitochondrial Tomm22 (green) in BAEC treated with $(PhSe)_2$, the white bars represent 50 μm. (B and G) Quantification of mitochondrial volume, (C and H) mitochondrial fission, (D and I) intensity of the perinuclear zone, (E and J) ratio of left/right intensity. Results were expressed as the percentage of treatment with $(PhSe)_2$ (white bars) with respect to vehicle group (dashed line). Data were represented as mean ± SEM (n=4). $*p < 0.05$, indicate statistical difference from vehicle group by unpaired t-test.

$(PhSe)_2$ prevents the mitochondrial superoxide production in BAEC exposed to oxidants.

To evaluate if $(PhSe)_2$ could modity the cellular capacity to respond to an oxidative challenge, we pre-treated BAEC with 1 μM of $(PhSe)_2$ and then exposed the cells to H_2O_2 (500 μM) or DMNQ (80 μM), an inhibitor of the mitochondrial electron transport chain that drives the formation of mitochondrial superoxide (O_2^-).

Mitochondrial RS generation was evaluated using the MitoSOX probe. In BAEC cultured in glucose-containing medium, DMNQ induced a significant increase in O_2^-production. Importanly, $(PhSe)_2$ pre-treatment reduced mitochondrial O_2^- levels in DMNQ challenged cells. H_2O_2 in glucose cultured cells actually reduced mitochondrial O_2^- levels, and $(PhSe)_2$ pre-treatment partially rescued H_2O_2 inhibition (Fig. 3A-B), suggesting that $(PhSe)_2$ pre-treatment improved redox homeostasis in glucose cultured BAEC. In contrast, in BAEC cultured in galactose-containing medium DMNQ did not generate a significant increase in O_2^-production, possibly because in these conditions the mitochondria are more resistant to DMNQ action, while H_2O_2 did. Furthermore, pre-treatment with $(PhSe)_2$ reduced tmitochondrial O_2^- levels following H_2O_2 exposure (Fig. 3C-D). These results consistently support the idea that the $(PhSe)_2$ reduces the mitochondrial accumulation of O_2^- levels.

