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Influence of glutathione availability on cell damage induced by human immunodeficiency virus type 1 viral protein R.

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Highlights

Heterologous expression-based system was used for phenotypic analysis of Vpr protein.

GSH or ATP supplementation protected yeasts from Vpr-induced growth reduction

Metabolites that stimulate GSH biosynthesis similarly protected from Vpr effect

Vpr caused intracellular depletion of energy stores and subsequent oxidative damage

Data showed that *S. cerevisiae* may be useful in screening for specific Vpr inhibitors **Abstract**

The human immunodeficiency virus type 1 (HIV-1) encodes for accessory viral protein R (Vpr), which arrests the cell cycle of host cells at G2 and causes mitochondrial dysfunction and alterations in glycolysis. High-level expression of Vpr protein correlates with increased viral production and disease progression. Vpr causes structural and functional injury in many types of eukaryotic cells, whether or not they are permissive for viral replication; among them is the budding yeast

Saccharomyces cerevisiae. We hypothesized that the dramatic Vpr-induced injuries in yeast could be prevented by strengthening their redox response capacity. We show that exogenous addition of glutathione (GSH) or its prodrug, N-acetylcysteine (NAC), protected budding yeasts from Vprinduced cytopathic effects. Moreover, addition of adenosine triphosphate (ATP) to growing cultures of Vpr-producing yeast returned cellular growth to control levels, whereas the addition dehydroascorbic acid (DHA) had only a minor protective effect. The diminished protein levels of Cox2p and Cox4p in *wild type* Vpr-producing yeasts together with the acute sensitivity of *petite* yeasts to Vpr activity may have been caused by low intracellular ATP levels. As a consequence of this energy deficit, eukaryotic cells would be unable to synthetize adequate supplies of GSH or to signal the mitochondrial retrograde response. Our findings strongly suggest that the cytopathogenic effect of Vpr protein in eukaryotic cells can be prevented by increasing intracellular antioxidant stores or, alternatively, supplying external ATP. Furthermore, these results support a potentially promising future for *S. cerevisiae* expression as a modality to search for Vpr-targeted inhibitors.

Keywords: HIV-1; Vpr; phenotypic analysis; therapeutic target; heterologous expression; oxidative stress

Abbreviations: CFU: Colony forming unit; COX: Cytochrome c oxidase; DHA: Dehydroascorbic acid; ETC:

Electron transport chain; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GSH: Glutathione; GSSG: Glutathione disulfide; HIV-1: Human Immunodeficiency Virus type 1; NAC: N-acetylcysteine; PGK, Phosphoglycerate kinase; VATPase: Vacuolar ATPase; Vpr: Viral protein R; YNB, Yeast Nitrogen Base

1. Introduction

Viral protein R (Vpr) is a 14 kDa accessory protein of human immunodeficiency virus type 1 (HIV1) encoded by *vpr* late gene (Cohen et al., 1990). Vpr protein can be found either inside or outside of host cells, localizing in the nucleus, cytoplasm and mitochondria, packed into virions or free in cerebrospinal fluids and plasma (Lu et al., 1993; Jacotot et al., 2000; Levy et al., 1994). Although dispensable in some *ex vivo* infections, Vpr protein increases viral replication of T cells, is essential for HIV-1 infection of macrophages and determines disease progression (Dedera et al., 1989; Connor et al., 1995; Goh et al., 1998; Mologni et al., 2006).

Newly synthesized Vpr causes pleiotropic effects in eukaryotic cells. Accordingly, Vpr prevents proliferation of infected cells by arresting them in G2 of the cell cycle, increases viral expression, provokes cell death of lymphoid target cells and induces a mitochondria-dependent apoptotic pathway (Goh et al., 1998; Muthumani et al., 2002; Rogel al., 1995). Extracellular and *de novo* synthesized Vpr protein has been used in a number of cellular models. Among them, the budding yeast *Saccharomyces cerevisiae* was demonstrated to be sensitive to Vpr activity (Zhao and Elder, 2000). The fission yeast *Schizosaccharomyces pombe* reproduces Vpr-induced effects such as cell cycle G2 arrest, changes in cell shape and cell death (Zhao et al., 1998). Similarly, cell growth arrest, size alterations, transient respiratory deficiency and cell death by endogenously expressed or extracellular protein were Vpr-induced effects reported in *S. cerevisiae* (Macreadie et al., 1995; Macreadie et al., 1996; Macreadie et al., 1997; Jacotot et al., 2000).

