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NADH-Linked Metabolic Plasticity of MCF-7 Breast Cancer Cells Surviving in a Nutrient-Deprived Microenvironment

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ABSTRACT

Characteristic of the tumor microenvironment are fluctuating gradients of reduced nutrient levels and released lactate. A fundamental issue is how tumor cells modulate their metabolic activity when both glucose and glutamine levels become limiting in the presence of high exogenous lactate. For functional analyses, the activities of pyruvate kinase, lactate dehydrogenase (LDH) and plasma membrane NADH oxidase (NOX) as well as cell growth were measured in breast cancer MCF-7 cells cultured in medium containing various concentrations of these metabolites. After 3 days at glucose concentrations below 2.5 mM, cell number was higher with 0.1 mM than with 1.0 mM glutamine, indicating that the glucose/glutamine balance is important for growth. On the other hand, NOX activity increased with increasing glucose >2.5 mM, but only with low glutamine (0.1 mM). Pyruvate kinase activity also increased, with LDH activity remaining 2–3-fold lower. Here NOX could have a complementary role in reoxidizing NADH for glycolysis. Exogenous lactate supported cell survival at limiting concentrations of glucose and glutamine while increasing NOX and pyruvate kinase activities as well as NADH levels. It is proposed that lactate supports cell survival by fuelling gluconeogenesis and/or the TCA cycle in mitochondria, from where NADH could be shuttled to the cytosol and reoxidized by NOX. Cell survival and the metabolic phenotype are thus interrelated to the dynamics of NADH and plasma membrane NOX activity, which are regulated by the balance of glucose/glutamine levels, in conjunction with lactate in a precarious tumor microenvironment. J. Cell. Biochem. 116: 822–835, 2015.

KEY WORDS: TUMOR METABOLISM; NUTRIENT DEPRIVATION; GLUCOSE; GLUTAMINE; LACTATE; PLASMA MEMBRANE NADH OXIDASE; GLYCOLYTIC ENZYMES

Tumor metabolism is characterized by shifts in catabolic and anabolic pathways with respect to normal cells, a prominent feature of many cancer cells being a high rate of aerobic glycolysis and production of lactate, that is, the Warburg effect (recent aspects are reviewed by Jose et al. [2011] and Porporato et al. [2011]). This effect is being exploited clinically by various technologies for diagnosis and therapeutic response monitoring, for example, positron emission tomography (PET) using ^{18}F-2-deoxyglucose and, more recently, with hyperpolarized ^{13}C-labeled metabolites such as pyruvate [Witney and Brindle, 2010]. Also, therapeutic strategies are being developed to subvert tumor specific metabolic derangements, for example, by inhibition of a monocarboxylate transporter (MCT1) [Sonveaux et al., 2008] or a key enzyme such as lactate dehydrogenase (LDH-A) [Granchi et al., 2011].

These strategies are highly dependent on our present understanding of tumor metabolism, which has been gained from investigations on various tumor cells models. In cancers, tumor cells are embedded in a microenvironment of fluctuating conditions depending on the distance from the blood supply and their interaction with stroma and other cells. With increasing tumor mass and even when new, but functionally inept, vessels are formed, the cells will be exposed to an increasing deprivation of oxygen and

Abbreviations: LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; NOX, plasma membrane NADH oxidase; PK, pyruvate kinase; PEP, phosphoenolpyruvate; PDH, pyruvate dehydrogenase; PMS, 1-methoxy-5-methyl phenazinium methyl sulphate; TCA, tricarboxylic acid cycle; SOD, superoxide dismutase.

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nutrients as well as an increasingly acidic environment [Sutherland, 1986; Kallinowski et al., 1988; Vaupel, 2009]. Such gradients in the microenvironment will have immediate effects on the metabolism of individual cells and contribute to the functional heterogeneity of a tumor, which has not always been considered in in vitro investigations.

Among the metabolic substrates, glucose and glutamine are considered to be the major ones for maintaining energy metabolism and growth of tumor cells. Changes in the metabolic flux of tumor cells are found particularly in glycolysis, tricarboxylic acid cycle (TCA) and in oxidative metabolism. An increase in glucose uptake and glycolytic rate is concordant with an upregulation of hexokinase, phosphofructokinase, aldolase, enolase and pyruvate kinase as well as lactate dehydrogenase (LDH), as reported, for example, in breast cancer tissues [Hennipman et al., 1987]. Moreover, expression and activity of glycolytic enzymes are under the influence of various signaling oncoproteins and transcription factors, foremost Myc, Akt, TOR and HIF1-α [DeBerardinis et al., 2008; Dang and Semenza, 1999]. In many tumors there is an upregulation of the pyruvate kinase isoform M2, which in its prevalent dimeric state has low specific activity; the resulting accumulation of glycolytic phosphometabolites could serve synthetic processes [Mazurek et al., 2002]. Under limiting conditions with attenuated oxidative metabolism, glycolytic flux is strictly controlled and maintains a stable ATP level, thereby ensuring the survival of tumor cells [Mazurek et al., 2002]. Generally, a high glycolytic rate is also coupled to a high activity of lactate dehydrogenase, that is, lactate production [Dang and Semenza, 1999].

The role of glutamine in tumor metabolism depends on the tumor cell type and the microenvironmental conditions. A high level of glutaminase activity in tumor cells ensures the production of glutamate and α-ketoglutarate, and thus an anaplerotic input into the TCA cycle. Some tumor cell lines appear to be highly dependent on this amino acid, where glutamine can maintain energy metabolism under glucose deprivation [Baggetto, 1992]. When glycolysis is downregulated by extracellular AMP in MCF-7 cells, glutamine appears to be even the main source for lactate [Hugo et al., 1992]. Metabolic profiling of tumor cell lines representing different cancerous stages has shown that the intracellular levels of glucose and glutamine increase and decrease in opposite ways and that their metabolisms may be uncoupled by the oncogene K-ras [Ramanathan et al., 2005; Gaglio et al., 2011]. Therefore, the balance of glucose and glutamine plays an important role in the metabolic phenotype, and this is expected to be particularly true at limiting availability of these metabolites in nutrient deprived microregions of a tumor.

Being a product of both glycolysis and glutaminolysis, pyruvate is a metabolite at the intersection of anaerobic and aerobic pathways [Yuneva, 2008]. Under aerobic conditions, pyruvate enters the mitochondria, where it is decarboxylated by the enzyme complex pyruvate dehydrogenase (PDH). The resulting acetyl CoA enters the tricarboxylic acid cycle, where further reactions spin off NADH and FADH, which fuel the respiratory chain and provide reducing equivalents for other metabolic pathways. Under anaerobic conditions, PDH activity is attenuated, and indeed, in tumor cells PDH expression is downregulated [Koukourakis et al., 2005b]. In this case, cytosolic pyruvate is converted mainly to lactate. This reaction also reoxidizes NADH to NAD^+, the latter being required to sustain the glycolytic flux. Lactate as well as pyruvate can leave the cells through their monocarboxylate transporters (MCT1–4), in the blood reaching concentrations of 0.5–14 mM and 0.04–0.1 mM, respectively. Moreover, both metabolites can also be released by tumor-associated fibroblasts in to the microenvironment, where lactate concentrations over 25 mM have been reported [Pavlides et al., 2009; Vaupel, 2009]. Lactate is released by actively growing tumor cells in particular by the low affinity MCT-4, which is often upregulated [Dimmer et al., 2000; Ganapathy et al., 2009]. Since both tumor and neighboring cells can release lactate, it is reasonable to assume that aerobic tumor cells may utilize extracellular lactate produced by stroma or anaerobic tumor cells [Feron, 2009]. At which glucose level a tumor cell would switch to lactate to fuel essential metabolic pathways is an aspect addressed in this paper.

Genetic alterations and changes in protein expression of many metabolic enzymes have been described, including LDH and pyruvate kinases [Koukourakis et al., 2005a; Mazurek, 2011], While valuable information on the role of glucose and glutamine on cell growth and in the oncogenic regulation of metabolism has been acquired by depleting or limiting either glucose or glutamine [Collins et al., 1998; Chen and Shtivelman, 2010], studies using combinations of both substrates at limiting tumor relevant levels have been lacking. Moreover, little information is available in quantitative terms on metabolic activities of cells growing in low-nutrient conditions. Our present knowledge is based mainly on biochemical studies, for example, with ^{14}C-labeled metabolites in cell cultures [Clegg and Jackson, 1990] and NMR-techniques using various non-radioactive isotope-labeled metabolites, such as ^{13}C-glucose, in selected tumor models with different metabolic phenotypes (e.g., with breast cancer cells [Kaplan and Cohen, 1994; Forbes et al., 2006]). More quantitative studies are required on metabolic dynamics under more tumor-like nutrient conditions to better understand how such conditions will modulate the metabolic phenotype. These will be of relevance not only for the evaluation of metabolic imaging in the clinic, but also for developing new antimetabolites and enzyme inhibitors as chemotherapeutic agents.

