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Comparative Biochemistry and Physiology, Part C 140 (2005) 395 – 402

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Substrate preferences and glucose uptake in glibenclamide-resistant *Leishmania* parasites

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Received 14 September 2004; received in revised form 28 March 2005; accepted 1 April 2005

Abstract

Several drug-resistant mammalian cell types exhibit increased glycolytic rates, preferential synthesis of ATP through oxidative phosphorylation, and altered glucose transport. Herein we analyzed the influence of parasite growth phase on energy substrate uptake and use in a *Leishmania* strain [NR(Gr)] selected for resistance against glibenclamide. Glibenclamide is an ABC-transporter blocker which modulates the function of glucose transporters in some mammalian cells. Our results demonstrate for the first time that compared to glibenclamide-sensitive *Leishmania*, exponential phase glibenclamide-resistant parasites exhibit decreased use of glucose as energy substrate, decreased glucose uptake and decreased glucose transporter expression. However, compared to glibenclamide-sensitive cells, stationary phase resistant parasites display an increased use of amino acids as energy substrate and an increased activity of the enzymes hexokinase, phosphoglucose isomerase, and especially NAD^+-linked glutamate dehydrogenase. These results suggest that drug resistance in *Leishmania* involves a metabolic adaptation that promotes a stage dependent modulation of energy substrate uptake and use as a physiological response to the challenge imposed by drug pressure.

Keywords: Amino acid; Drug resistance; Glibenclamide; Glucose; Glucose uptake; *Leishmania*; Metabolic substrate; MDR; MRP; P-gp

1. Introduction

Leishmania parasites develop resistance against a variety of compounds. This plasticity stimulated the discovery of the gene *ltpgpA*, for *Leishmania* (*L.*) *tarentolae* P-glycoprotein, and its protein product PGPA, as the first membrane system linked with drug resistance in *Leishmania* (Ouellette et al., 1990, 1991). Since then, a group of genes belonging to the ATP-binding-cassette (ABC)-transporters family, and whose gene products confer low levels of resistance to vinblastine, arsenite and trivalent antimoniates, has been characterized in *Leishmania* (Légaré et al., 1994; Papadopoulou et al., 1994; Borst and Ouellette, 1995). PGPA is defined as the most divergent eukaryotic ABC transporter (Ouellette et al., 1990), with close relation to the multi-drug resistance associated protein (MRP) (Cole et al., 1994). Of note, MRP belongs to a subgroup of structurally and functionally related proteins that include the mammalian sulfonylurea receptors SUR1 and SUR2 (Kast and Gros, 1997).

Glibenclamide was originally described as a K^+-$_{ATP}$ transporter blocker that interacts with sulfonylurea receptors (Inagaki et al., 1995); more recently it was established that glibenclamide inhibits anion transporters such as the ABC1 (Becq et al., 1997; Hamon et al., 1997), the AtMRP5 (Lee et al., 2004), the P-glycoprotein (Golstein et al., 1999) and the cystic fibrosis transport regulator (Schultz et al., 1996). The fact that different ABC proteins with dissimilar functions are inhibited by glibenclamide suggests that they exhibit a

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doi:10.1016/j.cca.2005.04.002

conserved glibenclamide-binding motive (Golstein et al., 1999) and that glibenclamide acts upon them by a common mechanism.

We demonstrated that *Leishmania* are susceptible to glibenclamide (Ponte-Sucre et al., 1998) and we selected and characterized a glibenclamide-resistant *Leishmania* strain (Ponte-Sucre et al., 1997; García et al., 2000; Silva et al., 2004). Glibenclamide-resistant parasites can also be selected in *L. amazonensis* LTB0016 (C. Machuca, A. Rodríguez, M. Herrera and A. Ponte-Sucre, unpublished); the resulting phenotype is reproducible according to multiple biochemical and physiological parameters. Levels of resistance reached in our in vitro model are modest (3–5 fold), but lie within those exhibited by *L. tarentolae* or *L. major* made resistant by either drug pressure selection (Singh et al., 2001), cosmid transfection (Cotrim et al., 1999; Coelho et al., 2003) or gene inactivation (Richard et al., 2004). The clinical relevance of modest drug resistance levels might be associated with poor prognosis and might be indicative of clinical failure.