Vpr protein is an apoptogenic protein that induces mitochondrial membrane permeabilization *via* interaction with the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (Jacotot et al., 2001; Vieira et al., 2000). Studies using exogenous Vpr in microglial cells showed a reduction in the cellular levels of GSH and ATP and suggested an oxidative role for the extracellular form of Vpr protein, which is detected in serum and cerebrospinal fluid of HIV-infected patients (Ferrucci et al., 2012). Moreover, the oxidative status of host cells modulates the disease progress and the Vpr-induced death in the *Sc. pombe* cellular model (Antal and Pesti, 2006; Perl and Banki, 2000). While it is generally accepted that virus production increases in the presence of Vpr protein, the significance for Vpr-induced cytopathicity, in the context of the viral infection, is still debated (Ferrucci et al., 2013; Guenzel et al., 2014). Nevertheless, HIV-1-infected individuals display multiple symptoms of redox imbalance and Vpr is one of the viral proteins that can be involved in these phenomena (Antal and Pesti, 2006; Baruchel and Wainberg, 1992; Porter and Sutliff, 2012; Stromajer-Racz et al., 2010). Thus, HIV-1 infected cells endure elevations in reactive oxygen species (ROS), including superoxide and hydrogen peroxide, and show a decrease in their detoxification mechanisms. Reduction of total antioxidant capacity entails a significant decrease of GSH level in host cells (Nakamura et al., 2002) and recent studies have associated HIV-1 infection with GSH levels (Nguyen et al., 2014; Pang and Panee, 2014; Bhaskar et al., 2015). Notably, drugs that replenish intracellular GSH also counteract oxidative stress and inhibit HIV replication in models of acute and latent infection (Staal et al., 1992).

In this study, we show that exogenous addition of ATP or GSH protected growing yeast from Vprinduced damage. The positive response of *petite* mutants to these treatments pointed to an impairment of an energetic pathway by Vpr. Moreover, the suppression of cell growth and resulting cell death induced by Vpr protein correlated with a downregulation of protein expression of the Cox4p subunit of cytochrome c oxidase.

Therefore, we propose that Vpr expression leads to an ATP deficit in yeast that impairs the mitochondria retrograde response, a mechanism that would be expected to remedy the mitochondrial ATP deficit and consequent reduction in GSH stores. This Saccharomyces heterologous expression system might be a useful tool in searching for inhibitors targeted to Vpr protein.

2. Materials and Methods

2.1. Plasmid construction and microbial strains.

E. coli DH5α strain (Sambrook and Rusell, 2001) was used for the construction of the *E.coli*-yeast shuttle vector. The *vpr* gene sequence from the NL4-3 clone of HIV-1 (Adachi et al., 1986) was cloned into the *Sma*I and *Bam*HI sites of pEMBLyex, a high copy number yeast expression vector carrying a galactose inducible and glucose repressible promoter (Cesareni and Murray, 1987). Exogenous gene expression is controlled by the *CYCGAL1* promoter, which is tightly repressed by glucose and strongly induced by galactose. Two clones of *S. cerevisiae* W303-1B strain (Thomas and Rothstein, 1989) were used; a wild type clone and a *petite* mutant isolated after growth in the presence of ethidium bromide (Goldring et al., 1970), which was generously provided by M. Remacha, (CBMSO. Universidad Autónoma de Madrid. Spain). The petite mutant preserved characteristics of respiratory deficient mutants, such as slow growth and formation of small (*petite*) colonies in fermentable media (Ephrussi, 1949).

2.2. Yeast media, transformation and induction.

Yeast cells were grown at 30ºC, with orbital shaking at 300 rpm, in standard YNB glucose medium supplemented with 20 mg/l L-tryptophan, 40 mg/l adenine and 20 mg/l L-histidine. A standard lithium acetate protocol was used for production of competent yeast and transformation (Burke et al., 2000). Transformants of the W303-1B strain (carrying pEMBLyex or pEMBLyex-*vpr* plasmids) were maintained in selective medium (containing 20 mg/l L-leucine). For *vpr* expression, yeast cells were cultured in YNB medium containing 2% galactose plus required supplements (inducing medium). The pH of media was adjusted with Trizma base. When required, solid medium was prepared by the addition of 1.5 % purified agar (Difco) to the broth.