In many in vitro investigations, the tetrazolium-based WST-1 assay is frequently used as a measure of cell growth and viability. As a commercially available kit, the WST-1 assay actually measures the activity of a plasma membrane electron transport system consisting of a cell surface and an inner-membrane NADH oxidase [Herst and Berridge, 2007; Berridge and Tan, 1998]. While the cell surface NADH oxidase can directly utilize extracellular NADH (or NADPH), the inner membrane NADH depends on cytosolic NADH, passing on the reduction equivalents via ubiquinone to drive the extracellular enzyme [Scarlett et al., 2005; Berridge and Tan, 1998]. There exist several isoforms of this NADH oxidase complex, including a constitutive form cNOX and those being expressed on the surface of different tumor cells (tNOX) [Morre et al., 2009]. In this paper, the term NOX will used generally to functionally denote plasma membrane NADH oxidase activity as measured by the WST-1 assay on MCF-7 cells.

This study was initiated by addressing two issues: (1) how do limiting glucose and glutamine concentrations, in variable

combinations, modulate tumor cell metabolism and affect survival; (2) at which level of nutrient deprivation could extracellular pyruvate or lactate be a substitute substrate? The human breast cancer cell line MCF-7 served as an in-vitro model for a carcinoma showing epithelial-like morphologically and low malignant potential. These cells express the monocarboxylate transporters MCT1, 2 and 4 as well as the LDH isotypes 1–4, with LDH-1 being typical of oxidative cells which convert lactate to pyruvate [Hussien and Brooks, 2011; Porporato et al., 2011]. The variables characterizing a tumor microenvironment are too numerous to include in a single investigation, therefore, this initial work focused on varying the concentrations of the metabolic substrates under standard culture conditions, that is, pH 7.4 and atmospheric O_2-condition. Since the metabolic role of the NADH oxidase system in tumor cells is not yet well understood, activities of pyruvate kinase and LDH as well as NADH levels were measured to further characterize the metabolic phenotype of MCF-7 cells. The results show that NOX activity is linked to changes in NADH resulting from glycolysis and lactate metabolism, and these metabolic activities are highly sensitive to the balance of low glucose and glutamine concentrations.

MATERIALS AND METHODS

CHEMICALS

Cell cultivation media, phosphate-buffered saline (PBS), glucose, glutamine, lactate, pyruvate, and insulin were obtained from Sigma–Aldrich, and fetal calf serum (FCS) was from Biochrom, Germany. Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle and the tetrazolium salt XTT (In Vitro Toxicology Assay Kit) were also obtained from Sigma–Aldrich, Germany; WST-1 (Cell Proliferation Reagent WST-1) was from Roche Applied Science, Germany.

CELL CULTIVATION

MCF-7 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L (25 mM) glucose, 0.58 g/L (4 mM) glutamine, 3.7 g $NaHCO_3$/L, supplemented with 5% fetal calf serum (FCS), and were incubated in a standard incubator (37°, 10% CO_2). For experiments, cells were first plated under standard conditions in 24-well-plates ($0.2–0.3 \times 10^5$ cells in 500 μl) or in 6-well dishes (0.8×10^5 cells in 2.5 ml). After 24–48 h, medium was changed to DMEM-Base (without glucose, glutamine and $NaHCO_3$) which was supplemented with 1–2% FCS, 50 nM insulin, 3.7 g $NaHCO_3$/L to maintain a pH of 7.4, and the substrate concentrations indicated in the experiments. These cultures were incubated for up to 4 days.

DETERMINATION OF CELL SURFACE NADH-OXIDASE ACTIVITY BY TETRAZOLIUM-BASED ASSAYS

Cells were incubated with the tetrazolium salt solution WST-1, which contains 1-methoxy-5-methyl phenazinium methyl sulphate (PMS), in the conditioned culture medium as prescribed by the kit. Initial experiments were performed with an XTT-assay. Both reagents are membrane impermeable salts and gave similar results. Formazan formation was measured after 4 h at 450 nm (FLUOstar Galaxy, BMG Labtechnologies; Victor 3, Perkin Elmer). Thereafter, cells were counted in the same well, and the specific optical density for formazan was calculated per cell number ($OD/10^5$ cells). Rotenone (10 μM), an inhibitor of mitochondrial NADH-oxidase, was added to cultures for up to 120 min prior to adding WST-1 solution as described by Berridge and Tan (1998). NADH (0.4 mM) as well as superoxide dismutase (SOD) (50 μg/ml), which removes possible O_2-radicals from the reaction, were added just before the WST-1 solution.

DETERMINATION OF CELL NUMBER

The procedure is based on counting nuclei in suspensions prepared from rinsed cell monolayers using a hypotonic buffer and a lysis agent as described before [Butler, 1984; Otto et al., 1996]. The nuclei suspension was counted in an electronic counter (Casy, Schärfe).

ACTIVITY OF PYRUVATE KINASE AND LACTATE DEHYDROGENASE

Whole-cell homogenates were prepared by incubating cells in the same hypotonic buffer used for preparing the nuclei suspension. The activity of LDH was determined using NADH and pyruvate as a substrate and monitoring the changes in absorption at 340 nm (Specord 210, Analytik Jena AG). Pyruvate kinase activity of the homogenate was measured likewise, except that the reaction buffer contained LDH, and phosphoenolpyruvate (PEP) and ADP as substrates. LDH activity was also determined directly in centrifuged culture supernatants. Enzyme activities were calculated from the regression line of the absorption slope and specific activities were calculated per cell number, or per ml supernatant.

DETERMINATION OF GLUCOSE CONSUMPTION

Aliquots of the conditioned media were frozen at −20° until analysis. Glucose levels were determined using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, Life Technologies). An aliquot was diluted depending on the expected glucose content and combined with the Amplex Red® solution. Fluorescence was measured after 20 min at the excitation and emission wavelengths of 530 and 590 nm, respectively (Victor 3, Perkin Elmer). Glucose concentrations were calculated from a standard glucose curve. To calculate glucose consumption, the glucose remaining in the culture medium was subtracted from the initial glucose content and normalized by the respective cell number in each well.

DETERMINATION OF RELEASED LACTATE

Aliquots of the culture supernatants were frozen at −20° until analysis. The assay was performed according to Maughan [1982]. An aliquot was added to a buffer containing 1.1 M hydrazine (pH 9.0), 5 mM NAD^+, L-LDH (2500 units/ml, Sigma) and the change in fluorescence measured after 20 min at the excitation and emission wavelengths of 355 and 460 nm, respectively (Victor 3, Perkin Elmer). Lactate concentrations were calculated from a standard lactate curve

STATISTICAL ANALYSIS

All experiments were performed at least three times, each in triplicates. Figures show averaged numbers and standard errors of representative experiments. Error bars in the figures are often smaller than the symbols. In support of biological relevance,

statistical analysis of key experiments was performed using the two-way *t*-test provided by the excel software.

RESULTS

NON-ADDITIVE EFFECTS OF VARYING RATIOS OF LOW GLUCOSE AND GLUTAMINE CONCENTRATIONS

To mimic selected parameters of poorly supplied tumor micro-regions, serum growth factors and hormone levels were reduced by supplementing the medium with 1% FCS plus 50 nM insulin for sustaining growth. The first question was how cell growth and metabolism would change when both glucose and glutamine were reduced to limiting concentrations in varying ratios. As Figure 1Aa shows, at low glucose concentrations (<2.5 mM) cell numbers after 3 days were unexpectedly higher in combination with 0.1 mM than with 1 mM glutamine, while above 2.5 mM glucose, cell number increased more with 1.0 mM glutamine. The XTT- and WST-1 assays, measuring plasma membrane NOX activity, were used as a first indicator for metabolic activity. When expressing tetrazolium transformation as optical density (OD) per cell number (Fig. 1Ab), the values show enhancement of NOX activity up to 4-fold with increasing glucose concentrations above 2.5 mM, but only in combination with 0.1 mM glutamine. With 1 mM glutamine NOX activity remained at a low basal level suggesting that glutamine metabolism produced little cytosolic NADH for NOX activity.

Testing cell growth and metabolism with varying glutamine concentrations below 0.5 mM (Fig. 1Ac, d), the cell number increased up to about 2-fold with 2.5 mM glucose as compared with 1 mM glucose, corresponding with the results in Figure 1Aa. Specific NOX activity was highest without glutamine and dropped markedly to a basal level at 0.1 mM glutamine when in combination with 1 mM glucose. However with 2.5 mM glucose, NOX activity was markedly higher at 0.1 mM than with 0.5 mM glutamine (Fig. 1Ad). Taken together, the results in Figure 1A indicate that in MCF-7 cells there is a switch in metabolic activity occurring with 0.1 mM glutamine at a glucose concentration >2.5 mM.