Malignant drug-resistant mammalian cells display increased glycolytic rates, preferential synthesis of ATP through oxidative phosphorylation, and alterations in GLUT1-and GLUT3-mediated glucose transport (Lyon et al., 1988; Vera et al., 1991; Martell et al., 1997). No data exist on the impact of parasite growth phase on energy substrate uptake and use in drug-resistant *Leishmania*. We herein explored if glibenclamide resistance is accompanied by a growth phase dependent modulation of *Leishmania* metabolic preferences and glucose uptake.

Our results demonstrate that, compared to glibenclamide-sensitive *Leishmania*, exponentially growing glibenclamide-resistant parasites depict decreased glucose use, glucose uptake and expression of glucose transporters. In stationary phase parasites, resistance to glibenclamide associates with increased amino acid used as energy substrate. Furthermore, at exponential and stationary phases of growth, glibenclamide resistance is linked with changes in the activity of key enzymes relevant for the incorporation of carbon substrates into the Krebs cycle. These results suggest that drug resistance may involve a growth phase dependent modulation of energy substrate uptake as a physiological response to the challenge of drug pressure.

2. Materials and methods

2.1. Drugs, strains and culture conditions

Glibenclamide-sensitive *Leishmania* NR(Gs) (MHOM/VE/80/NR) was kindly provided by Dr. Angel Hernández (Universidad Central de Venezuela). These parasites were originally isolated in 1980 from a Venezuelan patient suffering from tegumentary diffuse leishmaniasis. *Leishmania* NR, previously classified as *L. mexicana mexicana* (Correnti and Ortega, 1991; Rangel et al., 1996; García et al., 2000; Silva et al., 2004), has been recently identified by molecular tools as *L. amazonensis* (Luis et al., 1998; Mendoza-Leon et al., 2002). *Leishmania* promastigotes were grown as previously described (Ponte-Sucre et al., 1993). The glibenclamide-resistant strain NR(Gr) was selected in vitro by means of successive passages of the sensitive strain in the presence of 16–25 μM (free drug concentration) glibenclamide (Research Biochemical International, USA) (Ponte-Sucre et al., 1997). The final NR(Gr) strain was 3–5 fold more resistant to glibenclamide than the original NR(Gs) strain. The resistant cells were always grown under the pressure of the drug, but the experiments were performed in the absence of glibenclamide unless otherwise indicated.

2.2. Glucose, ammonium and protein determination

The D-glucose concentration was determined enzymatically by the hexokinase/glucose 6-phosphate dehydrogenase method (Seyfang and Duszenko, 1991). The ammonium concentration was determined with the 170-UV Kit (Sigma). Protein concentrations were estimated by the dye-binding method (Bradford, 1976) using serum albumin as standard. All the determinations were done in triplicate for each experiment.

2.3. Growth of Leishmania and changes in glucose and ammonium concentrations in culture medium

Growth of glibenclamide-sensitive and -resistant cells seeded in complete medium was followed up to day 10. Aliquots were harvested every two days from the cultures and both glucose and ammonium concentrations were determined in the supernatants. Changes in medium concentration of glucose and ammonium were calculated as the decrease in glucose or increase in ammonium concentration during each growth phase. For both cell lines the exponential phase was taken from day one to day 4 and the stationary phase from day 5 to day 10. The increase in ammonium concentration was used as an indicator of amino acid catabolism (Cazzulo et al., 1985; Louassini et al., 1999). Glucose concentration in fresh medium was 12.62 ± 0.15 mM, while ammonium concentration was 0.221 ± 0.054 mM.

