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Expression of hexose-6-phosphate dehydrogenase in rat tissues

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ABSTRACT

Hexose-6-phosphate dehydrogenase (H6PD) is the main NADPH generating enzyme in the lumen of the endoplasmic reticulum. H6PD is regarded as an ancillary enzyme in prereceptorial glucocorticoid activation and probably acts as a nutrient sensor and as a prosurvival factor. H6PD expression was determined in a variety of rat and human tissues by detecting mRNA and protein levels, and by measuring its dehydrogenase and lactonase activities. It was found that H6PD was present in all investigated tissues; both expression and activity remained within an order of magnitude. Correlation was found between the dehydrogenase activity and protein or mRNA levels. The results confirmed the supposed housekeeping feature of the enzyme.

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1. Introduction

Each subcellular compartment of eukaryotic cells is equipped with enzymes for the generation of reduced electron carriers, which are essential for biosynthetic pathways, antioxidant defense and biotransformation reactions. In this regard, maintenance of a pool of reducing equivalents in the form of NADPH is particularly important; hence, the NADPH/NADP$^+$ ratio is kept high in each subcellular compartment [1]. Although the cytosolic mechanisms for NADPH generation and utilization are well known, the redox state of the pyridine nucleotide pool as well as the source and fate of NADPH in the endoplasmic reticulum (ER) lumen have been scarcely explored.

Hexose-6-phosphate dehydrogenase (H6PD) – a microsomal counterpart of the cytosolic glucose-6-phosphate dehydrogenase (G6PD) – has emerged as a key NADPH generating enzyme of the ER lumen [see [2] for a recent review]. The luminal localization of the H6PD enzyme in the ER was confirmed in several studies demonstrating that the latent activity in microsomal preparations can be liberated only by detergent treatment [3–6]. Under physiological conditions, the native substrates for H6PD are glucose-6-phosphate (G6P) and NADP$^+$ in the lumen of the ER [see [2] for a recent review]. Because the ER membrane is practically impermeable to pyridine nucleotides [7], H6PD (and perhaps additional local dehydrogenases; see Ref. [8] utilizes NADP$^+$ produced by luminal reductases.

H6PD is a bifunctional enzyme [9]: in addition to its oxidative activity on G6P it can promote the hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate, catalyzing the first two steps of the pentose phosphate pathway within the ER [10]. The 6-phosphogluconolactonase (6PGL) activity was demonstrated in native H6PD purified from mouse liver [11], and in microsomes from adipose tissue [12] and human neutrophils [13].

A well-established function of H6PD is the electron supply for the reduction of (inactive) glucocorticoids, catalyzed by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [2]. Thus, the expression level of H6PD has been implicated in different prereceptorial glucocorticoid activation in various tissues. This latter observation resulted in the formulation of a hypothesis that H6PD expression during adipocyte differentiation is responsible for the switch from 11β-HSD1 dehydrogenase to reductase activity [14]. By contrast we observed a constant expression of H6PD during the differentiation to adipocytes of adipose-derived human stem cells and 3T3-L1 cells [15]. Moreover, H6PD activity seems to play a role as a prosurvival factor [16,17] and might act as a nutrient sensor of the cell [18,19].

Early studies [20–23] already suggested a wide tissue distribution, with the highest activity observed in liver. Antibody towards the lactonase domain of H6PD could reveal an immunoreactive protein at approximately 95 kDa in rat liver and adipose tissue [7,12,15] and in human neutrophils [13]. In another study, two different antipeptide antibodies towards short amino acid sequences of the dehydrogenase and the lactonase moieties revealed an immunoreactive protein at approximately 95 kDa expressed at different extents in various rat tissues [24]. The authors found an immunore-