GROWTH KINETICS AT VARYING RATIOS OF LIMITING CONCENTRATIONS OF GLUCOSE AND GLUTAMINE

To investigate how long cells can survive in low nutrient conditions, kinetics of cell growth were determined without medium replenishment for up to 4 days (Fig. 1Ba,b). Cell number increased up to day 2 and declined thereafter, except for cultures containing 0.1 mM glutamine/2.5 mM glucose, where cells survived for at least another 24 h. In contrast, in all conditions NOX activity was highest immediately after medium change and markedly decreased within 1 day thereafter, depending on the different combinations of glucose and glutamine. NOX activity fell to 10–20% of the initial value, except with the combination 0.1 mM glutamine/2.5 mM glucose, where this activity prevailed. There thus appears to be a relationship between NOX activity and cell survival.

A low cell number could be the result of reduced proliferation rate, cell death, or both due to nutrient deprivation during the cultivation period. Therefore, cells were cultured with a daily medium change (Fig. 1Bc–d). In this case, cell number increased continuously during the 4-day incubation at all conditions, albeit with a markedly higher rate for cells in 1 mM than in 0.1 mM glutamine. This indicates that the replenishment with nutrients maintained continuous cell growth. Nevertheless, within 1 day after the initial medium change, NOX activity declined to basal levels, as was observed for the cells without medium change (Fig. 1Bb). However, this decline was again much slower for cells in the combination 0.1 mM glutamine/2.5 mM glucose (Fig. 1Bd). Therefore, NOX activity does not reflect proliferation potential, but rather a variable metabolic state.

ACTIVITIES OF GLYCOLYTIC ENZYMES AT LOW GLUCOSE AND GLUTAMINE CONCENTRATIONS

To investigate if NOX activity correlated with glycolysis, the activity of the glycolytic enzymes pyruvate kinase and LDH were tested in homogenates of cells after their incubation in selected low-growth conditions (Fig. 2). While these determinations do not measure actual metabolic flux, they do give quantitative information on the potential enzyme activities available in cells grown in different metabolic conditions. Cells cultured with the combination 0.1 mM glutamine/2.5 mM glucose had comparatively high pyruvate kinase activity (Fig. 2Ac,Bc); but again for cells grown with 1 mM glutamine, enzyme activity was lower, especially with 1 mM glucose, implicating a status of reduced glycolytic rate. In these conditions, pyruvate kinase activity correlated well with NOX activity. However, when cells were grown in standard DMEM, containing physiologically saturating glucose and glutamine concentrations (25 and 4 mM, respectively), NOX activity remained low (Fig. 2Ab), while pyruvate kinase activity was as high as for the combination of 0.1 glutamine/2.5 glucose (Fig. 2Ac). Therefore, these two enzyme activities are not directly coupled.

Also in cell homogenates LDH activity, measured as the conversion of pyruvate to lactate, was higher at 0.1 mM than at 1 mM glutamine, regardless of the glucose concentration. This suggests that there is an enhanced rate of anaerobic glycolysis at limiting glutamine levels. However, even under these conditions, LDH activity was only about 50% of the pyruvate kinase activity, and the ratio LDH/pyruvate kinase activity was lowest with the combination of 0.1 mM glutamine/2.5 mM glucose. Therefore, higher LDH activity did not simply correlate with available glucose levels, but also depended on the glutamine concentration. The highest LDH activity was measured in cells grown in standard DMEM, there being 5-fold higher than with cells in low-growth medium at 1 mM glutamine and 1 mM glucose (Fig. 2Ad, Bd).

The reduction in cell growth was attributed to cell death, since the culture supernatant contained a high number of detached and trypan blue-positive cells. This observation was confirmed by measuring high LDH activity as an indicator of cell damage in the culture supernatant (Fig. 2Bb). However, only marginal or no LDH activity was detected in supernatants from cells growing in 0.1 mM glutamine/2.5 mM glucose, corresponding with very few detached cells and a sustained cell number. Therefore, this glutamine-glucose combination maintained higher cell viability under these limiting conditions than with other low "unbalanced" nutrient combinations.

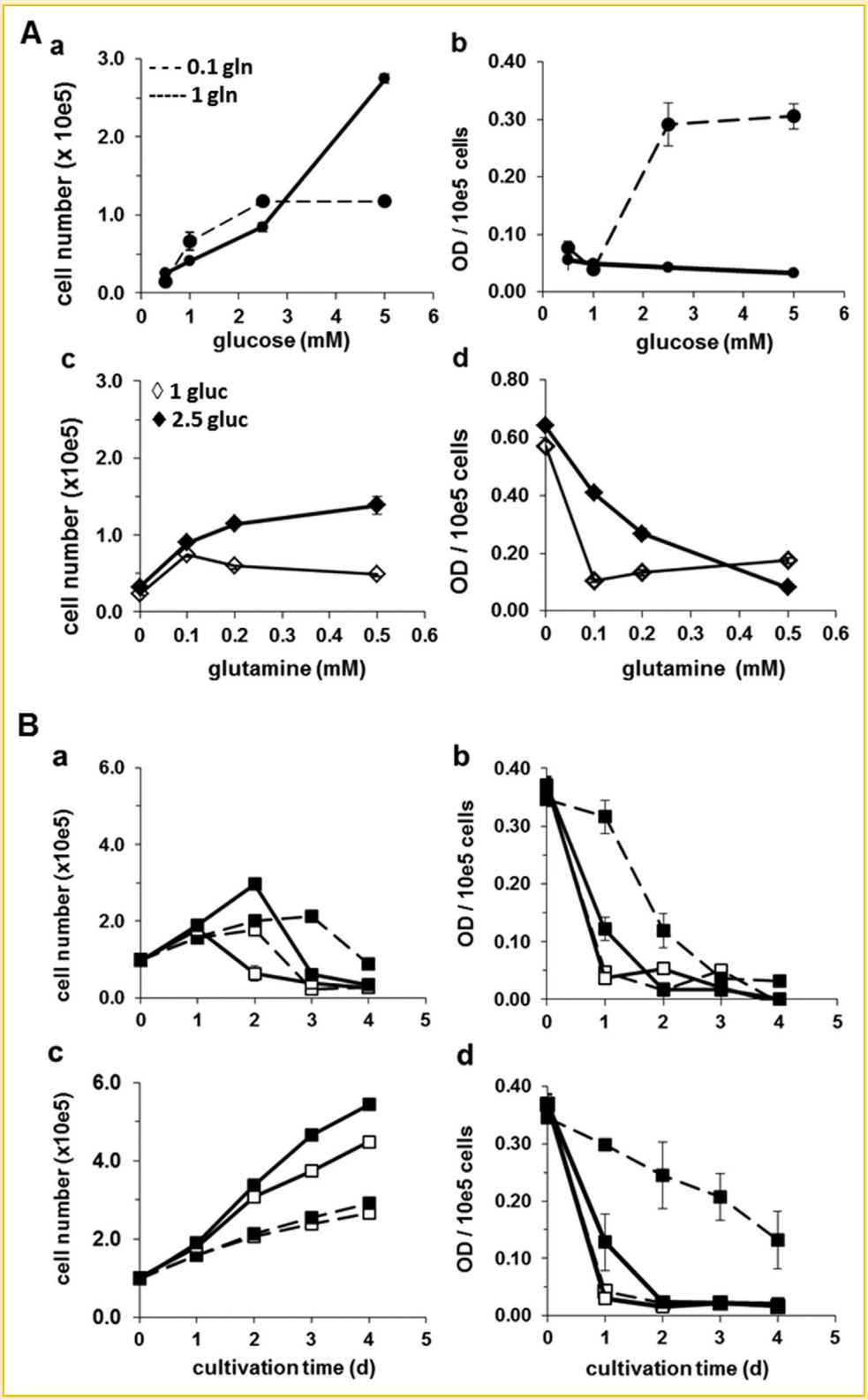

Fig. 1. Non-additive effects of glutamine and glucose combinations on growth and NOX activity. A: Concentration curves of glucose and glutamine in variable combinations. (a, b) glucose with 0.1 and 1 mM glutamine (dashed and solid line, respectively). (c,d) glutamine concentration curves with 1.0 and 2.5 mM glucose (open and closed symbols, respectively); (a,c) cell number per 500 μl culture, (b,d) NOX activity expressed as OD per cell number. B: Growth kinetics and changes in NOX activity without and with nutrient replenishment. Cells were cultivated with different combinations of glucose (at 1.0 and 2.5 mM, open and closed symbols, respectively) and glutamine (at 0.1 and 1.0 mM, dashed and solid line, respectively). (a,b): without medium change, (c,d) with medium changed daily.