2.4. Glucose uptake and its modulation by putative inhibitors

The term “glucose uptake” was used throughout this study to indicate the incorporation of substrate with or without further metabolism (Seyfang and Landfear, 1999). Glucose uptake was estimated by measuring the disappearance of glucose from medium (Seyfang and Duszenko, 1991). Cell aliquots were withdrawn from cultures at mid-exponential or stationary phases of growth, washed and resuspended (10^9 cells mL^{-1}) in 20 mM Hepes, 132 mM

NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3, with or without glucose uptake inhibitors. After 5 min pre-incubation at 4 °C, and 5 min at 30 °C, glucose uptake was started by adding glucose (5 mM final concentration) at time zero. An aliquot of 150 μL was withdrawn immediately thereafter and then at 5, 10 and 15 min. Glucose uptake was terminated by centrifugation of the cell suspension for 30 s at 13,000 $\times g$, 20 °C, and addition of 7.5 μL 70 percent (by vol.) perchloric acid to the supernatants. These were further stored at 4 °C until glucose determination (Seyfang and Duszenko, 1991). The following inhibitors of glucose uptake were obtained from Sigma-Aldrich Chem. Co. (USA): cytochalasin B, phloretin, monensin, ouabain, oligomycin, and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). These inhibitors were dissolved in 70 percent ethanol (by vol.). Glibenclamide was dissolved in dimethyl sulfoxide.

2.5. Electrophoresis and western blotting

Crude cell homogenates were separated by PAGE and transferred into nitrocellulose membranes by standard procedures. The blots were assayed with a polyclonal antibody against an extracellular loop of the *Leishmania enrietii* Pro-1 glucose transporters (P1L) (1:500) (Burchmore and Landfear, 1998), as well as with a monoclonal antibody against *Leishmania mexicana* flagellar proteins (B7.1) (1:10,000) (Ismach et al., 1989). Diluted peroxidase-coupled goat anti-rabbit (1:2500) (Gibco Invitrogen Co. USA) was used as the secondary antibody. As *Leishmania* promastigotes show stable expression of flagellar proteins, their evaluation is convenient both as an internal control for protein loading into the gel and for normalizing the intensities of P1L with respect to B7.1. The intensities of the protein bands were analyzed by the Lab Image software.

2.6. Enzyme assays

Aliquots of 10^9 cells were withdrawn from cell cultures at mid-exponential or stationary phases of growth, and homogenized as previously described (Ponte-Sucre et al., 1993). Triton X-100 (0.2 percent final concentration) was added to the homogenate. Aliquots were also taken for protein determination. Hexokinase (EC 2.7.1.1) activity was assayed by monitoring the reduction of $NADP^+$ through a coupled reaction with glucose-6-phosphate dehydrogenase (Adroher et al., 1990). Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activity was assayed by monitoring the oxidation of NADH (Bergmeyer, 1984). Phosphoglucose isomerase (EC 5.3.1.9) activity was assayed in a coupled reaction with glucose-6-phosphate dehydrogenase by monitoring the reduction of $NADP^+$ (Hart and Coombs, 1982). Pyruvate kinase (EC 2.7.1.40) activity was assayed in a coupled reaction with lactate dehydrogenase by monitoring the oxidation of NADH (Ponte-Sucre et al., 1993). Alanine aminotransferase (EC 2.6.1.2) activity was assayed by the conversion of alanine to pyruvate in the presence of NADH (Mottram and Coombs, 1985). Glutamate dehydrogenase (EC 1.4.1.3) activity was determined by the conversion of 2-oxoglutarate to L-glutamate in the presence of ammonium acetate and NADH (Mottram and Coombs, 1985). The spectrophotometric measurements were carried out at room temperature. Enzyme activities were expressed as International Units (IU) mg^{-1} protein. 1 IU corresponds to the amount of enzyme needed to catalyze the conversion of 1 μmol of substrate min^{-1}.

2.7. Data analysis

Data on glucose utilization and ammonium production, glucose uptake inhibition and enzyme activities are expressed as mean ± SD of at least three independent experiments done in triplicate. Differences between glibenclamide-sensitive and glibenclamide-resistant parasites regarding enzyme activities, glucose utilization and ammonium production, were tested for statistical significance with the two way ANOVA (Schefler, 1981). Differences between glibenclamide-sensitive and glibenclamide-resistant parasites regarding glucose uptake, were tested for statistical significance with the unpaired-sample Student *t* test (Schefler, 1981).