2.3. Assay conditions.

All experiments were repeated at least three times using at least three cultures derived from independent colonies, with consistent results. Growth curves represent average data from three experiments. Before initiation of experiments, cells were grown in pH 7-adjusted non-inducing medium to the exponential phase. Following a washing step in MilliQ water, cells were diluted in fresh inducing medium and cell density was adjusted. Cell growth was estimated by measuring optical density at 600 nm (OD_{600}) in a spectrophotometer (Genesys 10 VIS Thermo Scientific). Culturing was carried out at 30 ºC, with shaking at 300 rpm. Liquid cultures were initiated with exponentially growing cells diluted in inducing medium to an OD_{600} of 0.01 (corresponding to 2 x 10^5 cells/ml) or 0.1 (*petite* mutants). In growth kinetic assays, samples were collected after 24 h of incubation (t= 0 h) and OD_{600} was determined. Agar plate assays were performed with exponentially growing cells. After a washing step, cultures were diluted with fresh medium to an OD_{600} of 0.2 (glucose) or 0.4

(galactose). Drop tests were carried out by spotting 5 µl of serial dilutions of the cell suspension onto agar plates. Drop tests used the same (non-inducing or inducing) pH- adjusted medium for dilutions and also to prepare agar plates. Plates were incubated for the indicated time at 30 ºC and then scanned.

2.4. Protein extraction and western blotting.

Exponentially growing cells were diluted in YNB (2 % galactose) to an OD_{600} of 0.005. Samples (1 ml) were collected at OD_{600} 1.0 and protein extraction was performed using the following procedure: cells were pelleted and suspended in 1 ml lysis buffer (0.2 M NaOH, 0.1 M β-mercaptoethanol and 0.1 mM PMSF). After 5 min on ice, 2 µl of 100 % TCA was added and cell lysates were incubated at 65 ºC for 5 min followed by incubation at 4 ºC for 5 min. The non-soluble fraction was collected by centrifugation and washed with cold (-20 ºC) acetone (Merck). The pellet was dried in a Speed-vac concentrator (Savant) and the extract was suspended in 100 µl sample buffer (0.16 M Tris-HCl pH 6.8, 13.3 % glycerol (Merck), 2 % SDS, 1.5 % DTT and 0.033 % bromophenol blue). Lastly, samples were briefly sonicated and heated to 100 ºC for 5 min. Total protein content of each extract was measured using the Protein Assay Kit from Bio-Rad and concentrations were normalized to 1mg/ml protein prior to loading onto SDS/20 % polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by wet blotting. Protein transfer efficiency was monitored by Ponceau S staining. Membranes were subsequently cut into strips with the aid of molecular weight marker bands. Membranes were quickly destained in distilled water and blocked before addition of primary and secondary antibodies (Gonzalez and Carrasco, 2001). Labelled proteins were detected using ECL™ Western Blotting Detection Reagents (Amersham). Membrane striping was carried out in a buffer containing 100 mM 2-Mercaptoetanol, 2 % SDS and 62.5 mM TrisHCl (pH 7) at 50 ºC for 30 min. Membranes were subsequently reprobed.

2.5. Antibodies.

The Vpr monoclonal antibody was kindly provided by Dr. J. Koop (Provided by the EU Program

EVA Centre for AIDS Reagents, NIBSC, UK. AVIP Contract Number LSHP-CT-2004-503487). The monoclonal antibodies, anti-yeast 3- phosphoglycerate kinase (PGK), anti-yeast mitochondrial porin (Por1), anti-yeast COX complex IV subunit II (Cox2p) anti-yeast COX complex IV subunit IV (Cox4p) and anti-yeast H+-ATPase 60 kDa subunit were purchased from Mitosciences. A primary monoclonal antibody to GAPDH and peroxidase-conjugated goat secondary antibodies (anti-rabbit or anti mouse) were purchased from Pierce.

2.6. Transmission electron microscopy:

Wild-type yeasts were cultured in induction medium and 48 h later cells were collected. Cells were fixed (5 min) in glutaraldehyde in 0.1 M cacodylate buffer, added as a 2x stock to the culture. Cells were collected by centrifugation and resuspended at 1 OD_{600}/ml in fresh 1x fixative to complete fixation while gently rotating at room temperature (15 min). After potassium permanganate staining, samples were dehydrated and embedded in epoxy resin. Thin sections were prepared and mounted on mesh grids as described (Wright, 2000).

3. Results

3.1. Heterologous expression of HIV-1 Vpr protein causes cell death in Saccharomyces cerevisiae

We used the same *S. cerevisiae*-based expression system with which we previously characterized the impairment of potassium transport by the HIV-1 Vpu viroporin (Gonzalez, 2015; Herrero et al., 2013). An advantage of this system is its tight control of protein expression for extremely toxic