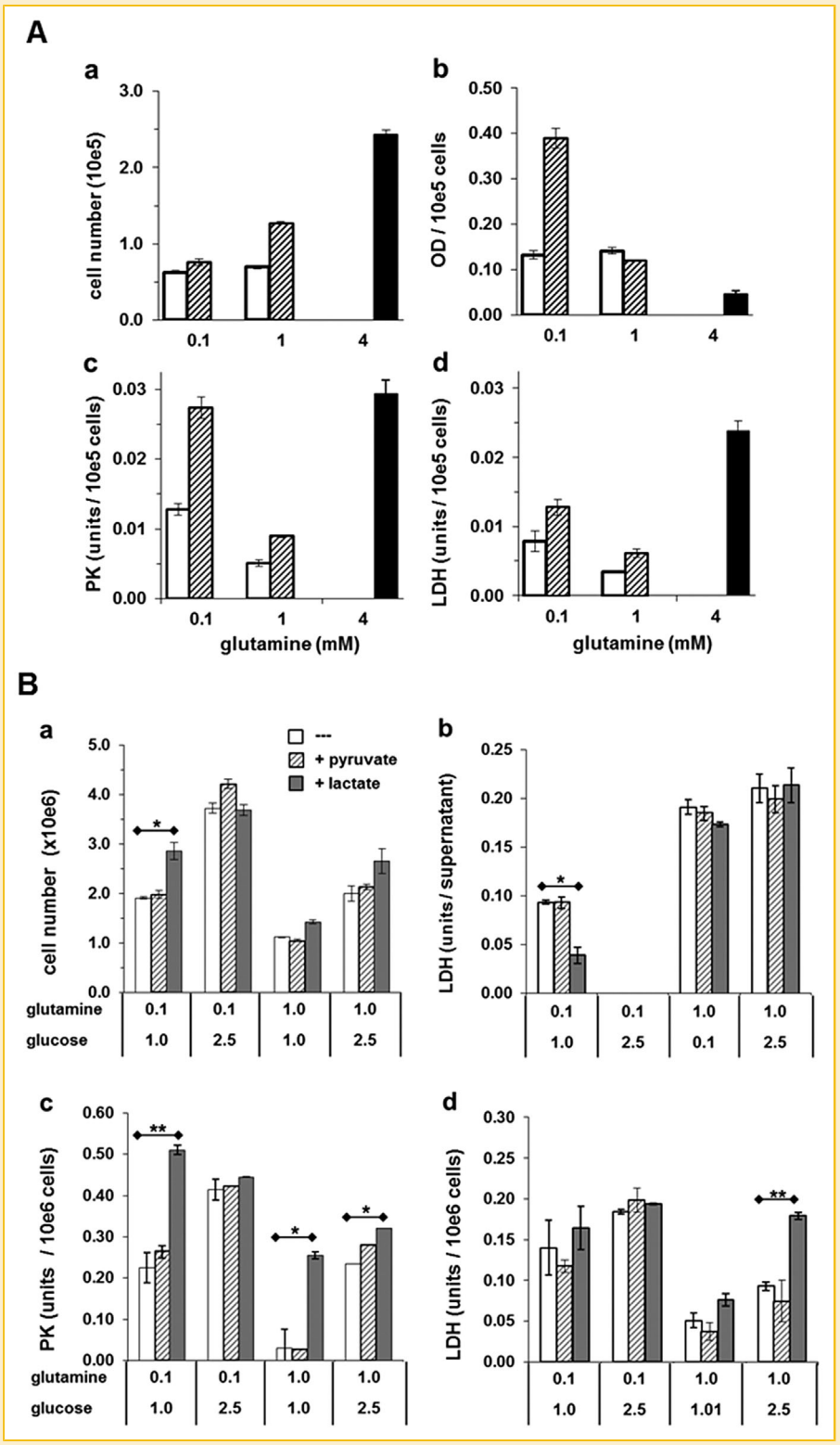

Fig. 2. Relationship between growth and enzyme activities of NOX, pyruvate kinase and LDH. A: Comparison of cells in standard medium and in low-growth medium with the indicated glucose and glutamine concentrations: (a) cell number per 500 μl culture, (b) NOX activity, expressed as OD per cell number, (c) pyruvate kinase activity per cell number; (d) LDH activity per cell number. Open bars: 1.0 mM glucose, hatched bars: 2.5 mM glucose. The standard DME (with 5% FCS) contained 25 mM glucose (solid bars). B: Activities of pyruvate kinase and LDH in cells and their culture supernatants from variable nutrient conditions including pyruvate and lactate additions. Cells were cultured in low-growth medium with the indicated combinations of glutamine and glucose (concentrations in mM) without (empty bars), and with addition of 1 mM pyruvate (hatched bars), or 25 mM lactate (filled bars). (a) cell number per 2 ml culture, (b) LDH activity in the culture supernatant; (c) pyruvate kinase and (d) LDH activity in cell homogenates. In B, statistical differences between data points with and without lactate are indicated by *($P \leq 0.05$) and **($P \leq 0.005$).

GLUCOSE CONSUMPTION AND LACTATE RELEASE

For further information on the metabolic activity under low glucose and glutamine conditions, the amounts of glucose consumed and lactate released were measured in identical cultures after 3 days of incubation (Fig. 3). By this time, cells cultured with 1 mM glutamine had consumed all exogenous glucose, regardless of the initial concentrations (Fig. 3c). In contrast, cultures with 0.1 mM glutamine retained up 1.1 mM glucose in the supernatant, that is, 20% of the initial 5.6 mM. For comparison, in standard culture medium (5% FCS) with 25 mM glucose, the conditioned medium after 3 days still contained about 9 mM, that is, 36% of the initial 25 mM glucose (not shown). An increase in glucose consumed per culture is not simply explained by an increase in cell number, even though both parameters are higher at 1 mM than at 0.1 mM glutamine. Instead, the amount of glucose consumed per cell number correlated almost linearly with the initial glucose concentration (Fig. 3d), suggesting that in this concentration range glucose availability was a rate-limiting factor. Moreover, cellular glucose consumption was higher in medium containing 0.1 mM glutamine than with 1.0 mM glutamine. A possible explanation could be that with low glutamine, more glucose is required as a metabolic precursor for anabolic processes otherwise provided by anaplerotic metabolites derived from sufficient glutamine. Interestingly, there was no direct correlation of glucose consumption with either growth (Fig. 3a) or NOX activity (Fig. 3b).

The amount of lactate in the supernatant showed an expected glucose-dependent increase (Fig. 3e,f). At high glutamine (1 mM), lactate levels reached concentrations up to 5.5 mM, amounting to 50% lactate compared to the initially 5.56 mM glucose provided in the medium (each glucose molecule theoretically giving two lactate). In contrast, at 0.1 mM glutamine there was no further increase in supernatant lactate with glucose concentrations >2.5 mM in the