3. Results

Under our experimental conditions growth of *Leishmania* NR(Gs) and NR(Gr) lasted 10 days, with minor differences between the two strains. Changes in glucose and ammonium concentrations in the culture media, an indication of glucose utilization and amino acid catabolism, were followed throughout. NR(Gs) parasites used 2.730 ± 0.107 mM glucose and NR(Gr) cells used 1.860 ± 0.280 mM glucose, that is, 30 percent less, during their exponential growth ($p < 0.05$). During their stationary phase of growth, NR(Gs) used 7.560 ± 0.485 mM glucose, while NR(Gr) used 7.728 ± 1.102 mM. NR(Gs) parasites produced 0.688 ± 0.088 mM ammonium during their exponential growth phase, while NR(Gr) parasites produced 0.737 ± 0.077 mM. Through their stationary phase of growth NR(Gs) produced 1.833 ± 0.013 mM ammonium, while NR(Gr) produced 2.304 ± 0.056 mM, that is, 25 percent more ($p < 0.01$). These data indicate that glibenclamide-resistant parasites use less glucose as energy substrate during their exponential phase of growth than glibenclamide-sensitive *Leishmania*. Additionally these data indicate that, during the stationary phase of growth, use of amino acids as energy substrate is up-regulated in glibenclamide-resistant cells.

To further substantiate these results, we determined the activity of enzymes involved in the parasite's glycolysis

such as hexokinase (HK), phosphoglucose isomerase (PGI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 1). At the exponential phase of growth, HK activity was 1.289±0.118 and 1.152±0.034 IU mg^{-1}, respectively in NR(Gs) and NR(Gr), while PGI activity was 2.911±0.083 and 2.353±0.148 IU mg^{-1} ($p<0.001$), respectively in NR(Gs) and NR(Gr); in stationary phase NR(Gr) cells these activities were 1.349±0.105 IU mg^{-1} for HK and 3.290±0.174 IU mg^{-1} for PGI that is, 49 percent and 44 percent higher than in sensitive cells ($p<0.001$). GAPDH activity was similar for NR(Gs) and NR(Gr) in exponential phase, and increased equally for both in stationary phase. The activity of pyruvate kinase (PK) was measured in an enriched cytosolic fraction obtained from stationary phase parasites. A 25 percent

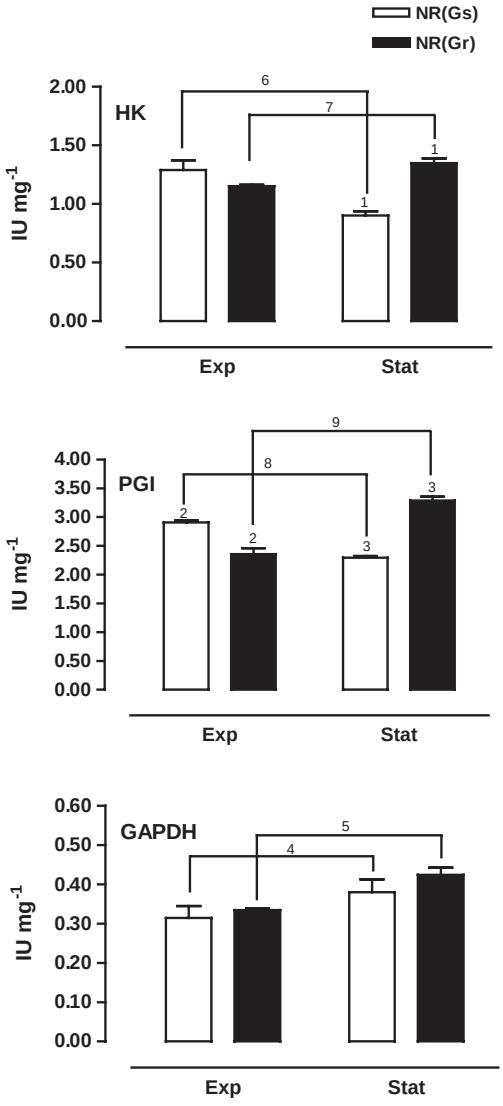

Fig. 1. Activities of hexokinase (HK), phosphoglucose isomerase (PGI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in exponential (Exp) and stationary (Stat) phase NR(Gs) and NR(Gr) ($^{1, 2, 3, 4, 5}p<0.001$; $^{6, 7, 8, 9}p<0.05$). IU, international units.