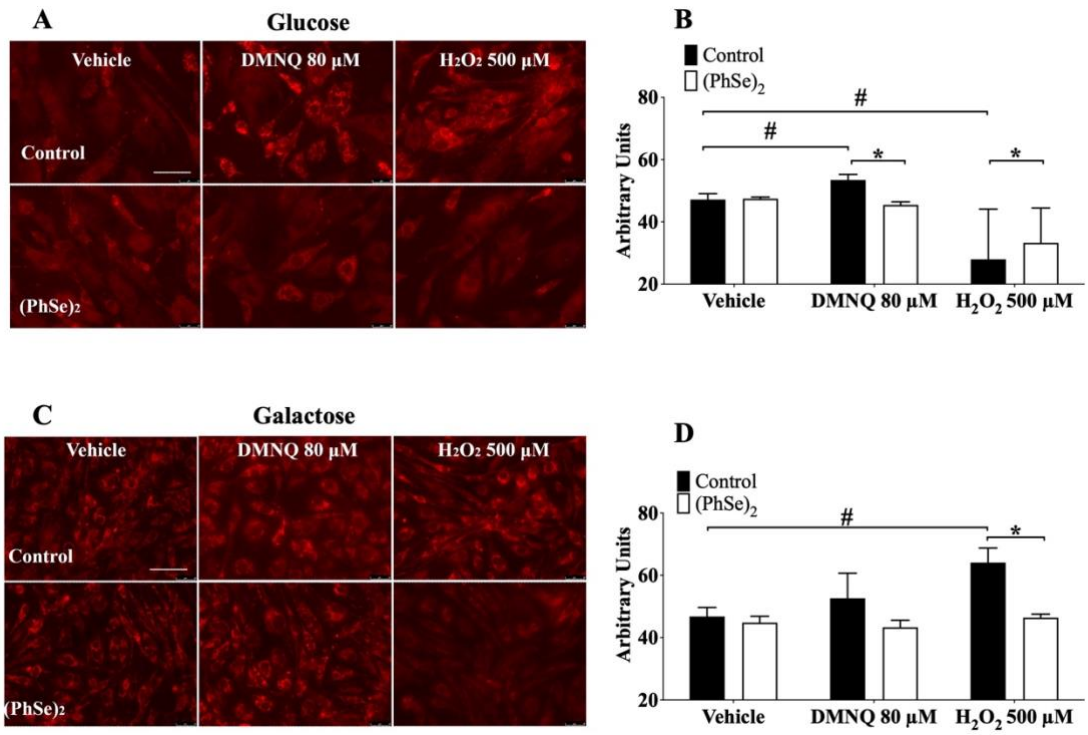

Fig. 3 Effect of $(PhSe)_2$ on mitochondrial O_2^- production in BAEC conditioned in glucose- or galactose-containing medium. BAEC were pretreated with $(PhSe)_2$ (1 µM) or vehicle (DMSO) for 24h in medium containing glucose (A-B) or galactose (C-D) and subjected to oxidants as DMNQ or H_2O_2. The $O_2^{\bullet-}$ production was determined by confocal

microscopy through the MitoSOX probe. (A and C) Representative image of mitochondrial O_2^- production in BAEC pretreated with $(PhSe)_2$ and exposed to DMNQ (80 μM) or H_2O_2 (500 μM) (for 2 or 4h, respectively), the white bars represent 50 μm. (B and D) Quantification of mitochondrial O_2^- production (values x 10^4) in BAEC pretreated with $(PhSe)_2$ and exposed to DMNQ or H_2O_2. * $p < 0.05$, indicate statistical difference between the groups exposed to oxidants and $(PhSe)_2$, # $p < 0.05$, indicate statistical difference between vehicle and DMNQ or H_2O_2 groups by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

Time-course effect of $(PhSe)_2$ in the expression of antioxidant proteins in BAEC conditioned in glucose or galactose medium.

Next, we decided to evaluate if the observed effect of $(PhSe)_2$ on mitochondrial $O_2^{\bullet-}$ levels could be derived from an increased cellular detoxification capacity. To that end we used targeted gene expression and protein analysis of some mayor antioxidant coding genes regulated by the two important redox sensitive transcription factors, NRF2 and FOXO3. In BAEC cultured in glucose-containing medium, following 6h of treatment with $(PhSe)_2$ we observed a significant increase in MnSOD and *SOD2* gene expression levels (Fig. 4A-B) as well as an increase in PRDX3 protein levels (Fig. 4C-D). Both MnSOD and PRDX3 are antioxidants proteints that localize in the mitochondrial