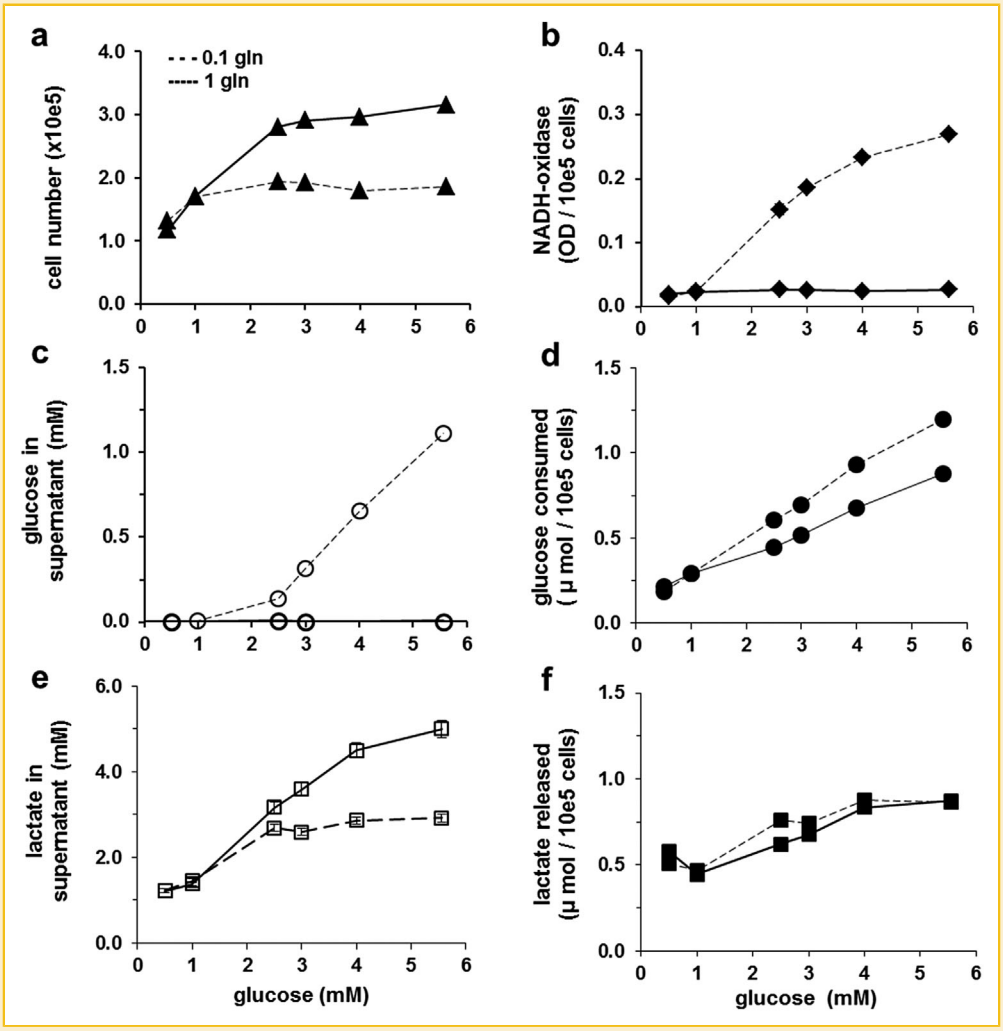

Fig. 3. Concentration-dependent glucose consumption and lactate release by cells in variable nutrient conditions. MCF-7 cells were cultured for 3 days in low-growth medium with increasing glucose concentrations at 0.1 mM and 1 mM glutamine (dashed and solid line, respectively). (a) Cell number per 500 µl culture; (b) NOX activity per cell number; (c) glucose concentration remaining in the culture medium and (d) glucose consumed per cell number; (e) concentration of lactate released to the culture medium; (f) lactate detected per cell number. All results are from the same experiment. Errors of triplicates are less than 5%, the error bars are generally smaller than the symbols. The statistical differences between 0.1 and 1.0 mM glutamine for the data points of 3–5.6 mM glucose were in (c,e) $P < 0.02$ and in (d,f) $P = 0.36$ and 0.74, respectively.

medium. This glucose dependency of lactate levels in the conditioned medium was similar to that of the cell number (Fig. 3a). Accordingly, the amounts of lactate released per cell number, were not different between low and high glutamine, suggesting that glutamine did not increase lactate release in these cells, supporting the assumption that glucose is here used for anabolic processes. Again, specific NOX activity showed no correlation with lactate release.

EFFECTS OF EXTRACELLULAR PYRUVATE

Blood-born pyruvate could serve as a supplementary metabolite under limiting nutrient conditions. Therefore, cell growth and metabolic activity were assayed in cultures with varying levels of pyruvate (0.1–1.0 mM) in conditions containing various concentrations of glucose and glutamine. Although in some experiments pyruvate enhanced cell number by 10–20%, in general it showed no preferential effect at either low glucose or low glutamine concentrations (Fig. 2Ba). Also, pyruvate did not consistently affect the specific NOX activity (data not shown). Moreover, as shown in Figure 2Bc–d, adding pyruvate did not reproducibly affect either the pyruvate kinase or LDH activity measured in cell homogenates, suggesting that the availability of extracellular pyruvate is not a modulating factor for metabolic activity in MCF-7 cells.

EFFECTS OF EXTRACELLULAR LACTATE

As to the question whether these cells utilize extracellular lactate when glucose and glutamine are limiting, the answer is conditional: the addition of lactate (25 mM) with various combinations of low glucose and glutamine had only marginal effects on cell growth, with inhibition being maximally 20% in combination with 1 mM glutamine (Fig. 4a,c). In contrast, NOX activity was consistently enhanced by lactate, notably in the presence of 0.1 mM glutamine, and this enhancement was most pronounced again at glucose >2.5 mM (Fig. 4b). Lactate enhanced basal NOX activity also at

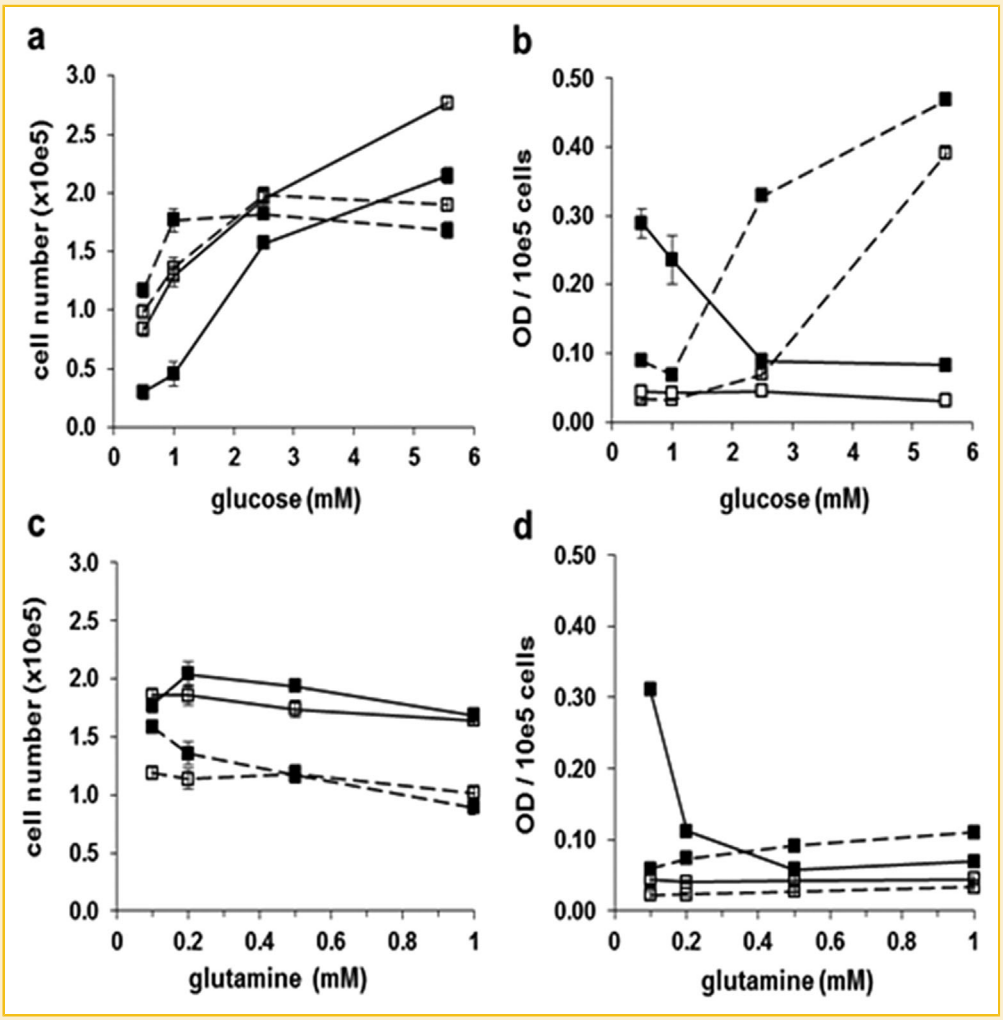

Fig. 4. Effects of exogenous lactate with increasing concentrations of glucose and glutamine on cell growth and NOX activity. Cells were cultured in low-growth medium with the indicated combinations of glutamine and glucose without and with lactate (25 mM). (a,b) Glucose concentration curves with glutamine at 0.1 mM (dashed line) and 1.0 mM (solid line). (c,d) Glutamine concentration curves with glucose at 1.0 mM (dashed line) and 2.5 mM (solid line). (a,c) cell number per culture, (b,d) NADH oxidase activity. Open symbols: no lactate; closed symbols: with lactate.

glutamine concentrations above 0.1 mM (Fig. 4d), corroborating that extracellular lactate modulates NADH-linked metabolic activity.

Extracellular lactate unexpectedly enhanced pyruvate kinase activity of cells maintained in low glucose (1 mM) in combination with low glutamine (0.1 mM) (Fig. 2Bc). No further enhancements were observed for the highest activity achieved with the combination 0.1 mM glutamine/2.5 mM glucose. The increase in pyruvate kinase activity at a limiting glucose concentration may be an indication for lactate being channeled to gluconeogenesis, that is, for providing metabolites upstream in the glycolytic pathway. LDH activity was also enhanced by extracellular lactate, but only in the presence of higher glutamine concentrations (e.g., 1 mM) and 2.5 mM glucose (Fig. 2Bd). Whether this enhanced activity reflects a change in LDH isoforms needs further analysis. Since maximal enzyme activities were already achieved with the 0.1 mM glutamine/2.5 mM glucose combination, lactate had an enhancing effect only under "unbalanced" nutrient conditions; this argues for a facultative metabolic role of lactate in these cells.