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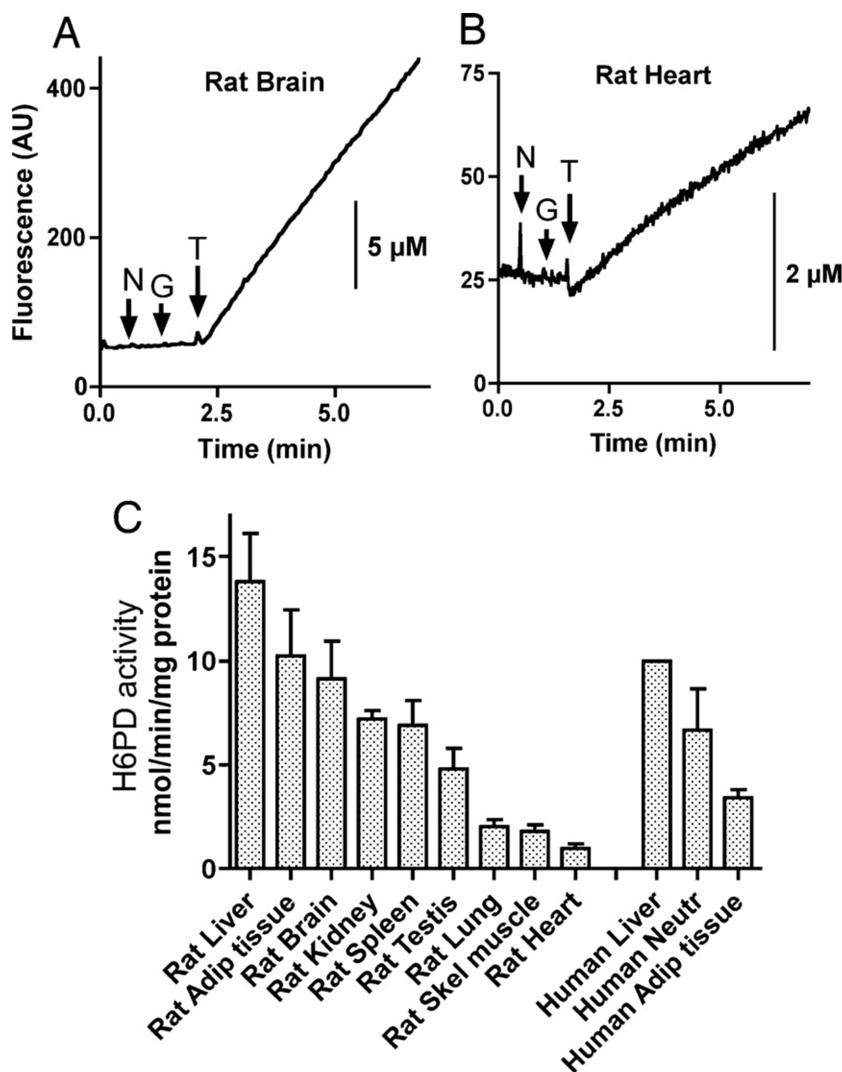

Fig. 1. Microsomal dehydrogenase activity of H6PD in rat and human tissues. Microsomes, were incubated at 37 °C in the KCl/MOPS buffer at a protein concentration of 0.5 mg/ml. NADPH formation was measured fluorimetrically (excitation and emission wavelengths at 350 and 460 nm, respectively) following the subsequent addition (arrows) of 2 mM $NADP^+$ (N), 1 mM G6P (G), and 1% Triton X-100 (T). Two fluorescence traces as examples are shown: panel A, rat brain and panel B, rat heart. Panel C shows the H6PD activity in microsomes from various rat and human tissues calculated from different fluorescence traces as reported in Section 2. Data are means ± SE of three to five independent experiments. In the case of human liver microsomes the mean of two independent experiments is shown.

active protein of 50 kDa in the whole brain, and a 95 kDa faint band in the cerebellum. Detailed immunohistochemical analysis showed intense staining of Purkinje cells, whilst some neurons and the epithelial cells of the pia and choroid plexus were also stained [24]. An immunohistochemical screening also indicates a widespread distribution of the dual enzyme, including central nervous system (Human Protein Atlas; website: www.proteinatlas.org). RT-PCR analysis of mRNA levels revealed a similar expression in liver, adipose tissue, and neutrophils [12,13,25]. A previous study reported a wide tissue distribution of the H6PD mRNA, but with large variation among the different tissues, and a correlation between the mRNA levels of 11β-HSD1 and H6PD [24].

The aim of the present study was the reinvestigation of tissue distribution of H6PD by using a variety of rat (and human) samples. The dual enzymatic activity of H6PD was measured first time and compared with protein and mRNA data. Evident dehydrogenase and lactonase activities of H6PD were found in the microsomal fraction of all the investigated tissues. Consistently, the mRNA and protein levels statistically correlated with the H6PD dehydrogenase activity. Taken together, the results indicate that H6PD may possess housekeeping functions not solely related to the 11β-HSD1 activity.

2. Materials and methods

2.1. Preparation of microsomal fractions

Microsomes from rat tissues (liver, adipose tissue, brain, lung, hide limb skeletal muscle, kidney, spleen, heart and testis) and human tissues (liver and adipose tissue) as well as from human neutrophils were prepared as previously reported [12,13,15]. Human adipose tissue microsomes were prepared from omental adipose tissue obtained from patients undergoing elective lipectomy procedures [15]. Human liver microsomes were prepared from liver samples showing normal hepatic structure (controlled by pathologists) obtained from patients undergoing partial hepatectomy. Human neutrophils were isolated from the blood of healthy volunteers. Ethical approval was guaranteed for this study by the Scientific and Research Ethics Council of the Semmelweis University, Budapest. Microsomes were resuspended in Mops-KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$ and 20 mM Mops) pH 7.2