matrix. We also monitored the effect of $(PhSe)_2$ on GCL, the enzyme of the limiting step for the synthesis of the antioxidant tripeptide glutathione, and GPx1 the main enzyme involved in the recovery of reduced gluthatione following its oxidation, in glucose-containing medium. GCL is made up of two subunits. We noted that GCLC protein and *GCLC* gene expression levels increased following 24h of treatment with $(PhSe)_2$ (Fig. 4E-F) as well as *GCLM* levels at a 6h of treatment (Fig. 4 G-H). Furthermore, *GPx1* levels were increased at 12 and 24h of treatment with $(PhSe)_2$ in BAEC cultured in in glucose-containing medium (Supplementary Fig. 1A). Similar results were observed in BAEC maintained in galactose-containing medium. We observed an increase in the mRNA levels of *GCLC* after 12h and GCLC protein content at 24h (Fig. 4 M-N), as well as an

increase in GCLM protein levels after 6h of treatment with $(PhSe)_2$ (Fig. 4 O-P). $(PhSe)_2$ also increase the mRNA expression of GPx1 at 12 and 24h of treatment in galactose-containing medium (Supplementary Fig. 1B). In contrast, *SOD2* mRNA levels and protein of MnSOD were unaltered (Fig. 4 I-J), while the PRDX3 levels decreased after 24h of treatment with $(PhSe)_2$ in galactose-containing medium (Fig. 4 K-L). The mRNA levels of *CAT*, *UCP2*, *CYTC*, *GSS* and *NRF2* were unaltered by the treatment with $(PhSe)_2$ of BAEC maintained in either glucose- or galactose containing medium (Supplementary Fig. 1 C-L). Overall these results suggest that in glucose containing media $(PhSe)_2$ induces both the main mitochondrial and cytosolic antioxidant systems, while in galactose media $(PhSe)_2$ induces mainly the cytosolic antioxidant systems, a result consistent with the expected pre-activation of OXPHOS and mitochondrial antioxidant capacity in galactose media.

