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Maintenance of luminal NADPH in the endoplasmic reticulum promotes the survival of human neutrophil granulocytes

Tamás Kardon[a,1], Silvia Senesi[b,1], Paola Marcolongo[b], Balázs Legeza[a], Gábor Bánhegyi[a,b,*], József Mandl[a], Rosella Fulceri[b], Angelo Benedetti[b]

[a] *Semmelweis Egyetem Orvosi Vegytani, Molekuláris Biológiai és Patobiokémiai Intézete & MTA-SE Patobiokémiai Kutatócsoport, Budapest, Hungary*

[b] *Dipartimento di Fisiopatologia, Medicina Sperimentale e Sanità Pubblica, Università di Siena, Viale Aldo Moro 53100, Siena, Italy*

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Abstract The present study demonstrates the expression of hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase type 1 in human neutrophils, and the presence and activity of these enzymes in the microsomal fraction of the cells. Their concerted action together with the previously described glucose-6-phosphate transporter is responsible for cortisone–cortisol interconversion detected in human neutrophils. Furthermore, the results suggest that luminal NADPH generation by the cortisol dehydrogenase activity of 11β-hydroxysteroid dehydrogenase type 1 prevents neutrophil apoptosis provoked by the inhibition of the glucose-6-phosphate transporter. In conclusion, the maintenance of the luminal NADPH pool is an important antiapoptotic factor in neutrophil granulocytes.

Keywords: Apoptosis; 11β-Hydroxysteroid dehydrogenase type 1; Hexose-6-phosphate dehydrogenase; Glucose-6-phosphate transporter; Glucocorticoid

1. Introduction

The glucose 6-phosphate transporter (G6PT) of the endoplasmic reticulum (ER) allows the entry of the cytosolic metabolite glucose-6-phosphate (G6P) into the luminal space. G6P is the substrate for at least two luminal enzymes: glucose-6-phosphatase (G6Pase) and hexose-6-phosphate dehydrogenase (H6PDH). The activity of the former enzyme is crucial for the maintenance of blood glucose level [1,2]. Recent evidence show that H6PDH has a role in regulating the redox state of an ER pool of pyridine nucleotides, as well as in supplying the cofactor NADPH to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [3–7]. This enzyme, highly represented in liver and adipose tissue, is located within the ER lumen and plays a key role in the prereceptorial activation of glucocorticoids [8–10].

The genetic deficiency of G6PT, named glycogen storage disease type 1b (GSD 1b), causes – in addition to an inventory of symptoms ultimately due to the lack of G6Pase activity – a severe pathology of neutrophils/monocytes [11–14]. A recent report shows that G6PT knock-out mice also present with defects of neutrophils/monocytes largely mimicking those of GSD 1b patients, such as alterations of cell differentiation and growth, neutropenia, impairment of superoxide anion generation and chemotaxis [12]. We have previously observed that the chemical ablation of G6PT activity (with the selective inhibitor S3483) [15,16] results in an impaired differentiation of the promyelocytic HL-60 cell line, as well as in apoptosis of differentiated HL-60 cells and human neutrophils (PMNs) [17]. Consistently, other authors observed that apoptotic PMNs are present in the peripheral blood of GSD 1b patients [18].

In our aforementioned study [17] it was also observed that the PMN apoptosis induced by S3483 is fully prevented by the antioxidant vitamin E analogue, trolox C, as well as by the inhibitor of NADPH oxidase, diphenylene iodonium. On this ground, we hypothesized that G6P entry into the ER compartment mediated by G6PT might be required for the sufficient antioxidant protection of PMNs. The G6PT-dependent H6PDH activity in other tissues (i) keeps luminal pyridine nucleotides in reduced state and (ii) allows the reductase activity of 11β-HSD1. However, neither H6PDH nor 11β-HSD1 has been reported to be present and functional in the neutrophil ER. Therefore, experiments were undertaken to investigate the expression and activity of these enzymes in the ER of human PMNs, with special emphasis on the possibility of modulating neutrophil apoptosis through their activity.