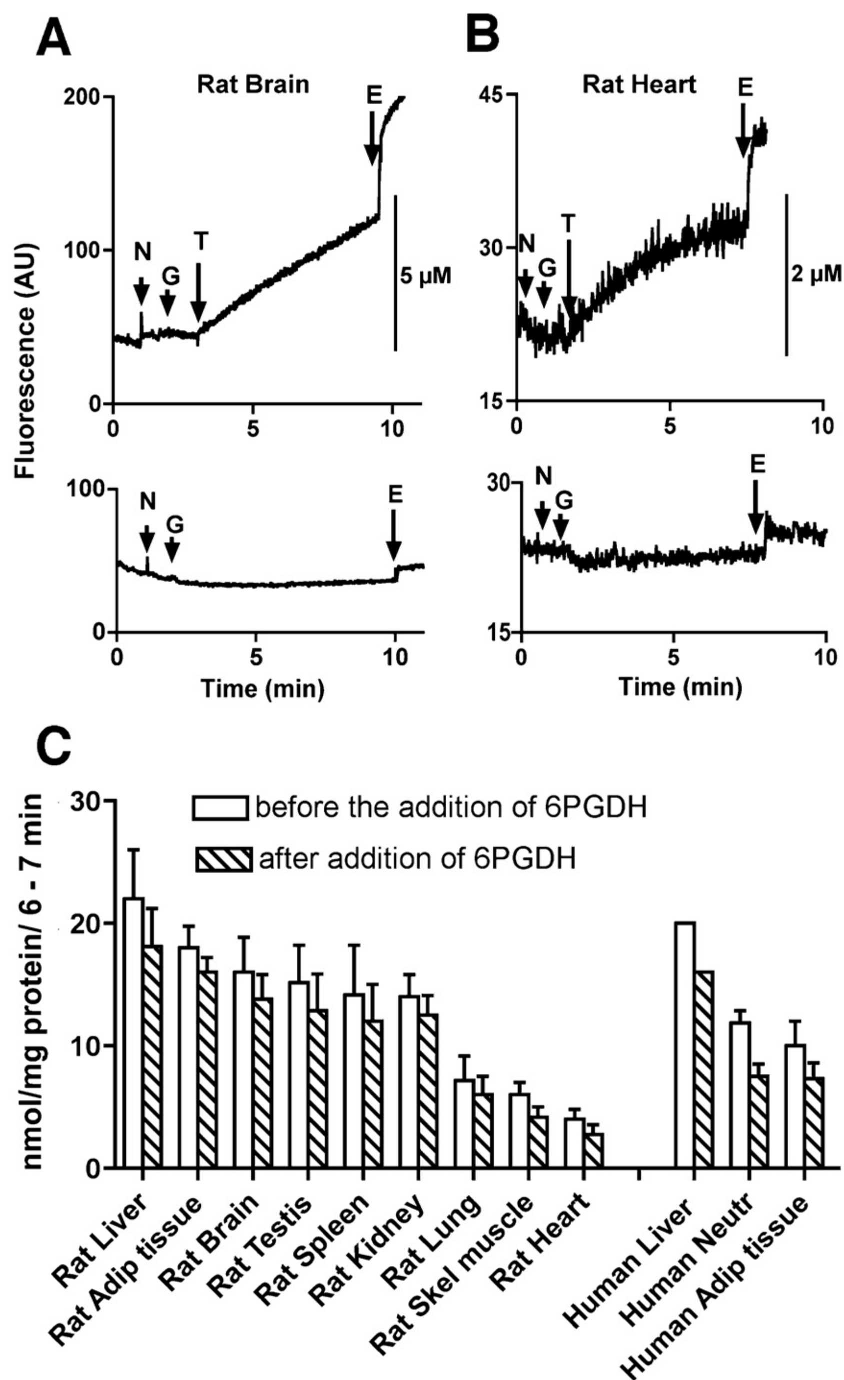

Fig. 2. Microsomal lactonase activity of H6PD in rat and human tissues. The product of lactonase activity, 6-phosphogluconic acid, was measured enzymatically with 6-phosphogluconate dehydrogenase (6PGDH) on the basis of NADPH formation. Microsomes were incubated at 37 °C in the KCl/MOPS buffer at a protein concentration of 0.5 mg/ml. NADPH formation was measured fluorimetrically (excitation and emission wavelengths at 350 and 460 nm, respectively) following the subsequent addition (arrows) of 2 mM $NADP^+$ (N), 10 μM G6P (G), 1% Triton X-100 (T), and 6PGDH enzyme (E). Two fluorescence traces as examples are shown: panel A, rat brain and panel B, rat heart. Control traces were performed in the absence of the permeabilizing agent Triton X-100 (panels A and B, lower traces). Panel C shows the values of NADPH formation, before and 1 min after the addition of the 6PGDH enzyme, which should reflect the production of 6-phosphogluconic acid, in the microsomal incubates from different rat and human tissues. NADPH formation was calculated on the basis of NADPH pulse additions to each trace, and expressed as nanomoles/mg protein/at 6 or at 7 min. Data are means ± SE of three to five independent experiments. In the case of human liver microsomes the mean of two independent experiments is shown.