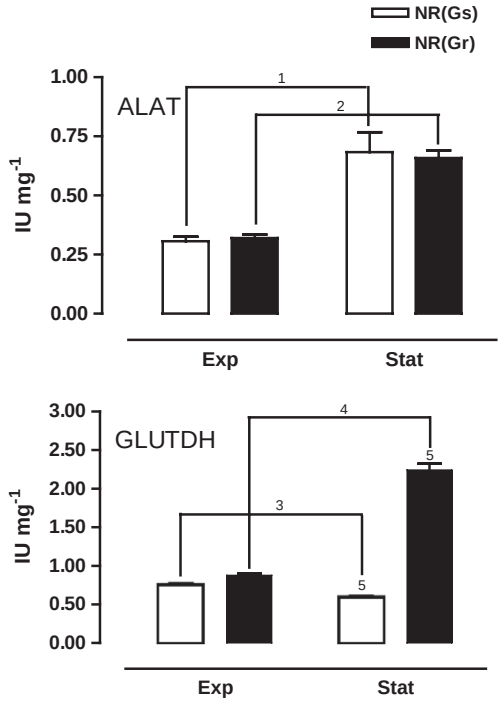

Fig. 2. Activities of alanine amino transferase (ALAT) and glutamate dehydrogenase (GLUTDH) in exponential (Exp) and stationary (Stat) phase NR (Gs) and NR (Gr) ($^{1, 2, 3, 4, 5}p<0.001$). IU, international units.

higher activity was observed in glibenclamide-sensitive vs. -resistant cells (23.09±0.35 IU mg^{-1} vs. 17.05±0.42 IU mg^{-1}, $p<0.001$). The previous experiments were performed in the absence of glibenclamide; of note, in similar experiments done in the presence of 100 μM glibenclamide, activities of HK, PGI and GAPDH were not affected. Altogether these results indicate that the activity profile of the glycolytic enzyme PGI is decreased in exponential cells by glibenclamide resistance and that, compared to sensitive parasites, the activity of HK and PGI considerably increases, the activity of GAPDH increases modestly, and the activity of PK decreases considerably in stationary phase resistant parasites.

The activity of key enzymes for the conversion of different substrates into Krebs cycle intermediaries, i.e., alanine transferase (ALAT) and NAD^+-linked glutamate dehydrogenase (GLUTDH) was evaluated (Fig. 2). The activity of ALAT was similar for glibenclamide-sensitive and -resistant cells in either the exponential phase (0.306±0.034 vs. 0.320±0.026 IU mg^{-1}) or the stationary phase (0.683±0.146 vs. 0.658±0.056 IU mg^{-1}), and for both cell lines it was higher in stationary than in exponentially growing cells ($p<0.001$). In glibenclamide-resistant cells, the activity of the NAD^+-linked GLUTDH was 17 percent higher in the exponential phase (0.752±0.045 vs. 0.880±0.054 IU mg^{-1}), and 378 percent higher in the stationary phase (0.592±0.023 *vs.* 2.242±0.200 IU mg^{-1}) ($p<0.001$), than in the corresponding growth phase of glibenclamide-sensitive cells. Again, the previous experiments were performed in the absence of

glibenclamide but, in similar experiments done in the presence of 100 μM glibenclamide, activities of ALAT and GLUTDH were not affected. Altogether these results demonstrate that the activity profile of the NAD^+-linked GLUTDH considerably increases in stationary phase resistant parasites.

To further evaluate if the previous results are related to the modulation of glucose transporter expression during glibenclamide resistance, we used P1L and Western blot analysis to determine the expression of glucose transporters in both exponential and stationary phase cells (Fig. 3, panel A, above). P1L recognized a 55 kDa band that represents the glucose transporters LmGT2 and LmGT3 in exponentially growing NR(Gs). The intensity of this band was 25 percent smaller in NR(Gr) (Fig. 4, panel A, below). Furthermore, down-regulation of the 55 kDa band happened in NR(Gs) but not in NR(Gr) upon reaching the stationary phase (Fig. 3, panel A, below).