ENERGETIC STATE AND ACTIVITY OF CELL SURFACE NADH-OXIDASE

To describe the energetic state of these tumor cells in variable nutrient conditions, ATP as well NADH and NAD^+ levels were determined. ATP content did not consistently vary with the different substrate concentrations provided in the medium (Fig. 5Ab), suggesting that even at low nutrient levels a certain energy status was preserved. Also, there was no correlation of ATP levels with the activities of NOX (Fig. 5Ba) or pyruvate kinase and LDH (Fig. 2Bc,d). Presumably these increased enzyme activities were required for non-catabolic metabolic pathways. Similarly, there were little glucose and glutamine-dependent differences in NADH-levels (Fig. 5Ac) and NAD^+ (not shown). However, with all combinations, lactate increased total NADH levels by 1.5–2-fold, thereby reducing the NAD^+/NADH ratio (Fig. 5Ad). However, NADH levels did not correlate with changes in NOX activity, possibly due to a different compartmentalization of additional NADH.

While WST-1 (in conjunction with PMS) measures plasma membrane NOX activity depending on electrons from cytosolic NADH, the cell surface NOX can also oxidize extracellular NADH [Berridge and Tan, 2000; Scarlett et al., 2005). Adding NADH to the cells with the WST-1 assay should therefore give an estimate of the total NOX activity in the plasma membrane. For cells incubated with the different glucose/glutamine combinations, extracellular NADH increased the specific absorption values by 3–4-fold (Fig. 5Bb), with the exception of 2.5 mM glucose/0.1 mM glutamine, where there was no effect. The increase in NOX activity indicates that the cytosolic NADH had not exploited the full activity of the cell surface enzyme. When lactate was included in the growth medium, NOX activity increased even more ($\geq$5-fold) with various glutamine/glucose combinations. These results suggest that cell surface NOX activity is more sensitive to lactate metabolism than the transmembrane NOX activity requiring cytosolic NADH.

It has been shown that the transfer of electrons from the plasma membrane system to WST-1 (via PMS) is mediated by oxygen radicals, which is inhibited by superoxide dismutase (SOD) [Herst and Berridge, 2007]. In MCF-7 cells this electron transfer was likewise inhibited by the addition of SOD (Fig. 5Bc). This inhibition, averaged over the four different combinations of glucose/glutamine, amounted to $82 \pm 4.8\%$ and $69 \pm 10.4\%$, without and with lactate additions, respectively. The increase in cell surface NOX activity by addition of extracellular NADH was also inhibited by SOD. However, this was true mainly in growth conditions including lactate, where the extent of inhibition was $64.1 \pm 7.45\%$, averaged over the combinations (Fig. 5Bd). The SOD-insensitive NOX activity was the same with and without lactate, which suggests that lactate had increased mainly cell surface NOX activity.

Finally, to test whether metabolic processes in mitochondria contributed to the activity of cell plasma membrane NOX, cells grown with different combinations of glucose and glutamine were exposed to rotenone, an inhibitor of the mitochondrial NADH-Q-reductase, for variable times prior the incubation with WST-1 [Berridge and Tan, 1998]. As Figure 6 shows, plasma membrane NOX activity was not inhibited for up to 2 h of pre-incubation with rotenone; on the contrary, there was an immediate but transient rise in WST-1 absorption. Under limiting conditions this effect was further enhanced by lactate. Taken together, the results suggest that a block in mitochondrial NADH oxidation leads to increased NADH oxidation at the plasma membrane, which can be explained by an increase in cytosolic NADH fuelled by a mitochondrial surplus.

DISCUSSION

The first question of this study was how variable combinations of limiting metabolic substrates modulate metabolic enzyme activities required for survival in a changing tumor microenvironment. Normal plasma levels of glutamine are in the range of 0.6–0.8 mM, while those of glucose normally range between 5 and 16 mM, depending on the nutritional status, and tumor concentrations fall well below these values [Vaupel, 2009]. Cell cultures with 25 mM glucose, as provided in DMEM with 4.5 g/L, more likely resemble a diabetic condition. Therefore, combinations of glucose and glutamine at low concentrations which might occur in a tumor microenvironment were used in the protocols of this work. The results show that in MCF-7 cells glucose utilization is highly dependent on glutamine concentrations for both growth and metabolic activity. Glucose concentrations at $>$2.5 mM maintained cell survival longer in combination with 0.1 mM glutamine than with high (1.0 mM) glutamine, since cells with a lower growth rate will deplete less glucose from the culture medium. However, even though with 1 mM glutamine, glucose consumption per cell is slightly lower than with 0.1 mM glutamine, these cells have a higher growth rate (Fig. 1Ba,c), leading to more rapid glucose depletion when there is no replenishment (Fig. 3c) and therefore to cell death (Figs. 1Ba, 2Bb). In combination with low glutamine (0.1 mM), a glucose concentration of 2.5 mM marks a threshold for a substantial increase in the activity of pyruvate kinase and NOX. Such increases in enzyme activity were not observed with combinations of low glucose and 1 mM glutamine, indicating that an excess of glutamine may lead to a reduction in the glycolytic rate. Thus, saturating glutamine could not replace glucose deficiency in these cells, as has been reported for other tumor cell types (reviewed by McKeehan [1982]); and also argues against glutamine generally being a major substrate for energy metabolism.