including a cocktail of protease inhibitors, and maintained under liquid N_2 until used. The microsomal fractions were enriched in the ER-specific protein calnexin.

2.2. H6PD enzyme assays

The dehydrogenase activity of H6PD was evaluated by measuring NADPH formation upon the addition of 2 mM $NADP^+$ and 1 mM G6P to microsomes. Microsomes were incubated at 37 °C in the KCl/MOPS buffer at a protein concentration of 0.5 mg/ml. Limited access of the cofactor compound to the intraluminal enzyme proved the intactness of the microsomes. Microsomes have been subsequently permeabilized with Triton X-100 (1% final concentration) to allow the free access of the cofactor to the intraluminal enzyme. NADPH pulse addition (5 μM each) allowed the calculation of the enzyme activity in the linear initial phase (5 min) of the reaction.

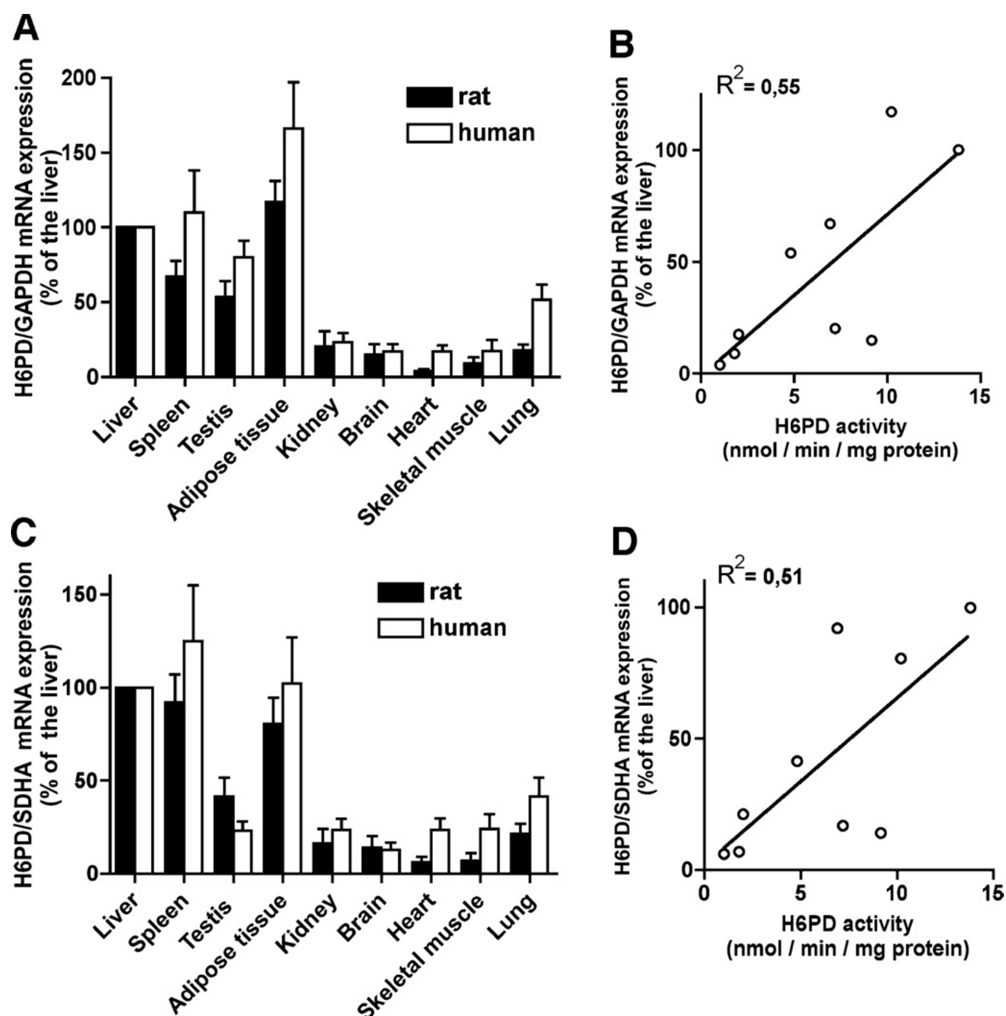

Fig. 3. H6PD mRNA expression levels in rat and human tissues. Total RNA from rat tissues and from human tissues was reverse transcribed and subjected to real-time PCR, as described in Section 2. The Ct value, the threshold cycle number at which fluorescence is detected above the baseline, is used to calculate the relative expression, according to Ref. [26]. The relative expression levels among the different tissues, calculated on the liver as 100%, were corrected for reference gene GAPDH (panel A) or SDHA (panel C). Data are means ± SE of three to five independent experiments. Rat H6PD mRNA expression levels (as % of the liver expression) were plotted towards H6PD enzymatic activities (panels B and D). In panel B, the proportional increase (slope = 7.23) was statistically significant: $p = 0.02$. In panel D, the proportional increase (slope = 6.35) was statistically significant: $p = 0.01$.

The lactonase activity of H6PD was assessed by measuring the product 6-phosphogluconic acid. This phosphoester was detected enzymatically with 6-phosphogluconic acid dehydrogenase on the basis of NADPH formation. To this end, microsomes were incubated as above and after 6 min preincubation, 6-phosphogluconic acid dehydrogenase was also added. The prompt formation of NADPH upon the addition of the enzyme indicates the amount of 6-phosphogluconic acid accumulated during the incubation. In control experiments with purified (yeast) glucose-6-phosphate dehydrogenase (but without microsomes) spontaneous hydrolysis of 6-phosphogluconolactone to 6-phosphogluconic acid was not observed.

NADPH fluorescence was monitored at 350 nm excitation and 460 nm emission wavelengths by using a Cary Eclipse fluorescence spectrophotometer (Varian).

2.3. Western blot

Microsomal proteins were loaded on polyacrylamide gels and blotted onto nitrocellulose. Immunoblots were probed with a rabbit polyclonal antibody against the lactonase domain (residues 537–653) of human H6PD (Atlas, Sigma Prestige Antibodies). After reacting with the horseradish peroxidase linked secondary antibodies, blots were analyzed by enhanced chemiluminescence (GE Healthcare).