The rate of glucose uptake during 15 min was also evaluated. Exponentially growing NR(Gs) depicted a higher rate of glucose uptake compared to NR(Gr) [(0.1298±0.0053) vs. (0.0782±0.0039) $mM \times 10^9$ $cell^{-1}$ min^{-1}] ($p<0.001$) (Fig. 3, panel B, above), but there was no difference between glibenclamide-sensitive [(0.0466±0.00035) $mM \times 10^9$ $cell^{-1}$ min^{-1}] and -resistant [0.0604±0.00423) $mM \times 10^9$ $cell^{-1}$ min^{-1}] stationary phase cells (Fig. 3, panel B, below). These results thus imply that glibenclamide resistance is related to a decreased expression of cell membrane glucose transporters and glucose uptake by exponentially growing cells.

Finally, the effect of glibenclamide and specific inhibitors of glucose uptake was evaluated in glibenclamide–sensitive and –resistant cells. The uptake of glucose was not significantly affected by ouabain (1 mM, a Na^+/K^+ ATPase inhibitor), monensin (1 μM, a ionophore with high specificity for Na^+ and K^+), FCCP (1 μM, a mitochondrial uncoupling agent) or oligomicin (20 μM, a proton translocator inhibitor) (data not shown). Glucose uptake by exponentially growing NR(Gs) and NR(Gr) (Fig. 4) was significantly decreased by cytochalasin B (300 μM), phloretin (150 μM) and glibenclamide (16 μM). Also, in stationary phase cells (Fig. 4) glucose uptake by NR(Gs) and NR(Gr) was inhibited by cytochalasin B, phloretin and glibenclamide. Inhibition of glucose uptake by glibenclamide in exponentially growing cells was greater in NR(Gr) than in NR(Gs) (49 percent vs. 25 percent) ($p<0.05$) (Fig. 4). These results thus confirm that glucose uptake in *Leishmania*