2. Materials and methods

2.1. Preparation of human PMNs

Human PMN cells of healthy volunteers were isolated from peripheral venous blood collected in tubes containing 16 IU heparin/ml blood. The neutrophils were separated by dextran sedimentation method according to Hjorth et al. [19] with small modifications as described in details earlier [17]. Preparations contained more than 95% PMNs and cell viability (evaluated by trypan blue exclusion method) exceeded 97%.

2.2. Preparation of microsomal fractions

Microsomes from PMNs and rat liver were prepared as previously reported [17]. Human liver microsomes were prepared as detailed earlier [20]. The Ethical Committee of the Semmelweis University

*Corresponding author. Address: Dipartimento di Fisiopatologia e, Medicina Sperimentale e Sanità Pubblica, Università di Siena, Viale Aldo Moro 53100, Siena, Italy. Fax: +39 (0)577 234009.
E-mail address: banhegyi@unisi.it (G. Bánhegyi).

[1]These authors contributed equally to the work.

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approved the study on the H6PDH/11βHSD1 in human liver specimens. Microsomes were resuspended in MOPS-KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$ and 20 mM MOPS) pH 7.2 including a cocktail of protease inhibitors, and maintained under liquid N_2 until used. Immediately before Western blot analysis or enzyme assay, the possible cytosolic or other contaminants loosely associated with the vesicles were removed by a rapid washing as follows: polyethylene glycol (at 7% w/v final concentration) was added to the microsomes and the suspension was centrifuged at $4000 \times g$ for 15 s. After discarding the supernatant, the microsomal pellet was resuspended in polyethylene-glycol-free MOPS-KCl buffer [21]. The microsomal fraction was enriched in ER-specific proteins (calnexin, IP_3R3, SERCA2b) as revealed by Western blotting (data not shown).

2.3. Western blot

Microsomal proteins were loaded on polyacrylamide gels and blotted on nitrocellulose. Immunoblots were probed with a rabbit polyclonal antiserum against the lactonase domain (residues 539-791) of human H6PDH kindly provided by Dr. E. van Schaftingen (ICP, Bruxelles, Belgium), or with rabbit polyclonal antibodies to mouse 11βHSD1 (Alpha Diagnostic International). After reacting with the secondary antibodies, blots were analyzed either by enhanced chemiluminescence or using fluorescent secondary antibodies (Amersham Biosciences). The blots were visualized by Typhoon 8600 multiimager (Amersham Biosciences).

2.4. RT-PCR

PMN RNA was isolated using the RNeasy Mini Kit (Qiagen) and human liver total RNA was from Biocat GmbH. 500 ng of RNA was reverse transcribed by using the SuperScript® II First-Strand Synthesis System (Invitrogen). For H6PDH, the oligonucleotide primers were: sense, 5′-CAACTGGGGACCTGGCTAAGAAGT-3′; antisense, 5′-GTTGATGAGAGGCAGGCTAAGGCT 3′. For 11βHSD1 the oligonucleotide primers were: sense, 5′-GAACATCAATAA AAAGAAGTCAGA-3′; antisense, 5′-CATTATTATTACATTTC-CATTTTG-3′. PCR products were separated on a 1% agarose gel. The identity of the RT-PCR products was confirmed by DNA sequencing.

2.5. Enzyme assays

H6PDH and isocitrate dehydrogenase activities were evaluated by measuring NADPH formation upon the addition of 1 mM $NADP^+$ and either 10 μM glucose-6-phosphate or 1 mM isocitrate to microsomes. Limited access of the cofactor compounds to the intraluminal enzymes 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. The product of lactonase activity, 6-phosphogluconic acid, was then measured enzymatically with 6-phosphogluconate dehydrogenase on the basis of NADPH formation. 11βHSD1 activity was measured in both directions, as cortisone reductase and cortisol dehydrogenase activity, upon the addition of 100 μM NADPH or $NADP^+$ and 20 μM cortisone or cortisol to microsomes. Microsomes have been subsequently permeabilized with Triton X-100 (1% final concentration) to allow the free access of the cofactors to the intraluminal enzyme. NADPH fluorescence was monitored at 350 nm excitation and 460 nm emission wavelengths by using a Cary Eclipse fluorescence spectrophotometer (Varian).