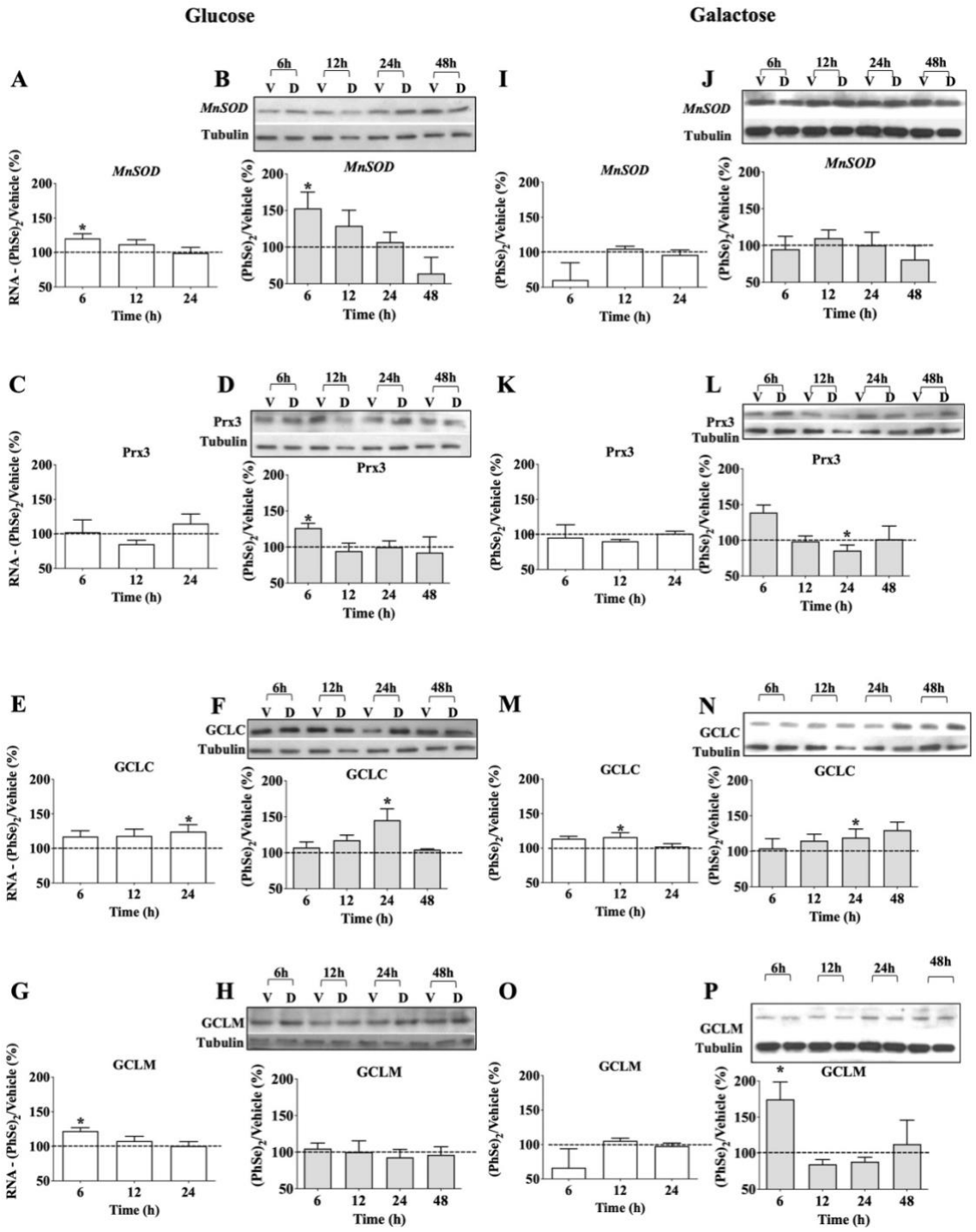

Fig. 4 Effect of $(PhSe)_2$ in time-course expression profiles of antioxidant proteins in glucose- or galactose-containing medium. BAEC were treated with $(PhSe)_2$ or vehicle (DMSO) for 6, 12, 24 or 48h in glucose or galactose medium. Protein expression by Western Blotting analyses and mRNA expression by qPCR. Results were expressed as protein /β-tubulin ratio and the data were presented as the percentage of treatment with $(PhSe)_2$ (bars) with respect to vehicle group (dashed line). V = vehicle, D = $(PhSe)_2$. Data were represented as mean ± SEM (n=3-4). *$p < 0.05$, indicate statistical difference from vehicle group by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

Effect of $(PhSe)_2$ on Nrf2 nuclear translocation of BAEC conditioned in glucose or galactose medium.

Since $(PhSe)_2$ increased the levels of antioxidant genes known to be targeted ty the transcription factor NRF2, we evaluated the effect of $(PhSe)_2$ on Nrf2 nuclear translocation, an thus activation, in BAEC cultured in glucose- or galactose-containing medium. We found that NRF2 nuclear localization was incrased following 3h of incubation with $(PhSe)_2$ in BAEC cultured in both glucose and galactose containing medium (Fig. 5A-B). The increased nuclear localization was gradually reduced overtime in both glucose and galactose cultured cells, but was maintained over a more extended period of time in galactose cultured cells (Fig. 5C-D), suggesting a more effective nuclear retention in galactose. The separate evaluation of nuclear and cytosolic levels of NRF2 showed a significant increase in cytosolic NRF2 levels following 3 h of treatment with $(PhSe)_2$ in BAEC maintained in glucose- containing medium but not in galactose cultured cells possibly suggesting that global NRF2 levels stabilized by $(PhSe)_2$ in glucose but not in galactose media (Supplementary Fig. 2). NRF2 degradation is a tighly regulated process, cytosolic retention of NRF2 by KEAP1 is linked to its proteasomal degradation, and therefore, KEAP1 release is generally associated not only to NRF2 nuclear translocation but also to its stabilization [26]. In sum, NRF2 activation is detectable in both glucose and galactose media, but in galactose media is likely to be effective for more extended periods of time.