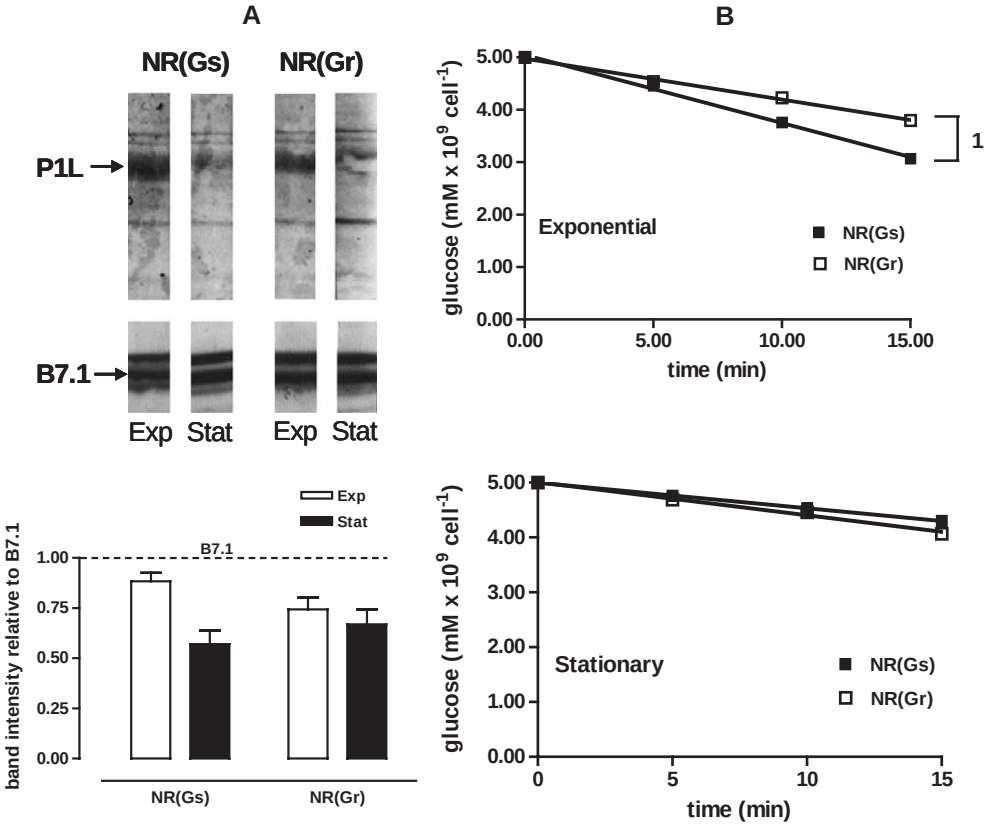

Fig. 3. (Panel A, above) Western blot analysis of glucose transporters identified by P1L in exponential (Exp) and stationary (Stat) phase NR(Gs) and NR(Gr) cells. (Panel A, below) Relative P1L band intensity (mean±SD of the mean from three independent blots) normalized to the B7.1 expression. (Panel B) Rate of glucose uptake by exponential (above) and stationary phase (below) cells ($^1p<0.001$ for differences between slopes).

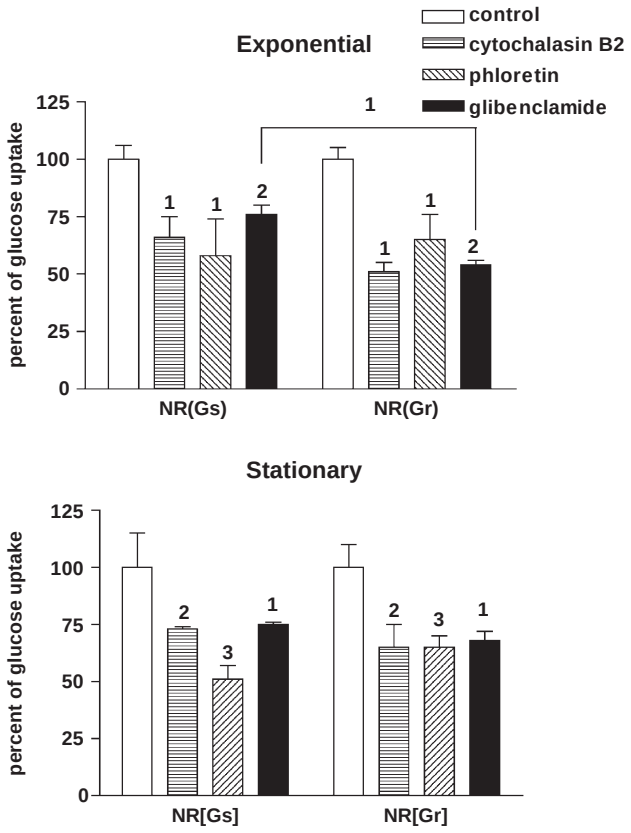

Fig. 4. Inhibition of glucose uptake in NR (Gs) and NR (Gr) by cytochalasin B, phloretin and glibenclamide in exponential and stationary phase cells. Number above the error bars indicates significant differences of glucose uptake in the same strain respect to control (without drug). Number above bracket indicates significant difference of glibenclamide effect on glucose uptake between the sensitive and the resistant strain. ($^1p<0.05$; $^2p<0.01$; $^3p<0.001$).

depends on systems sensitive to cytochalasin, phloretin and glibenclamide.

4. Discussion

Herein we investigated the influence of parasite growth phase on energy substrate uptake and use by drug-resistant *Leishmania*. Compared to glibenclamide-sensitive cells, exponentially growing glibenclamide-resistant parasites used less glucose and displayed lower glucose transporter expression and lower activity of the enzyme phosphoglucose isomerase. However, compared to glibenclamide-sensitive cells, stationary phase resistant parasites considerably increased their use of amino acids as a metabolic source, and showed increased activity of the enzymes HKPGI and NAD^+ linked GLUTDH.