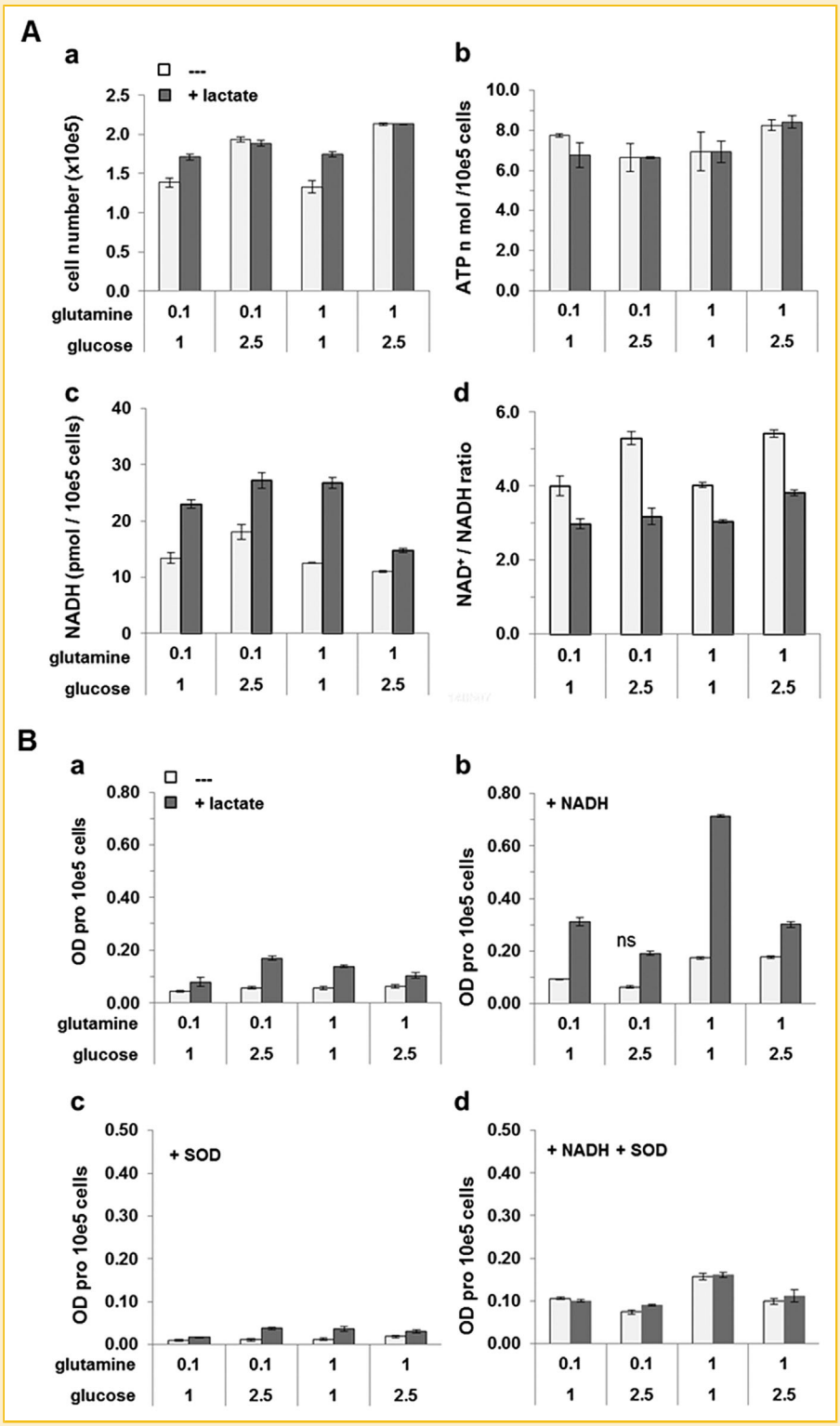

Fig. 5. Comparison of NOX activities with ATP and NADH levels in MCF-7 cells in variable nutrient conditions. Cells were cultured in low-growth medium with the indicated combinations of glutamine and glucose concentrations (mM) without (empty bars), and with addition of 25 mM lactate (filled bars). A: Energy and redox states (a) cell number per 500 μl culture, (b) ATP content; (c) NADH-content; (d) ratio of NAD^+/NADH. In A(c–d) all data points without and with lactate are significantly different by $P < 0.05$. B: Plasma membrane NOX activities measured with the WST-1 assay combined with (a) no additions; (b) NADH; (c) SOD; (d) NADH + SOD. In B (a), all data points without and with lactate are significantly different by $P \leq 0.02$. In B(b), all data points are statistical different to the corresponding value without NADH in B(a) by $P \leq 0.02$, except the one denoted by ns.

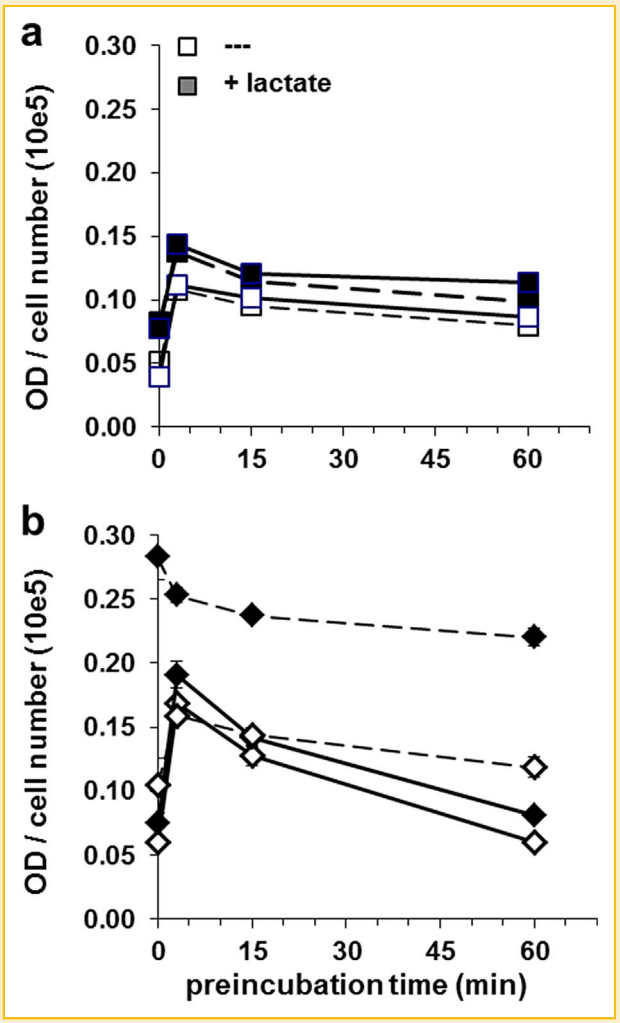

Fig. 6. Effects of rotenone on NOX activity in cells cultured in low-growth medium with different combinations of glucose and glutamine, without or with lactate, for 3 days. Cells were then pre-incubated with rotenone (10 μM) for the times indicated before addition of WST-1; absorption was measured after 4 h. (a) 1.0 mM glucose, (b) 2.5 mM glucose, combined with either 0.1 mM glutamine (dashed lines) or 1.0 mM glutamine (solid lines), without (open symbols) and with lactate (25 mM; closed symbols).

Moreover, this model study shows for MCF-7 cells that at low substrate concentrations there is an optimal ratio for glutamine/glucose of ≤1:25, demonstrating the importance of the balance of glucose and glutamine concentrations for tumor cell metabolism and survival at limiting nutrient levels. Preliminary results suggest that other tumor cell types may have a different balance of glucose and glutamine.

It should be noted that these results were obtained in medium at pH 7.4 and under physiologically hyperoxic conditions, that is, the standard normoxic culture conditions. Nevertheless, the highest metabolic plasticity for survival was found at concentrations of glucose and glutamine which match those found in tumors in vivo [Vaupel, 2009]. Depending on the nutritional state of the blood, it may be postulated that at about 50 μm distance from a blood vessel, where pH is in the range of 7.0 and oxygen is not yet depleted [Helmlinger et al., 1997], low nutrient niches exist in the tumor microenvironment characterized by combinations similar to 0.1 mM glutamine and 2.5 mM glucose. Moreover, fine-tuning of the metabolic program will depend also on the gradients of pO_2 and pH. Preliminary results with MCF-7 in acidic conditions show shifts in the metabolic dependencies on glucose and glutamine concentrations, and more changes are to be expected when including hypoxic conditions. The stringency required in controlling the culture conditions, in particular the pH, to obtain reproducible values is a connotation of high metabolic plasticity in adapting to minute variations in the microenvironment. It may explain also variations in results especially at the critical glucose concentration around 2.5 mM.

In the tumor microenvironment, cancer as well as stroma cells release lactate, which can have paracrine effects [Feron, 2009; Pavlides et al., 2009]. With increasing distance from the blood vessel, interstitial concentrations of glucose decrease down to 0.4 mM, while those of lactate can increase over 25 mM [Vaupel, 2009]. Such a situation was partially mimicked in MCF-7 cultures after 3 days of growth in non-replenished low nutrient condition, where glucose had been consumed and lactate had accumulated to a concentration up to 5.5 mM in the supernatant. Even though high extracellular lactate with low glucose had little supportive effect on cell growth, it did markedly enhance both NOX activity and potential pyruvate kinase activity, measured in cell homogenates with saturating substrates. Whether the tumor isotype pyruvate kinase M2 is responsible for the lactate-induced increase in pyruvate kinase activity in low glucose conditions will need further molecular analysis. An increase in pyruvate kinase activity would be expected to turnover more phosphenolpyruvate (PEP), which could be derived from oxaloacetate and is the metabolite for the first step in gluconeogenesis. Canonically, lactate could be a precursor for oxaloacetate both in the mitochondria and in the cytoplasm (Fig. 7), where respective isoforms of PEP carboxykinase are localized [Stark and Kibbey, 2014]. Even though the molecular and biochemical details are yet to be investigated, lactate is very likely a gluconeogenic metabolite in MCF-7 cells under glucose and glutamine limitations.

Another aspect of this study was what kind of metabolic information the reduction of the WST-1 assay can provide on the metabolic phenotype of the cells. With intact cells this assay measures the activity of a plasma membrane NADH oxidase system which uses intracellular reduction equivalents, probably derived from glycolysis [Scarlett et al., 2005]. NOX activity and isoform expression have also been reported for several tumor cells with different glycolytic and oxidative potential, including osteosarcoma cells and HeLa cells [Yantiri and Morre, 2001; Scarlett et al., 2005; Herst and Berridge, 2007]. A relationship between glycolysis and plasma membrane NOX activity in MCF-7 cells is substantiated by the enhancement of both NOX and pyruvate kinase activities by lactate, along with an increase in cellular NADH levels. The fact that exogenous superoxide dismutase markedly inhibited WST-1-reduction by about 85% shows that most of the plasma membrane NOX activity utilizes oxygen radicals and is suggestive of oxygen consumption on the cell surface [Herst and Berridge, 2007]. However, apparently the transmembrane activity measured with