2.4. Real-time RT-PCR

Total RNA from rat tissues was isolated using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. RNA from human tissues was from FirstChoice® Human Total RNA Survey Panel (Ambion-Applied Biosystems). One microgram of RNA was reverse transcribed in a final volume of 20 μl using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers. Expression levels of H6PD were quantified by fluorescent Real time PCR with a DNA engine thermal cycler (MJ Research, Waltham, MA) equipped with the Opticon Monitor 4 software Analyses were performed in triplicate in a 25 μl reaction mixture. cDNA (1 μl) was amplified with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 200 nM of the sense and antisense primers. For human H6PD, the oligonucleotide primers were: sense, 5′ ATGAAAGAGACCGTGGATGCTGAA 3′; antisense, 5′ CTCCATGGCCACGAGGGTGAG 3′. For rat H6PD, the oligonucleotide primers

were: sense, 5′ GGAGCTGATCTCCAAGCTGGC 3′; antisense, 5′ CCCTGACAGTGCCAGGTGGAA 3′. Amplification protocol was: 95 °C (10 min), 40 cycles of 95 °C (20 s), 57 °C (20 s), 72 °C (20 s). Real time PCR was performed using the same amplification protocol described above with human/rat primers for control genes changing annealing temperature when necessary. Oligonucleotide primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′ ACCATCTTCCAGGAGCGAGATC 3′; antisense, 5′ GAGCCCCAGCCTTCTCCATGGT 3′, annealing temperature 56 °C) and for rat succinate dehydrogenase complex, subunit A (SDHA) (sense, 5′ TGGGGAGTGCCGTGGTGTCAT 3′; antisense, 5′ CGCCCATAGCCCCCAGTAGC 3′, annealing temperature 55 °C) were designed with Primer-BLAST and checked for the absence of cross-reactivity by BLAST search. Primers for human succinate dehydrogenase complex, subunit A (SDHA) were those reported [26] and primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were those reported in [24].

The PCR amplification efficiency was evaluated by serial (10-fold) dilutions of the human/rat liver cDNA. Diluted and undiluted samples were then analyzed in duplicate. Amplification efficiency was calculated as reported [27]. The amplification efficiency for both human and rat H6PD was 90%. Every assay was run in triplicate and negative controls (no template, template produced with no reverse transcriptase enzyme) were always included. In the negative controls, no signal was detected in the investigated amplification range (40 cycles).

2.5. Statistical analysis

Differences in the measured variables between samples were evaluated by ANOVA and expressed as the mean ± SE where appropriate. Linear regression analysis (slope, p and R^2 values) was evaluated by Prism 4 GraphPad software.