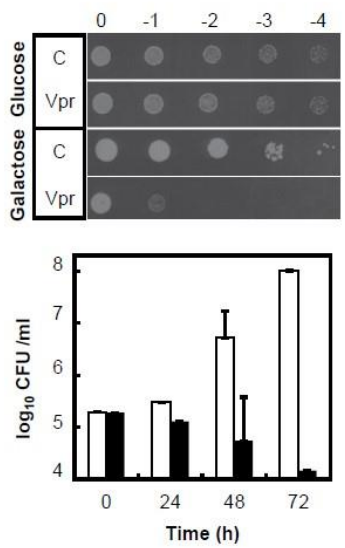

Figure 1. Analysis of Vpr-induced effects in *Saccharomyces* cultures. Exponentially growing yeast transformed with *control* or *vpr*-plasmids, were suspended in defined synthetic medium to 0.5 OD_{600} and a series of 10-fold dilutions were prepared. *Upper panel: Effect on culture growth.* Direct drop test plating serial dilutions onto agar-YNB medium supplemented with glucose or galactose. Plates were incubated for 2 days and were then digitally scanned. *Lower panel: Effect on cell survival*. Control (pEMBLyex) and Vpr (pEMBLyex*vpr*) cultures were diluted to 0.01 OD_{600} in inducing medium and incubated for the indicated periods before plating on rich medium supplemented with glucose. pEMBLyex yeasts (white) and pEMBLyex*vpr* -yeasts (black).

proteins such as HIV-1 Vpr, and transformed cells achieve high-level production of the viral protein. Plating of transformed pEMBLyex-yeast and pEMBLyex*vpr*-yeast in induction medium resulted in growth differences between the two cultures. Growth achieved by control yeast was 3-log greater than Vpr-expressing yeast (Fig. 1 upper panel). After adaptation to the induction medium for 24 h, the control culture grew over the next two days and increased by 3-log the number of colony forming units (CFU) while the Vpr-expressing culture completely failed to proliferate (Fig. 1 lower panel). Furthermore, culture of transformed yeasts in induction medium led to cell death of Vpr-expressing cells since the number of colony forming units CFU in rich medium was reduced over time. These results suggest that, in addition to inhibiting cell growth, the cytotoxic effect of Vpr protein causes cell death when *vpr* is inducible expressed in *S. cerevisiae* cells.

3.2. Protection from Vpr-induced growth arrest by antioxidant metabolites

Previous studies reported that extracellular Vpr protein impairs astrocytic levels of intracellular GSH and ATP. We tested for protection from Vpr-induced cell damage by exogenous exposure of Saccharomyces to GSH or other metabolites related with GSH metabolism, including the GSH prodrug N-acetylcysteine (NAC), adenosine triphosphate (ATP) and the oxidized form of the antioxidant ascorbic acid, dehydroascorbic acid (DHA). Dose

response analysis revealed that yeasts were protected from Vpr-induced growth reduction by GSH (Fig. 2). Compared with untreated cells, addition of GSH to levels just below the normal intracellular concentration of 10 mM (Penninckx, 2002) protected against Vpr-induced inhibition of growth almost completely (Fig. 2). Addition of 10 mM NAC also protected almost entirely against Vpr, although a higher concentration (15 mM) was cytostatic to the cultures. Addition of ATP offered poorer protection than GSH and NAC. The greatest protection was observed with 10 mM ATP, twice the physiological concentration. In contrast, the antioxidant DHA offered only minor protection from Vpr-induced growth arrest. Collectively, these results suggested that Vpr protein reduces intracellular ATP and GSH levels.

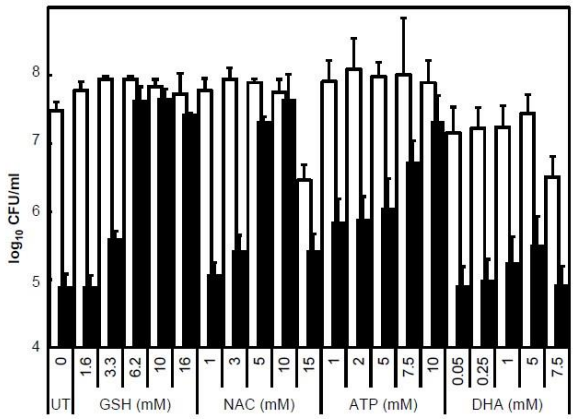

Figure 2. Comparison of the dose responses of cultures to metabolites involved in the antioxidant defense. Transformed yeasts were treated with the indicated concentration of each compound from the initiation of galactose induction. Viability of each culture was analyzed after 2 days of treatment by counting the number of CFU obtained in rich medium. pEMBLyex yeasts (white) and pEMBLyex*vpr* -yeasts (black). UT: untreated cultures.