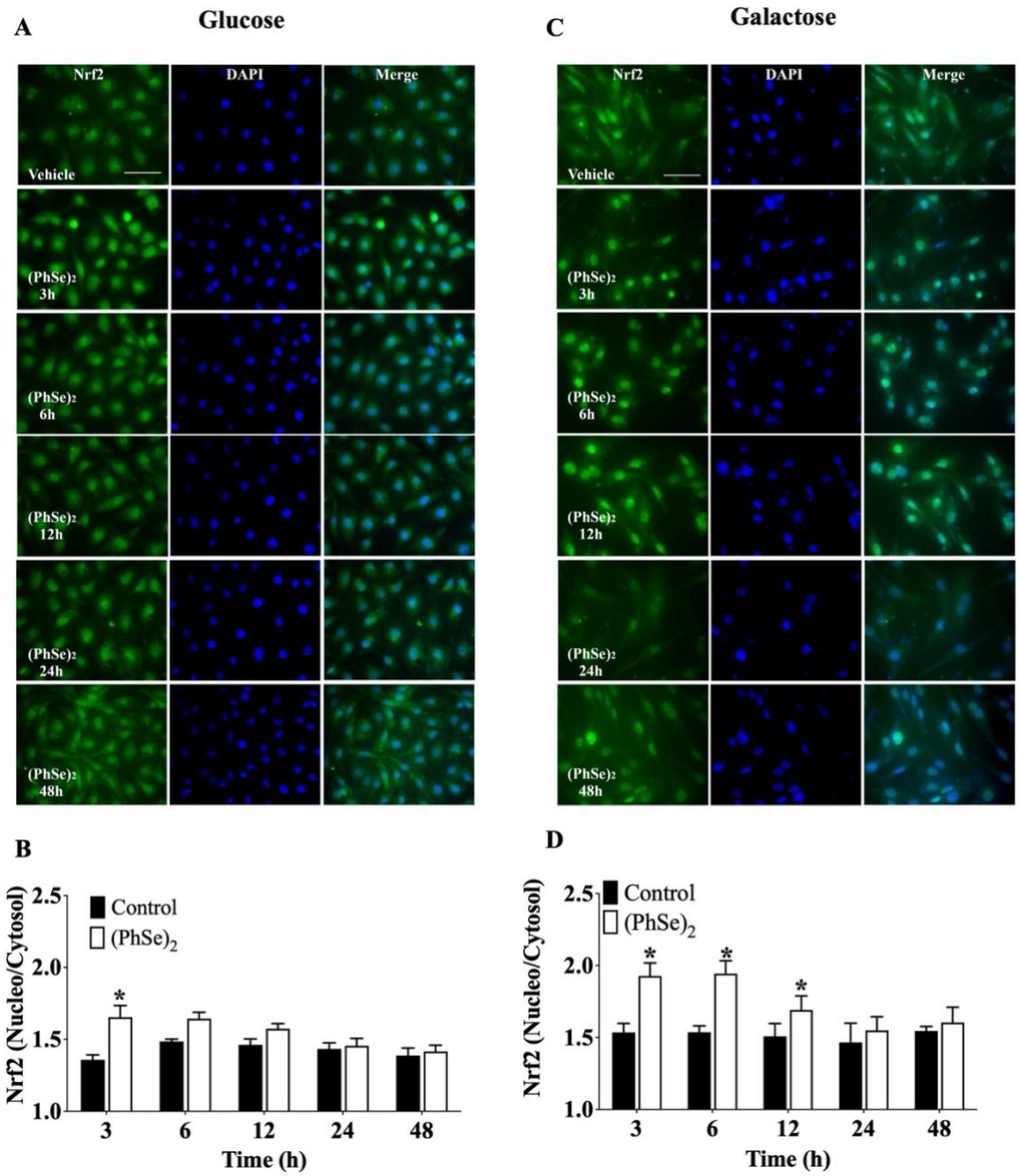

Fig. 5 Effect of $(PhSe)_2$ on Nrf2 translocation in BAEC conditioned in glucose- or galactose-containing medium. BAEC were treated with $(PhSe)_2$ (1 μM) or vehicle (DMSO) for 3, 6, 12, 24 and 48h in medium containing glucose (A-B) or galactose (C-D) and the immunofluorescence the Nrf2 were analyzed. (A and C) Representative image of immunofluorescence from Nrf2 (green) and DAPI (blue) in BAEC, the white bars represent 50 μm. (B and D) Ratio of Nrf2 in the nuclei/cytosol. Data were represented as mean ± SEM (n=4). * $p < 0.05$, indicate statistical difference from vehicle group by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

Effect of $(PhSe)_2$ on FOXO3 activation in BAEC conditioned in glucose or galactose medium.

Finally, we also analyzed the posible involvement of FOXO3 transcriptional factor in $(PhSe)_2$ activation of antioxidant gene expresion. Treatment of BAEC in glucose-containing medium with $(PhSe)_2$ for 6, 12 or 24h increased the level of FOXO3 phosphorylation (pFOXO3) at Thr32, a mark of AKT phosphorylation (pAKT), suggesting an inhibition of FOXO3 activity and nuclear extrussion (Fig. 6A). However, no significant changes in the nuclear/cytosolic ratio of FOXO3 (Fig 7) nor in the cytosolicy and nuclear levels when analyzed separately (Supplementary Figure 3), and, although an increase in AKT phosphorylation was detectable in response to $(PhSe)_2$, it could only be observed at 24 h of treatment in glucose media. (Fig. 6C). In contrast, although the nuclear/cytosolic ratio of FOXO3 transiently decreased at 3h of treatmen in galactose media, $(PhSe)_2$ decreased pFOXO3/FOXO3 ratio at 24h, suggesting an increase in FOXO3 activity and nuclear localization (Fig. 6B). In these conditions although the changes in pAKT levels did not reach statistical significant, a consistent tendency to lower pAKT/AKT ratio was detectable at 24h (Fig. 6D) as well as detectably higher FOXO3 levels in both the nuclei and the cytosol at 12h of treatment (Supplementary Figure 3). All together these results suggest that FOXO3 activity is more likely to play a

relevant role in antioxidant gene regulation in galactose than in glucose cultured cells.