3. Results

3.1. H6PD activity in microsomes from different tissues

H6PD activity was present in microsomes derived from all investigated rat tissues, namely liver, brain (see Fig. 1A, skeletal muscle, lung, kidney, (epididymal) adipose tissue, spleen, testis and heart (see Fig. 1B) as well as in microsomes from human liver, omental adipose tissue and neutrophils (Fig. 1C). As shown in example traces (Fig. 1A and B), in the presence of $NADP^+$, the G6P-dependent NADPH formation was evident upon permeabilization of the microsomal membrane (addition of Triton X-100, see arrow), whilst no activity was present before permeabilization. This was expected since $NADP^+$ cannot easily cross the ER membrane, and indicates the predicted luminal compartmentation of the enzyme [2]. Although a G6P-dependent H6PD activity could be demonstrated in all the investigated tissues, the activity appeared relatively lower in rat heart, skeletal muscle and lung (see Fig. 1C).

The ER H6PD is a dual enzyme possessing both G6P dehydrogenase and 6-phosphogluconolactonase activity [9,28]. Therefore, 6-phosphogluconolactone – derived from G6P oxidation – should be further metabolized by the same enzyme to 6-phosphogluconate. The latter metabolite was indeed formed, as revealed by the fact that the addition of 6-phosphogluconic acid dehydrogenase to all the microsomal incubates – in which G6P has been already oxidized and hydrolyzed to 6-phosphogluconic acid – resulted in a further rapid increase in NADPH level (Fig. 2A and B, as examples). The production of NADPH by the dehydrogenation of 6-phosphogluconic acid (reflecting the H6PD lactonase activity) was a phenomenon also observed in all the other tissues investigated as demonstrated in Fig. 2C. The amount of NADPH

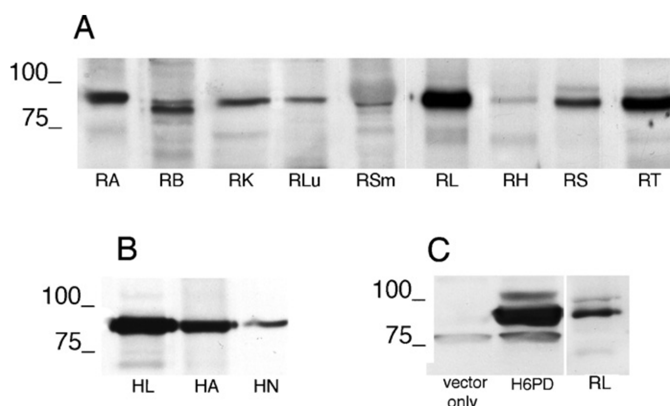

Fig. 4. H6PD protein expression in the different rat and human tissues. Microsomal proteins from rat adipose tissue (RA, 20 μg), rat brain (RB, 50 μg), rat kidney (RK, 50 μg), rat lung (RLu, 60 μg), rat hide limb skeletal muscle (RSm, 60 μg), rat liver (RL, 20 μg), rat heart (RH, 60 μg), rat spleen (RS, 60 μg) and rat testis (60 μg) (see panel A) as well as from human liver (HL, 10 μg), human adipose tissue (HA, 20 μg) and human neutrophils (HN, 40 μg) (see panel B) were separated by 10% SDS-PAGE gels. Gels were blotted on nitrocellulose membranes and the H6PD protein was immunorevealed as a protein with a Mr of approximately 95 kDa, by using an antibody raised against the lactonase domain (residues 537–653) of human H6PD. Representative Western blots are shown. Panel C: the same antibody was used to immunoreveal the H6PD protein in HEK293 (cell lysate, 50 μg) either transfected with an empty vector (lane 1) or with a H6PD construct (lane 2) and thus overexpressing the H6PD enzymatic protein; rat liver microsomes (20 μg, lane 3) are used as a reference.

produced in the latter reaction (i.e. the dehydrogenation of 6-phosphogluconic acid) was always higher than 80% of the NADPH amount derived from G6P oxidation (Fig. 2C). This indicates that 6-phosphogluconate accumulates in the lumen, possibly because the downstream enzymes of the pentose phosphate pathway are poorly represented in the microsomal fractions. It should be noted that human liver and adipose tissue have a lower H6PD activity as compared to the rat counterpart. The possibility that this is also due to the long time interval between the withdrawal of the tissues and the microsomal preparation procedure cannot be excluded.

3.2. Real-time PCR of H6PD in rat and human tissues.

As illustrated in Fig. 3, the H6PD messenger was present in all the tissues investigated although at different extent, but within the same order of magnitude. H6PD mRNA expression was normalized on the basis of two commonly used reference genes, GAPDH (Fig. 3A) and SDHA (Fig. 3C). The pattern of expression was similar in rats and humans. Rat H6PD mRNA expression data showed a good correlation to the enzymatic activity (reported in Fig. 1C) as shown in Fig. 3B and D.