To question whether Vpr affected mitochondria, we used western blotting to analyze distinct mitochondrial compartments in Vpr-treated cells with or without co-treatment with ATP, GSH, NAC or DHA. Mitochondrial markers used for this analysis were: mitochondrial protein localized in the outer membrane (OM, mitochondrial porin, Por1), inner membrane (IM, cytochrome c oxidase subunits 2 and 4, Cox2p and Cox4p), vacuolar protein (proton-ATPase 60 kDa subunit), cytosolic protein (PGK), a multi-localized protein (GAPDH), and Vpr protein. Striking differences were found between untreated Vpr cultures and Vpr cultures that were treated with ATP, GSH or NAC (Fig. 3). The relative levels of Cox2 and Cox4 proteins to the housekeeping proteins PGK and GAPDH were markedly different in treated than in untreated cultures, and levels of both mitochondrial IM proteins were clearly decreased in untreated Vpr-induced

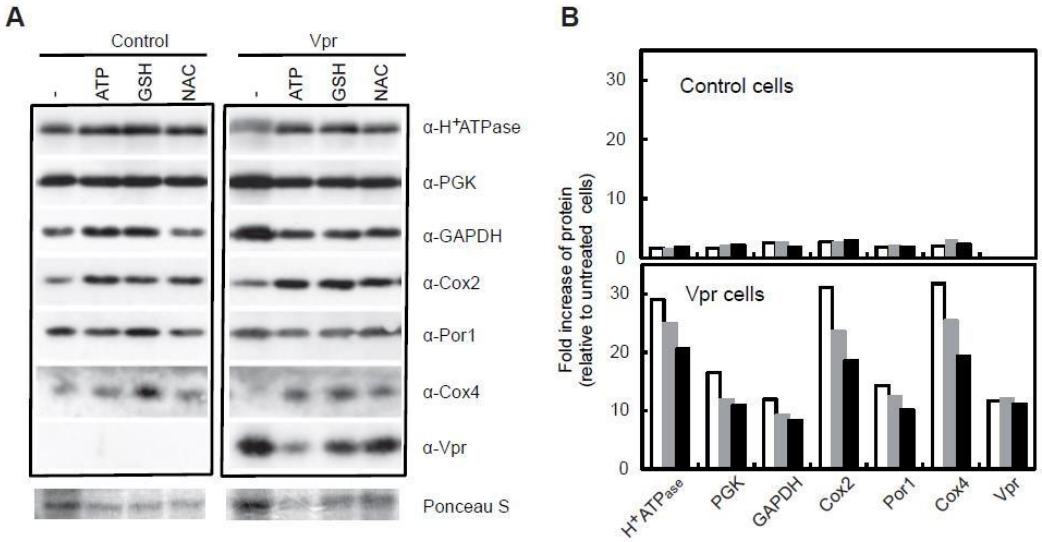

Figure 3. Western blot analysis of Vpr-induced changes in protein levels of endogenous proteins. Control (pEMBLyex) and Vpr (pEMBLyex*vpr*) cells were incubated in inducing medium supplemented or not (UT) with 5mM ATP, 10 mM GSH or 10 mM NAC as indicated. Cultures were collected during early exponential growth and cellular extracts were prepared. Equal concentrations (1mg/ml in PBS) were loaded on SDS-PAGE gels. All samples were analyzed on the same blot. *Panel A: Immunodetection of the proteins*. Resolution of each protein band is indicated in brackets. H^+-ATPase (60 kDa); PGK (45 kDa); GAPDH (38 kDa); Cox2p (35 kDa); Por1 (30 kDa); Cox4 (14 kDa); Vpr (14 kDa). The major band visualized after Ponceau S staining and expression of housekeeping genes *PGK* and *GADPH* help to compare each sample load and relative protein levels. *Panel B: Variations in protein contents by medium supplements*. Digitalized protein bands were quantified using PCBAS software and obtained values were normalized with corresponding Ponceau S stain values. Protein levels of treated cultures relative to untreated cultures are represented as follows: ATP (white), GSH (grey), NAC (black).

cultures. In contrast, similar differences were found between treated and untreated cultures in the level of the OM-localized mitochondrial protein, Por1, and glycolytic enzymes GADPH and PGK. It is noteworthy that levels of Cox2p, Cox4p and H^+ATPase were decreased despite the higher amounts loaded onto the gel as shown by Ponceau S staining (Fig. 3). The finding that the cytochrome c oxidase subunits 2 and 4 and vacuolar ATPase proteins were more sensitive to Vpr than Por1 pointed to a disturbed cytochrome c oxidase (COX) and V-ATPase assembly.

3.3. Vpr protein impairs the glutathione metabolism pathway

The above experiments were carried out in minimal synthetic medium, which ensured that exogenous GSH was not present unless specified. To guarantee that GSH was available when Vpr synthesis initiated, yeast cultures were pre-incubated with exogenous GSH or NAC prior to culture in galactose medium. We observed that cells were not protected against Vpr-induced damage by preloading with GSH or NAC (data not shown). Thus, protection against Vpr-induced cytopathic effects appeared to require the presence of intracellular glutathione simultaneously with Vpr protein.