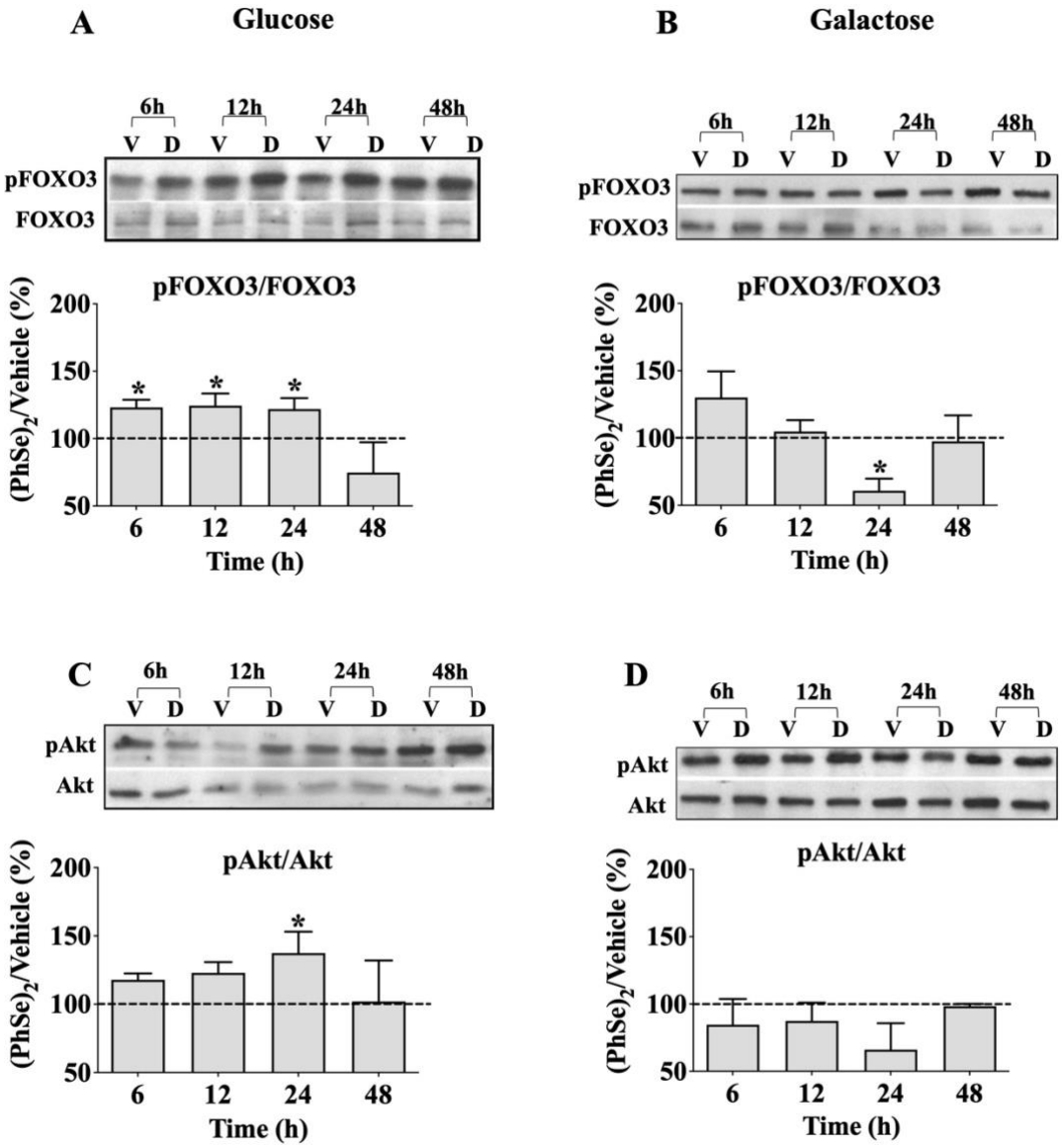

Fig. 6 Effect of $(PhSe)_2$ on pFOXO3 and pAkt levels in BAEC conditioned in glucose- or galactose-containing medium. BAEC were treated with $(PhSe)_2$ or vehicle (DMSO) for 6, 12, 24 and 48h in glucose (A-B) or galactose (C-D) medium to analyze pFOXO3 (A-B) and pAkt (C-D) protein content by Western blotting. Results are expressed as protein/β-tubulin ratio and the data is presented as the relative % of treatment with $(PhSe)_2$ (bars) *vs* vehicle. V = vehicle, D = $(PhSe)_2$. Data were represented as mean ± SEM (n=4-7). * $p < 0.05$, indicate statistical difference from vehicle group by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