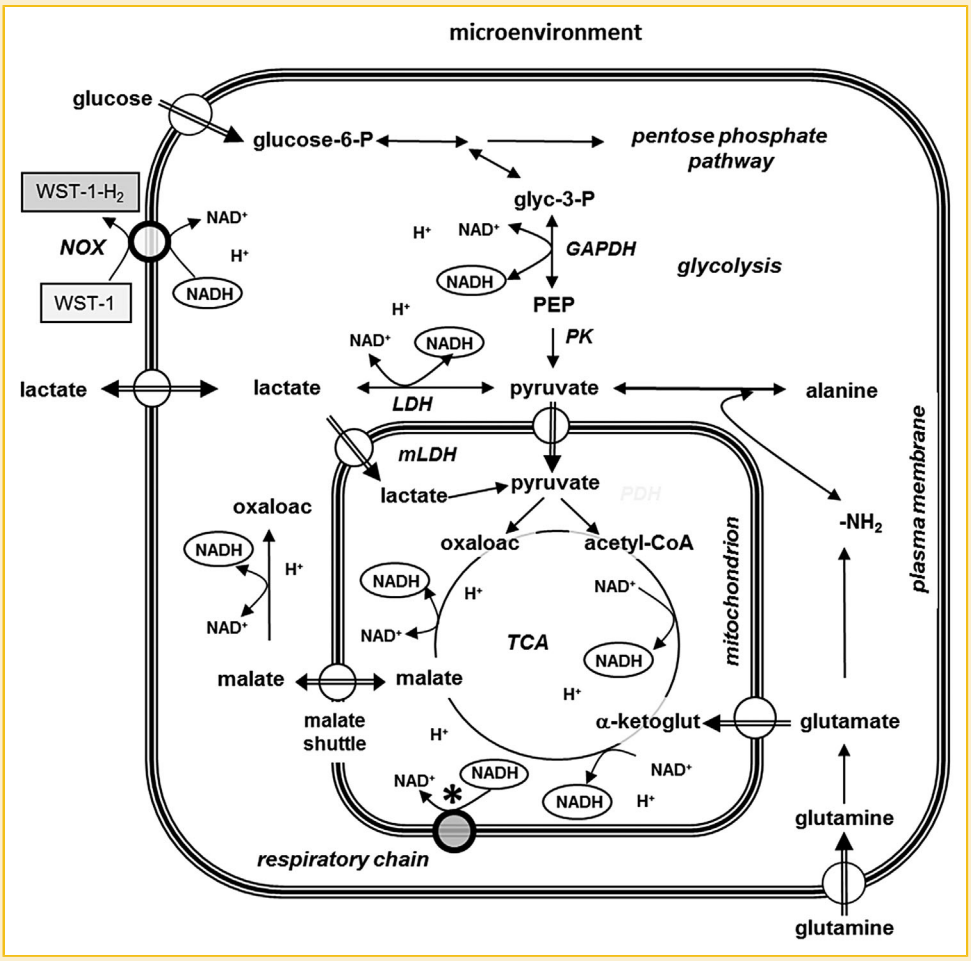

Fig. 7. Relationships between NADH-levels, the activity of plasma membrane NOX, and metabolic pathways of glucose and lactate. Shown are possible sites of NADH formation, namely glycolysis and the TCA cycle, the latter providing mitochondrial NADH equivalents, which can pass to the cytosol through the malate shuttle. Oxaloacetate can also be a metabolite for gluconeogenesis. In the plasma membrane system, electrons could be transferred to extracellular acceptors such as O_2 (or to WST-1 via PMS as an analytical substrate). For clarity, only selected reactions and key metabolites in glycolysis, the TCA-cycle, glutaminolysis and gluconeogenesis are shown. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; glyc-3-P: glyceraldehyde-3-phosphate; α-ketoglut: α-ketoglutarate; mLDH: mitochondrial lactate dehydrogenase; oxaloac: oxaloacetate; PK: pyruvate kinase; (•): site of mitochondrial NADH oxidase (complex I) inhibition by rotenone. Explanations on the role of lactate and variable NADH levels are provided in the discussion.

the WST-1 assay depended on a limited level of intracellular NADH, since adding extracellular NADH, a substrate for cell surface NOX activity, markedly enhanced the total activity. This total NOX activity also depended on the metabolite combination, but in particular on lactate supplementation (Fig. 5B), suggesting differences in the expression of cell surface NOX oxidase. SOD inhibited the NADH-induced increment of NOX activity by up to 60%, leading to a lactate independent activity. Together these functional results suggest that SOD insensitive isoform(s) of NOX exist, whose identity needs yet to be elucidated.

The insensitivity of WST-1 reduction to rotenone, an inhibitor of the mitochondrial NADH-ubiquinone oxidase (complex I), along with a transient increase in WST-1 reduction argues against mitochondria being the direct site and the primary NADH source fueling NOX activity. This latter effect, which has been reported also for other tumor cells [Berridge and Tan, 1998], can be explained by a shuttling of reducing equivalents from mitochondria to the cytosol to counteract NADH-accumulation resulting from respiratory inhibition. The required mitochondrial passage of reducing equivalents could operate via the malate shuttle, also proposed by Herst and Berridge (2006). The removal of excess NADH through the plasma membrane NOX is accompanied by an export of protons along with extracellular acidification [Herst and Berridge, 2007], thereby contributing to the maintenance of the redox balance and the cytosolic pH. At the same time, total NADH levels as well as the NAD^+/NADH ratio showed little variability with the different glucose/glutamine combinations, indicating that on the whole the cells maintain a stable energetic state under different low nutrient conditions. Therefore, further components must be involved in regulating NOX activity to explain the observed variability.

Combining the above observations, a model is proposed for a role of NADH in MCF-7 cell metabolism under precarious nutrient conditions (Fig. 7). Plasma membrane NADH oxidase activity, as measured with the WST-1 assay, could be driven by cytosolic NADH

resulting from glycolysis, as suggested by corresponding changes in potential pyruvate kinase activity. In tumor cells, the cytosolic re-oxidation of NADH to NAD^+ is attributed mainly to the activity of LDH-5, which converts pyruvate to lactate [Fantin et al., 2006]; but conditionally, this recycling process could also involve plasma membrane NOX. In fact, in MCF-7 cells LDH activity was always lower than that of pyruvate kinase by a factor of 2–3, suggesting that it may not the only candidate for NADH recycling. Furthermore, increased glycolysis in tumor cells need not necessarily increase lactate production when pyruvate is converted to other metabolites required for anabolic processes, such as amino acid and fatty acid synthesis. Considering that proliferating cells have a high demand for nucleotides, amino acids and lipids, lactate production (and release) could serve as means for removing surplus pyruvate when the anabolic processes have been satisfied. On the other hand, MCF-7 cells, which express plasma membrane MCT-1 as well as MCT-4 and MCT-2 [Hussien and Brooks, 2011] could utilize exogenous lactate for their metabolism when glucose becomes scarce. To explain the results with exogenous lactate, where both NADH content and plasma membrane NOX activity were increased, two scenarios can be envisaged: (1) Exogenous lactate is converted to pyruvate by LDH-A activity in the cytosol, and the pyruvate is then channeled in to the mitochondria to fuel the TCA cycle; (2) Exogenous lactate enters the mitochondria via MCT-2, where it is converted to pyruvate by a LDH-B-like isoform present in mitochondria of MCF-7 cells [Hussien and Brooks, 2011]. The decision will depend on the localization and specificity of the LDH isoforms and the intracellular pools of pyruvate and lactate. In either case, lactate would have an anaplerotic function in the TCA-cycle, resulting in more NADH, which can be transferred to the cytosol via the malate shuttle (Fig. 7). The increase in NOX activity upon inhibition of the mitochondrial NADH oxidation is coherent with this model. Therefore, the WST-1 assay is a measure of NADH-linked metabolism, which is reflected by plasma membrane NOX activity and plays a role in maintaining a cytosolic redox balance required for glycolysis and other metabolic reactions.

Many molecular aspects of this transmembrane electron transfer system in the metabolism of tumor cells need yet to be elucidated. This is of particular importance with respect to NOX playing a role in metabolic diseases [Morre and Morre, 2003] as well as being the target of old and new chemotherapeutic drugs, such as cisplatin, doxorubicin phenoxodiol and tamoxifen [Herst et al., 2007; Su et al., 2012]. Moreover, studying the quantitative effects of microenvironmental variables on metabolic activities has immediate relevance for metabolic imaging techniques for the clinic. Recent studies with new NMR technologies using hyperpolarized ^{13}C-pyruvate, ^{13}C-glucose, and other ^{13}C-subtrates show the dynamics of metabolite conversion. Examples are the production of ^{13}C-lactate and changes in [NAD]/[NADH] ratios as indicators of metabolic activity and necrosis in tumors [Kurhanewicz et al., 2011; Schilling et al., 2013; Christensen et al., 2014]. In this context, more biochemical quantitative data on metabolic activities from different types of tumor cells will be useful to develop algorithms and models which better describe how metabolic pathways are modulated by variable conditions of the microenvironment in vivo. Along with metabolic imaging technologies, such a data collection could also help to predict what kind of effects therapeutic strategies based on antimetabolites or silencing of metabolic enzymes might have on the metabolic phenotype of the tumor cells.

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