We next examined the ultrastructure of yeast cells after galactose-induced expression of Vpr using transmission electron microscopy (TEM). A dramatic destruction of cell membrane structures could be observed in Vpr-cells, including the formation of aberrant vacuoles with membrane-bound vesicles, an unusual accumulation of intermediate endocytic profiles, deep indentations of the

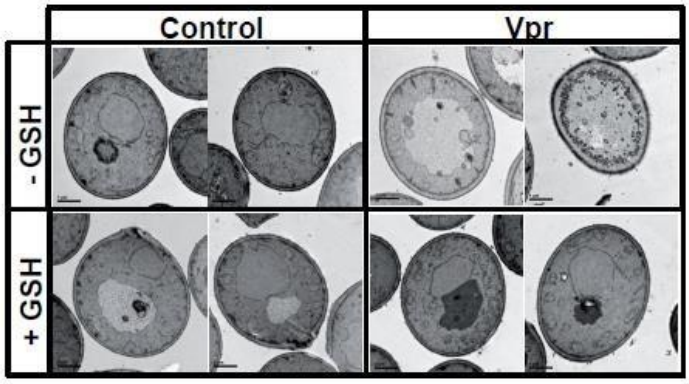

Figure 4. Ultrastructural analysis of Vpr-expressing cells. Representative transmission electron micrographs of ultrathin sectioned control (pEMBLyex) and Vpr (pEMBLyex-*vpr*) cells. Yeast cultures growing in inducing medium with/without 10 mM GSH were collected after 48 h of incubation. Samples were processed and stained with potassium permanganate to highlight membrane structures as described in the materials and methods section. Representative single cells of each sample were photographed.

vacuolar boundary membrane, autophagic bodies sequestered within, and cytoplasmic content engulfed by the vacuole (Fig. 4). Strikingly, the addition of GSH to the medium protected yeast from Vpr-induced morphological changes and the viral protein did not affect organelle membrane structures.

Glutathione can exist under two forms: the reduced form of glutathione (GSH) has antioxidant capacity by acting as a free radical scavenger, becoming oxidized in the process; the oxidized form of glutathione (GSSG) can be recycled back to GSH by the action of glutathione reductase, using NADH as an electron donor. Consequently, the intracellular GSH/GSSG ratio reflects the redox buffering capacity of cells, with optimal values ranging from 30-100:1. To examine whether the reducing capacity of glutathione was necessary for its protective effects on Vpr, we independently treated yeast cultures with oxidized and reduced forms of glutathione and compared their

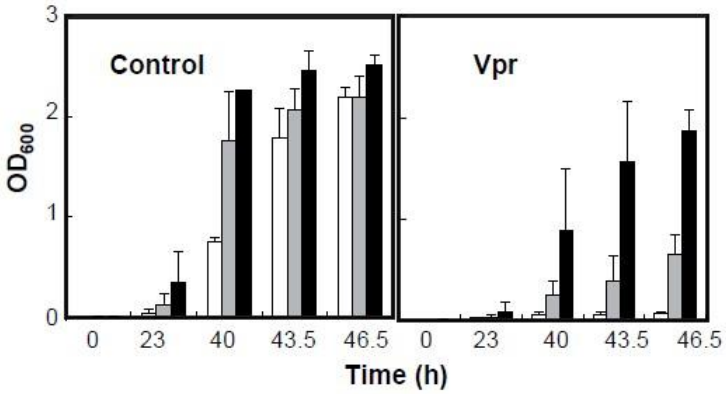

Figure 5. Protective capacity of the two redox forms of glutathione against Vpr-induced cytopathogenicity. Control (pEMBLyex) and Vpr (pEMBLyex-*vpr*) yeast were incubated in inducing medium. Cultures were untreated (white) or treated with 10 mM GSSG (grey) or 10 mM GSH (black) during the culture. At the indicated time after induction, aliquots were removed from cultures and the OD_{600} was measured. The results are representative of three experiments.

growth kinetics after induction of Vpr expression. At early stages of growth, less than 24 h after induction of *vpr* expression, a slight increase in Vpr-culture growth was observed in cultures supplemented with the reduced form of glutathione (Fig. 5). As incubation times increased, both reduced and oxidized forms caused increased culture growth relative to control cultures; however, GSH stimulated the growth of Vpr-induced cells 3-fold compared with GSSG-treated cells (Fig. 5), suggesting that the reducing power of GSH was used by cells as a defense against the cytopathic effects induced by Vpr.