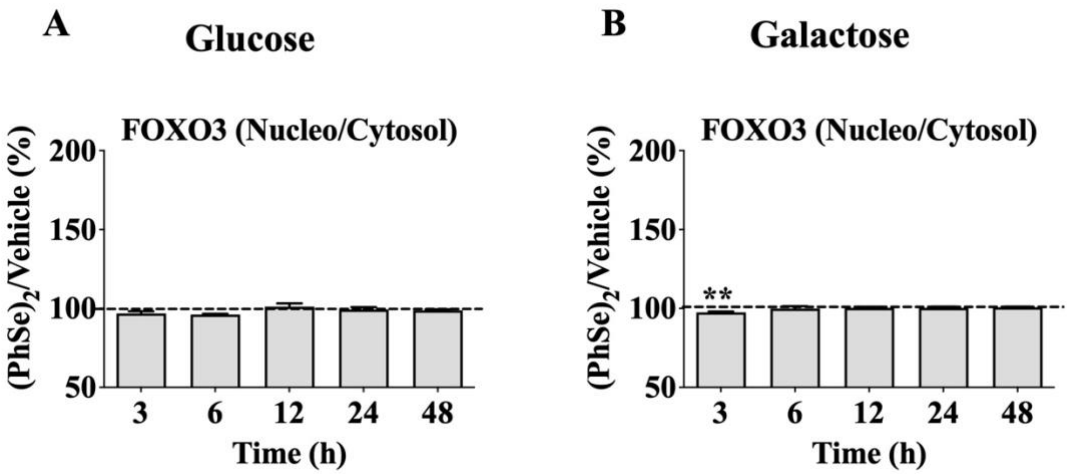

Fig. 7 Effect of $(PhSe)_2$ on the translocation of FOXO3 in BAEC conditioned in glucose- or galactose-containing medium. BAEC were treated with $(PhSe)_2$ (1 μM) or vehicle (DMSO) for 3, 6, 12, 24 and 48h in medium containing glucose (A) or galactose (B) and the immunofluorescence for FOXO3 were analyzed. Ratio of FOXO3 in the nuclei/cytosol. Data were represented as mean ± SEM (n=4). * $p < 0.05$, indicate statistical difference from vehicle group (dashed line) by two-way ANOVA, followed by the Bonferroni's *post hoc* test.

Discussion

Endothelial cells are the primary target of circulating redox-active molecules. Among these, primary bovine aortic endothelial cells (BAEC), since preserving their original metabolic plasticity, represent a relevant *in vitro* model for investigating the effects of pharmacological agents across diverse metabolic contexts. The choice of cell culture media profoundly influences the metabolic performance of cells *in vitro*. Our recent findings, particularly concerning the modulation of transcription factors NRF2 and FOXO3—key regulators of antioxidant gene expression—in BAECs, underscore the significance of celular metabolic state. Specifically, alterations in cell culture conditions, leading to heightened reliance on oxidative metabolism, demonstrate a consequential impact on the efficacy of redox-active agents on vascular cells. These observations suggest that the effectiveness of redox-active agents on endothelial cells may be intricately linked to their metabolic milieu.

Therefore, we investigate the effects of $(PhSe)_2$ on BAEC cultured in media containing glucose or galactose, assessing its impact on metabolism and antioxidant responses. $(PhSe)_2$ has been extensively studied for its pivotal role in vascular physiology and pathology, attributed to its capacity for redox modulation. This includes the promotion of mitochondrial respiration and the upregulation of antioxidant gene expression, partly mediated by the translocation of NRF2 to the nucleus [21]. Our findings reveal significant differences in the action of $(PhSe)_2$ depending on whether BAEC are culture in galactose and glucose media. These differences are likely to be relevant to their vascular effects *in vivo*.