2.6. Evaluation of apoptosis

Apoptosis was evaluated by annexin–propidium iodide staining as described in details earlier [17].

3. Results

3.1. H6PDH in microsomes of human neutrophils

H6PDH is expressed in human PMNs both at mRNA and protein level. As shown in Fig. 1A, the analysis of RT-PCR products showed a band whose apparent size (2094 bp) is consistent with a mRNA coding for a protein of approximately 90 kDa, which is the M_r of the ER H6PDH protein; human liver RNA gave the same band [22]. Accordingly, in Western blot analysis of microsomal proteins from PMNs, a band at an apparent M_r of ≈90 kDa was immunorevealed by antibodies towards the lactonase domain of H6PDH; this band was also present in human (and rat) liver microsomes (Fig. 1B).

Fig. 1C shows that H6PDH activity was also present in PMN microsomes. In the presence of $NADP^+$, a marked G6P-dependent NADPH formation was evident upon permeabilization of the microsomal membrane (addition of Triton X-100, see arrow), whilst a little activity was only present before permeabilization. This was expected since $NADP^+$ cannot easily cross the ER membrane, and indicates the predicted luminal compartmentation of the enzyme [22,23]. The ER H6PDH is a dual enzyme possessing both G6P dehydrogenase and 6-phosphogluconolactonase activity [24]. 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 microsomal incubates, in which G6P has been already oxidized to 6-phosphogluconolactone, resulted in a further increase in NADPH level (Fig. 1C). The amount of NADPH produced in the latter reaction (i.e., the dehydrogenation of 6-phosphogluconic acid) was roughly the same as NADPH amount derived from G6P oxidation (see Fig. 1C). This indicates that 6-phosphogluconate accumulates in the lumen, possibly because the downstream enzymes of the pentose pathway are not or poorly represented in the microsomes.

Since the presence of isocitrate dehydrogenase, another NADPH generating enzyme was reported in liver and adipose tissue microsomes [21], an attempt was made to demonstrate this activity in PMN microsomes. Very low $NADP^+$-dependent latent isocitrate dehydrogenase activity was detected (less than 5% of the observed H6PDH activity).

3.2. 11βHSD1 in microsomes of human neutrophils

Not only H6PDH, but also 11βHSD1 is represented in the ER of PMNs. The enzyme was expressed both at the mRNA and the protein level, as shown in Fig. 2A and B, respectively. Accordingly, 11βHSD1 activity could be also revealed in PMN microsomes. Upon the addition of cortisone or cortisol to permeabilized microsomes, NADPH was consumed or $NADP^+$ was reduced to NADPH, respectively (Fig. 2C). The activities appeared upon permeabilization of the microsomal membrane only. This is consistent with the luminal compartmentation of 11βHSD1 activity, as in the case of H6PDH [25,26].

3.3. Cortisol prevents the proapoptotic effect of the inhibition of microsomal G6P transport in human PMNs

As we have already shown, the addition of the G6PT inhibitor S3483 induced apoptosis in human granulocytes (Fig. 3). Addition of cortisol at high (20 μM) concentration did not alter the rate of apoptosis. Under these circumstances 11βHSD1 presumably acts as a dehydrogenase and generates NADPH in the ER lumen. When S3483 and cortisol were added together, cortisol significantly reduced the proapoptotic effect of the G6PT inhibitor. Inhibition of 11βHSD1 activity with carbenoxolone completely abolished the preventive effect of cortisol (data not shown). Physiological cortisol concentrations (20–100 nM) were ineffective. Cortisone addition did not increase