3.4. Respiratory deficient yeasts are vulnerable to Vpr-induced damage

We questioned whether respiratory-deficient yeast would also be damaged by Vpr protein. To address this, we used a *petite* mutant that is incapable of using non-fermentable carbon sources such as glycerol. Thus, *petite* Saccharomyces was transformed with control or *vpr*-containing constructs and growth rates were monitored. Compared with glucose-replete conditions, control cells grown in

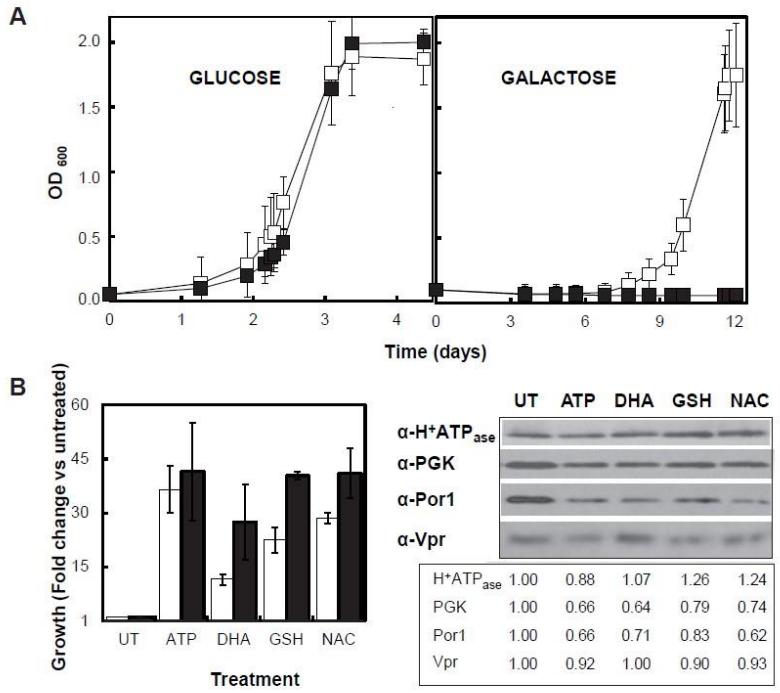

Figure 6. Vpr activity in respiratory-deficient mutants. *Panel A: Growth kinetics in non-inducing and inducing media.* Exponentially growing yeasts were diluted in synthetic medium containing glucose or galactose (0 days). OD_{600} was measured at the indicated periods of incubation. *Panel B: Vpr effect on respiratory-deficient yeast treated with metabolites of antioxidant defense:* Transformed yeasts (carrying pEMBLyex or pEMBLyex-*vpr* plasmids) were untreated (UT) or treated with the indicated compounds in inducing medium for 8 days. Cell density reached by each treated culture was measured by determining the OD_{600} and was represented as the fold change relative to untreated controls. Graph shows mean values from duplicate cultures. Error bars show standard deviation. Control yeast (white); Vpr yeast (black). Western blot analysis shows the immunodetection of indicated proteins after further incubation, for one day, of Vpr cells. Numbers in the table represent, in arbitrary units, the quantification of each protein band.

galactose-supplemented synthetic medium exhibited a long latency phase before initiating exponential growth (Fig. 6A). Indeed, galactose-grown control cells took 4-times longer to reach the equivalent growth level as glucose-grown control cells. Surprisingly, the growth of Vpr-induced cells was completely arrested by culture in inducing medium (Fig. 6A). These results imply that Vpr might not interfere directly with respiration since these cells are adapted to survive using nonrespiratory metabolism. We therefore decided to examine the effects of exogenous glutathione in the sensitivity of *petite* cells to Vpr. Treatment of this mutant with GSH, its prodrug NAC, ATP or DHA induced an early stimulation of growth in control cultures (Fig. 6B). The presence of Vpr protein was confirmed in all Vpr-cultures (Fig. 6B). After 9 days of treatment, control yeasts reached the maximum growth levels (data not shown). Because of this, the comparative growth levels were assessed while cells are still growing. The growth stimulation of Vpr-induced cultures by all treatments was even more remarkable than the growth stimulation of control cultures, though the differential stimulation of growth was less marked with ATP treatment (Fig. 6B). However, the level of protection from Vpr-induced damage was lower for the *petite* mutant than for wild type yeast, with all treatments. Collectively, these results suggested that the Vpr-induced growth arrest might be caused as a consequence of intracellular depletion of ATP stores.