3.3. Expression of the H6PD protein

A protein with an apparent Mr of approximately 95 kDa, which should correspond to H6PD, was immunorevealed in the microsomal fractions of all the rat and human tissues investigated (Fig. 4A and B, respectively). The antibody also immunorevealed the overexpressed H6PD in HEK293 cells and this confirms that the antibody indeed detects the enzyme protein (Fig. 4C). The expression level of the H6PD protein in the different rat tissues – relative to the liver – is reported in Fig. 5A. Data concerning human omental adipose tissue and neutrophils (expressed as percentage of the human liver) are reported in Fig. 5B. The expression of the H6PD protein in human liver microsomes was approximately 1.6-fold higher than in rat liver microsomes. This difference could be, at least in part, due to the fact that the polyclonal antibody used was raised against the

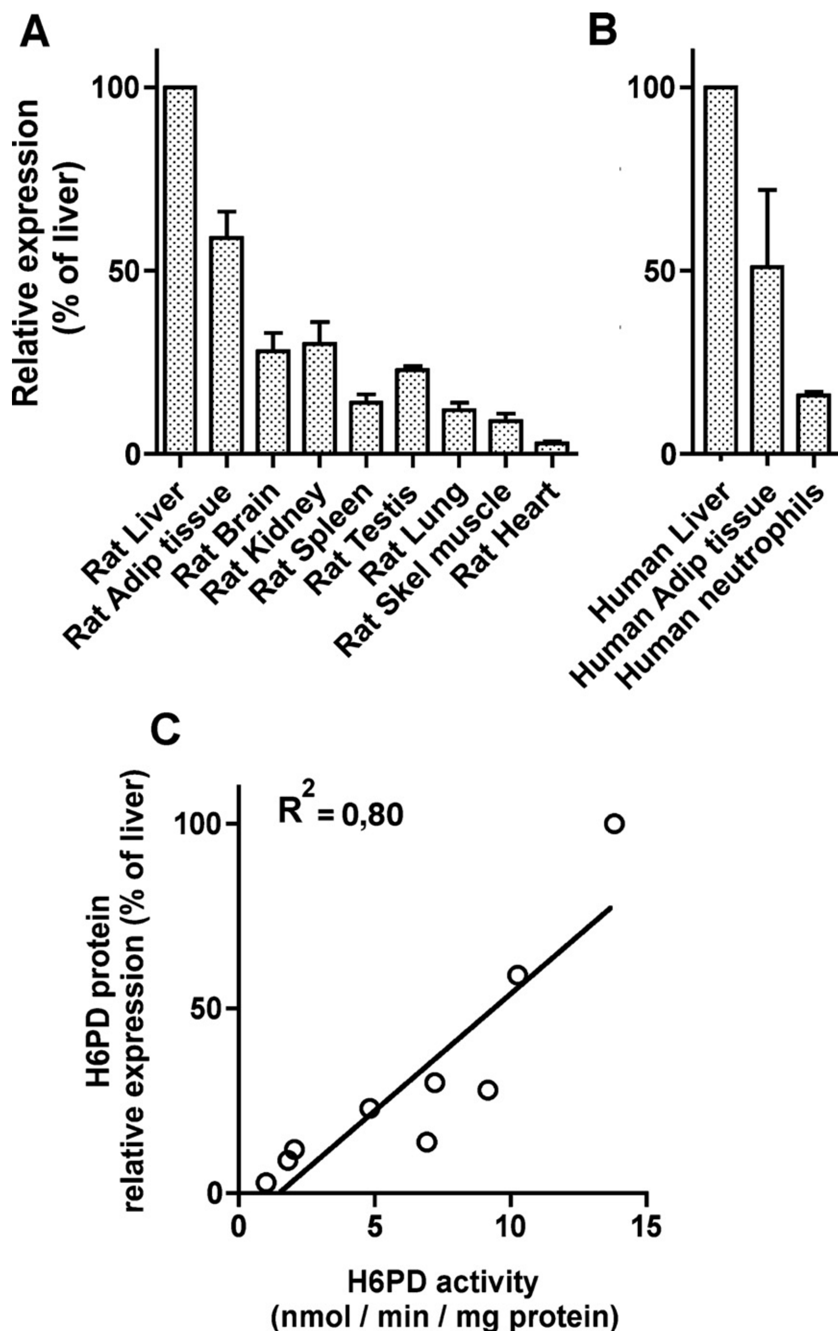

Fig. 5. Quantification of the H6PD protein expression and its correlation with H6PD dehydrogenase activity in rat tissues. Western blots were quantified using scanning densitometry (panels A and B). Normalized densitometry data are presented as % of liver intensity, and they represent the means ± SE of three to five different experiments. Rat H6PD protein expression levels were plotted towards H6PD enzymatic activities (panel C); the proportional increase (slope = 6.33) was statistically significant: $p = 0.001$.

human H6PD (residues 537–653) and the rat sequence of interest has a 90% sequence homology.