In cells cultured in glucose, treatment with $(PhSe)_2$ yielded a net positive impact on mitochondrial oxidative capacity, assessed by respirometry. This enhancement correlated with observed changes in mitochondrial cellular dynamics, characterized by more fused mitochondria and a uniform distribution of mitochondria throughout the cells, with reduced concentration in the perinuclear region. Conversely, these changes were not observed in cells cultured with galactose, where the cells were already compelled to utilize their oxidative capacity. Therefore, the modulation of mitochondrial function by $(PhSe)_2$ appears to be effective only in cells exhibiting limited utilization of their mitochondrial oxidative capacity.

Regarding $(PhSe)_2$ antioxidant capacity, we noted that although $(PhSe)_2$ induces the nuclear translocation of NRF2 in both glucose and galactose media, NRF2 remains longer in the nuclei of cells cultured in galactose media. This observation suggests a potential preference for nuclear retention in the galactose environment. However, any speculation regarding the underlying mechanism at this stage would be highly conjectural. Potential explanations could involve interactions with other transcription factors [27] or the inhibition of its nuclear extrusion [28].

The enhanced NRF2 activation profile observed in galactose media is even more pronounced in the case of FOXO3a, where its activation is solely detectable in galactose media, with reduced levels of pFOXO3a and increased nuclear protein levels. This shared pattern suggests a more efficient induction of antioxidant gene expression in galactose compared to glucose media. However, analysis of the gene expression changes elicited by $(PhSe)_2$ on crucial antioxidant genes controlling mitochondrial antioxidant capacity and glutathione synthesis and recycling reveals a stronger induction in glucose media. In galactose media, the changes induced by $(PhSe)_2$ are not only smaller but also predominantly focused on glutathione homeostasis, suggesting a more cytosolic, rather than mitochondrial, effect.

Therefore, the induction of antioxidants genes by $(PhSe)_2$ in glucose media likely requires additional transcription factors that are specially active in conditions of low mitochondrial activity. Potential candidates include NFkB [29] a redox-sensitive transcription factor controlling antioxidant gene expression, and c-MYC [30] among others. Additionaly, since $(PhSe)_2$ also enhances mitochondrial fission and maximal oxidative capacity, other pathways may be involved. Acute inhibition of mitochondrial respiration could drive compensatory mechanisms, such as the activation of PGC-1 α, which can induce both antioxidant gene expression and mitochondrial fussion [8] while supporting FOXO3 [13] and NRF2 [14] activation.

Despite the apparent lower capacity of $(PhSe)_2$ to induce antioxidant gene expression in galactose media, assessments of mitochondrial antioxidant capacity using the mitochondrial inhibitor DMNQ or the exogenous addition of H_2O_2 suggest a potentially heightened antioxidant response in galactose-treated cells. This inference arises from the substantial reduction elicited by $(PhSe)_2$ on mitochondrial superoxide ($O_2^{\bullet-}$) levels in galactose-cultured cells following a challenge with H_2O_2. However, drawing definitive

conclusions from these observations is challenging. In galactose media, cells exhibit greater resistance to DMNQ, while in glucose media, they are highly susceptible to H_2O_2-induced mitochondrial damage, resulting in under basal levels of mitochondrial $O_2^{\bullet-}$. Notably, $(PhSe)_2$ partially rescues these aberrant levels and restores them to normal, consistent with its observed capacity to enhance mitochondrial oxidative capacity.

Therefore, our data suggest that while $(PhSe)_2$ demonstrates greater efficacy in the activation of NRF2 and FOXO3a under pro-oxidative conditions, alternative pathways contribute to its enhancement of mitochondrial oxidative capacity and total antioxidant capacity when cells are cultured in glucose media. The observation that $(PhSe)_2$ induces acute inhibition of mitochondrial respiration shortly after treatment may indicate a significant role for mitochondrial function in this context. In sum, our findings underscore the critical influence of the cellular metabolic status on antioxidant capacity of redox-active molecules such as $(PhSe)_2$.

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