4. Discussion

Using *S. cerevisiae*, we here demonstrate that the cellular damage induced by *de novo* synthesized Vpr is blocked by extracellular GSH, NAC and ATP. Our results are consistent with previous observations showing that GSH levels in erythrocytes and T cells decreased as HIV disease progressed in a patient, and a significant restoration of both T cell GSH and total GSH in the whole blood occurred after oral administration of NAC (De Rosa et al., 2000). An indirect functioning of DHA might explain its modest protection against Vpr-induced cellular damage in comparison with ATP, GSH and NAC. DHA stimulates the pentose phosphate pathway and intracellular glutathione levels (Kim et al., 2005; Puskas et al., 2000), and Vpr might dampen the glycolytic pathway (Ferrucci et al., 2013). While exogenous DHA may have stimulated energetic metabolism of Vprtreated yeast, a toxic side effect was demonstrated at the highest concentration assayed (7.5 mM). Glutathione is the most abundant low molecular weight thiol involved in antioxidant defense in eukaryotic cells. In eukaryotic cells, the greatest concentration of GSH is found in mitochondria and subsequently in the cytosol, nuclei, cell walls and vacuoles (Zechmann et al., 2011). In minimal media, yeast need to synthesize GSH in order to survive and grow (Baudouin-Cornu et al., 2012). Thus, growth stimulation caused by exogenous GSSG might be explained by its exploitation as a sulfur/nitrogen source after degradation. Another fact worth mentioning is that glutathione concentrates in the nucleus, where it promotes cell cycle progression *via* an ATP-dependent mechanism. In yeast, the half-life of GSH is approximately 90 min and two molecules of ATP are consumed during the synthesis of one GSH molecule from cysteine (Mehdi and Penninckx, 1997). Thus, the potential inhibition of ATP synthesis in the mitochondrion by Vpr should be reflected by an intracellular depletion of GSH. In contrast, exogenous addition of ATP would benefit GSH synthesis and transport to the nucleus, and subsequently yeast growth. Additionally, GSSG is stored in the vacuole for further catabolism. Therefore, replenishment of the vacuole with exogenouslysupplied GSSG may explain the growth stimulation of Vpr-yeast by oxidized glutathione. Here we observed that autophagic bodies accumulated in the vacuole of untreated *wild type* cells but not in

GSH-treated cells. The recovery of vacuole functioning might be related with increased levels of H^+ATPase demonstrated under these conditions.

Mitochondria are the major sites of metabolic energy production in eukaryotic cells *via* the process of oxidative phosphorylation. COX subunits are significantly induced during respiration and are necessary for assembly of complex IV of the electron transport chain (ETC) (Ohlmeier et al., 2004).

Conversely, unassembled subunits of the enzymatic complex undergo posttranslational degradation.

Of note is the high recovery of Cox2p and Cox4p levels by treatment with ATP, GSH or NAC in Vpr-expressing cells. COX is the terminal enzyme of the ETC and reduces molecular oxygen to water to generate ATP by the action of ATP synthase. The variations in the levels of subunits 2 and 4 were much higher in Vpr-yeast than in control yeast, suggesting that Vpr protein directly affects ATP production in mitochondria. Similarly, diminished levels of vacuolar ATPase by Vpr and high restoration of protein levels in treated cells suggest a Vpr-induced depletion of cytosolic ATP. During exponential growth, cells can change nuclear gene expression by using the mitochondrial retrograde response. Thus, low ATP levels may activate the mitochondrial retrograde signaling pathway (Liu and Butow, 2006). The inability of Vpr-expressing yeast to stimulate expression of the nuclear-encoded Cox4p suggests that mitochondrial signaling is impaired by Vpr protein. Furthermore, as a response to Vpr expression, steady-state levels of the GSH channel, Por1, might not be sufficient to ensure GSH ingress

into the intermembrane mitochondria space from the cytosol, where it is synthesized (Kojer et al., 2012). The levels of GSH are equivalent in normal and *petite* yeasts (Grant et al., 1997). It is important to consider that the oxidant sensitivity of slow growing *petite* mutants has been related to their depleted ATP levels rather than with any lack of antioxidative stress response. Indeed, disruption of the respiratory ETC results in sensitivity to oxidative stress. This sensitivity may explain the growth stimulation of mitochondrial mutants observed with antioxidant treatments.

Mechanisms mediating the pleiotropic functions of Vpr have been investigated for many years, but how this multitasking protein interacts with cells remains enigmatic (Guenzel et al., 2014; Muthumani et al., 2005). Heterologous overexpression *in S. cerevisiae* is a useful model to analyze the influence of viral proteins on cellular functioning (Alves-Rodrigues et al., 2006). Previous results have shown that this eukaryotic model is susceptible to the cell damage induced by the HIV-1 Vpr protein (Macreadie et al., 1995). Here we show that the *S. cerevisiae* model permits the analysis of Vpr interactions with the oxidative response and energy metabolism of eukaryotic cells and, consequently, should facilitate the search for Vpr targeted inhibitors.

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