PGI activity was significantly lower in exponential phase NR(Gr) vs. NR(Gs); HK and PGI activities increased significantly in stationary phase NR(Gr) but decreased in NR(Gs) cells; Interestingly however, changes in GAPDH activity were modest and paralell over the growth curve and for both strains. These results suggest that in resistant cells, the rate of glycolysis is decreased at the exponential growth phase, but that at the stationary growth phase, the rate of early glycolysis steps exceeds the rate at which metabolites are converted at steps below the aldolase reaction. The activity of PK (see also García et al., 2000), a cytosolic enzyme which in *Leishmania* controls the flux of metabolites into the Krebs cycle, was 25 percent higher in stationary phase NR(Gs) vs. NR(Gr). In stationary phase glibenclamide-resistant cells, a higher HK and PGI activity, with modest changes in GAPDH and with a decreased PK activity could lead to higher dihydroxyacetone phosphate (DHAP) production and increased D-lactate formation via the glyoxalate pathway (Martin et al., 1976; Simon et al., 1978; Darling et al., 1989). This is an operative mechanism that acts against the toxic effect of glycolytic derivates such as methylglyoxal (Blum, 1993, 1994; Irsch and Krauth-Siegel, 2004) and serves as an overflow mechanism under conditions of rapid glucose catabolism (Darling et al., 1989). The excess of DHAP would lead to the production of appreciable amounts of glycerol (Blum, 1993, 1994) and a decreased production of pyruvate (since PK activity is decreased), disturbing the rate at which carbon substrates enter the Krebs cycle through glycolysis. Stationary phase NR(Gr) vs. NR(Gs) cells exhibited an increased activity of GLUTDH (about 378 percent), but not of ALAT. The NAD^+-linked GLUTDH is involved in the incorporation of amino acid derived metabolites into the Krebs cycle (Cazzulo et al., 1985; Urbina, 1994).

Previous data suggest that glibenclamide resistance is related to the modulation of *Leishmania* metabolic steps. Therefore, we next evaluated glucose transporter expression and glucose uptake in glibenclamide-sensitive and -resistant parasites. Our results demonstrate a higher glucose uptake rate and a higher expression of a 55 kDa band, which represents the glucose transporters (Piper et al., 1995; Burchmore and Landfear, 1998; Burchmore et al., 2003) in NR(Gs) vs. NR(Gr) cells. These results could explain the overall behavior of glycolysis in resistant parasites during the exponential phase. In our experiments, differentiation to stationary phase decreased 3 uptake during 15 min and the expression of the 55 kDa band, a 50 percent decrease in glucose metabolism has been previously described in other *Leishmania* species during late exponential-stationary phase differentiation (Blum, 1990, 1993; Mukkada, 1985).

The data presented herein are in line with facts reported for *Saccharomyces cerevisiae.* In this organism, HXT11 overexpression increases drug sensitivity even in the wild-type strain and is involved in pleiotropic drug resistance. HXT11 is a permease which allows glucose uptake, of note, this putative hexose transporter expression is regulated by transcription factors known to control the ABC transporter production required for drug resistance in yeast (Nourani et al., 1997).

Glucose uptake in NR(Gs) and NR(Gr) was partially inhibited by cytochalasin B and phloretin, a result which supports the presence of a facilitated diffusion transport system for glucose, as in other species of *Leishmania* and in *Trypanosoma brucei* (Seyfang and Duszenko, 1991; ter Kuile and Opperdoes, 1993).

From our results it is thus tempting to speculate that the altered glucose and amino acid utilization observed in glibenclamide-resistant cells may reflect a modulation of metabolic key control points which would affect the rate at which carbon substrates enter the Krebs cycle. Given the flexibility of *Leishmania* metabolism, drug resistance may thus involve a growth phase dependent modulation of energy substrate uptake as a physiological response (Ponte-Sucre, 2003) to the challenge of drug pressure.

Acknowledgment

Supported by Venezuelan grants, FAC.MED, 000054/2000 and CDCH, PI-09.33.5520.2003. The antibody P1L was kindly donated by Dr. Scott Landfear. We would like to acknowledge Dr. Michael Duszenko and Dr. Julio Urbina for critical comments on the manuscript.

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