As demonstrated in Fig. 5C, the level of the H6PD activity statistically correlated with the level of protein expression in rat tissues.

4. Discussion

H6PD has been described several decades ago [reviewed in 2], and it has long been suggested that the role of this enzyme is to supply NADPH to ER reductases involved specifically in steroid and drug metabolism [29]. More recently a direct cooperation with 11β-HSD1, within the ER network, has been proved [6,30]. In addition, deletion of H6PD induces skeletal myopathy with activated unfolded protein response in mice [16], and sensitizes HepG2 cells towards oxidative injury [17]. Therefore, the missing function of H6PD can also impair cell viability, possibly altering the redox state of luminal pyridine nucleotides in the ER/SR. As an additional function, H6PD might act as a nutrient sensor of the cell [18,19].

The tissue distribution of H6PD in rat has been investigated in a comprehensive study [24]. The authors found mRNA expression in a broad range of tissues and protein expression in many tissues; enzymatic activities of H6PD were not measured. Here we clearly show the presence of the enzyme activity in a wide variety of rat tissues (and in some human tissues), together with mRNA and protein expressions. Relatively high activity and expression were also observed in those tissues where 11β-HSD1 is poorly represented (e.g. in spleen or testis). Moreover, both H6PD activity and immunoreactive bands close to the theoretical 95 kDa Mr could be

also demonstrated in whole brain microsomes. These discrepancies might be due to the antibody used: a commercially available polyclonal antibody raised against a large portion of the lactonase domain (116 aminoacid residues) was used in the present study, whilst in the previous study [24] antibodies raised against short peptides (14 aminoacid residues) of both dehydrogenase and lactonase domains of H6PD were used.

Notably, a relative high activity was present in (total) brain microsomes. Although H6PD mRNA and protein were less represented in whole rat brain than in rat liver (Figs. 3 and 4), a comparable H6PD activity was evident in liver and brain microsomes (Figs. 1 and 2). The reasons for this discrepancy were not further investigated. However, in brain microsomes the immunoreactive H6PD protein appeared split into two bands with a Mr somehow lower than the liver H6PD. H6PD has been shown to be a glycoprotein [31] and tissue differences in glycosylation status might explain both the difference in migration on SDS-PAGE gels as well as in activity.

The high activity of H6PD in brain microsomes is consistent with the presence of the reductase activity of 11β-HSD1 in several areas of the brain, which requires reduced pyridine nucleotides to activate glucocorticoids [[32,33] and refs. therein]. The H6PD activity, however, was also present independently of the expression level of 11β-HSD. For example, the latter enzyme is poorly expressed in skeletal muscle, heart and spleen [24].

H6PD expression, either normalized for GAPDH or SDHA (Fig. 3A and C), shows a similar pattern in the investigated rat and human tissues. Moreover H6PD mRNA shows a correlation to H6PD activity in the rat tissues taken into consideration (Fig. 3B and D). A significant correlation was also observed between protein levels and enzymatic activities in various rat tissues (Fig. 5).

The widespread distribution of the enzyme might suggest that its absence would cause pathological alterations in many tissues. However, it has been reported that H6PD knockout mice are apparently viable and present only an ER stress-associated myopathy as a main symptom. It can be speculated that besides H6PD other enzymes contribute to the maintenance of the ER NADPH pool, which are presumably less represented in the skeletal muscle. Alternatively, H6PD activity might be required for skeletal muscle-specific functions. Pyridine nucleotide dependent regulation of the ryanodine receptor calcium channel [34–36] might be a base of this assumption. Further studies are needed to clarify these possibilities. As a third possibility, defect in maintenance of the muscle SR/ER NADPH pool might result in sensitization to oxidative stress. In this respect, we recently reported that silencing of H6PD or oxidation of ER luminal pyridine nucleotides sensitizes HepG2 cells towards oxidative injuries, leading to ER stress-dependent autophagy [17].

In conclusion, the widespread tissue distribution strongly suggests a housekeeping function for H6PD, which much likely allows the maintenance of normal NADPH levels and redox environment inside the ER, not solely related to the activation of glucocorticoids. Accordingly, very recent studies indicate an involvement of H6PD in multiple sclerosis [37] and in myogenic differentiation [38].

Acknowledgements

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