Failure to eliminate a phosphorylated glucose analog leads to neutropenia in patients with G6PT and G6PC3 deficiency

Maria Veiga-da-Cunha a,b,1, Nathalie Chevalier a,b, Xavier Stephenne c, Jean-Philippe Defour d, Nicole Paczia a, Alina Ferster f, Younes Achouri b, Joseph P. Dewulf a,b, Carole L. Linster a, Guido T. Bommer a,b, and Emile Van Schaftingen a,b,1

aWalloon Excellence in Lifesciences and Biotechnology, B-1200 Brussels, Belgium; bGroupe de Recherches Metaboliques, de Duve Institute, UCLouvain (Université Catholique de Louvain), B-1200 Brussels, Belgium; cService de Gastro-Enterologie et Hepatologie Pediatrique, Cliniques Universitaires Saint-Luc, UCLouvain, B-1200 Brussels, Belgium; dBiologie Hématologique, Cliniques Universitaires Saint-Luc, UCLouvain, B-1200 Brussels, Belgium; eLuxembourg Centre for Systems Biomedicine, Université du Luxembourg, L-4367 Belvaux, Luxembourg; and fDepartment of Hematology/Oncology, Hôpital Universitaire des Enfants Reine Fabiola, Université Libre de Bruxelles, B-1020 Brussels, Belgium

Neutropenia represents an important problem in patients with genetic deficiency in either the glucose-6-phosphate transporter of the endoplasmic reticulum (G6PT/SLC37A4) or G6PC3, an endoplasmic reticulum phosphatase homologous to glucose-6-phosphatase. While affected granulocytes show reduced glucose utilization, the underlying mechanism is unknown and causal therapies are lacking. Using a combination of enzymological, cell-culture, and in vivo approaches, we demonstrate that G6PT and G6PC3 collaborate to destroy 1,5-anhydroglucitol-6-phosphate (1,5AG6P), a close structural analog of glucose-6-phosphate and an inhibitor of low-Km hexokinases, which catalyze the first step in glycolysis in most tissues. We show that 1,5AG6P is made by phosphorylation of 1,5-anhydroglucitol, a compound normally present in human plasma, by side activities of ADP-glucokinase and low-Km hexokinases. Granulocytes from patients deficient in G6PC3 or G6PT accumulate 1,5AG6P to concentrations (~3 mM) that strongly inhibit hexokinese activity. In a model of G6PC3-deficient mouse neutrophils, physiological concentrations of 1,5-anhydroglucitol caused massive accumulation of 1,5AG6P, a decrease in glucose utilization, and cell death. Treating G6PC3-deficient mice with an inhibitor of the kidney glucose transporter SGLT2 to lower their blood level of 1,5-anhydroglucitol restored a normal neutrophil count, while administration of 1,5-anhydroglucitol had the opposite effect. In conclusion, we show that the neutropenia in patients with G6PC3 or G6PT mutations is a metabolite-repair deficiency, caused by a failure to eliminate the nonclassical metabolite 1,5AG6P.

Significance

Neutropenia presents an important clinical problem in patients with G6PC3 or G6PT deficiency, yet why neutropenia occurs is unclear. We discovered that G6PC3 and G6PT collaborate to dephosphorylate a noncanonical metabolite (1,5-anhydroglucitol-6-phosphate; 1,5AG6P) which is produced when glucose-phosphorylating enzymes erroneously act on 1,5-anhydroglucitol, a food-derived polyol present in blood. In patients or mice with G6PC3 or G6PT deficiency, 1,5AG6P accumulates and inhibits the first step of glycolysis. This is particularly detrimental in neutrophils, since their energy metabolism depends almost entirely on glycolysis. Consistent with our findings, we observed that treatment with a 1,5-anhydroglucitol-lowering drug treats neutropenia in G6PC3-deficient mice. Our findings highlight that the elimination of noncanonical side products by metabolite-repair enzymes makes an important contribution to mammalian physiology.

Author contributions: M.V.-d.-C., G.T.B., and E.V.S. designed research; M.V.-d.-C., J.-P.D., N.P., and Y.A. performed research; M.V.-d.-C., X.S., A.F., J.P.D., and C.L.L. contributed new reagents/analytic tools; X.S. and A.F. contributed patient material; J.-P.D. contributed useful discussions; M.V.-d.-C., J.-P.D., N.P., C.L.L., G.T.B., and E.V.S. analyzed data; and M.V.-d.-C., G.T.B., and E.V.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. R.G.K. is a guest editor invited by the Editorial Board.

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: maria.veiga@uclouvain.be or emile.vanschaftingen@uclouvain.be.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1816143116/-/DCSupplemental. Published online January 9, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1816143116

PNAS | January 22, 2019 | vol. 116 | no. 4 | 1241–1250

neutrophenia | SLGT2 inhibitors | 1,5-anhydroglucitol | metabolite repair | glucose-6-phosphatase-β
Neutrophils derived from patients with G6PC3 or G6PT deficiency are characterized by reduced glucose utilization, which is likely responsible for their decreased capacity to produce superoxide anions, decreased protein glycosylation, and increased endoplasmic reticulum stress (10, 11). It has been proposed that lack of functional G6PC3 or G6PT prevents endogenous glucose formation from glucose-6-P in neutrophils and thereby decreases the amount of glucose available for glycolysis and the pentose-P pathway in a way that is vital for neutrophil function (11). What is unsatisfying with this explanation is that blocking the conversion of glucose-6-P to glucose is expected on the contrary to increase glucose-6-P availability for glycolysis and the pentose-P pathway (see also ref. 12).

These considerations led us to hypothesize that the critical role of G6PC3 and G6PT in neutrophils is to dephosphorylate a noncanonical phosphate ester that inhibits glucose utilization in these cells. Many enzymes of intermediary metabolism catalyze side reactions and produce noncanonical metabolites that are then destroyed by metabolite-repair enzymes (13, 14). Mutations in metabolite-repair enzymes lead to the accumulation of side...
products (15–19) and in some cases cause metabolic diseases, like 1,2-hydroxyglutaric aciduria (20, 21) and NAD(P)HX epimerase deficiency (22).

In the current work, we present compelling evidence that the neutropenia in patients with G6PC3 or G6PT mutations is caused by a failure to eliminate the noncanonical metabolite 1,5-anhydroglucitol-6-phosphate (1,5AG6P), which results from the phosphorylation of 1,5-anhydroglucitol (1,5AG), a glucose analog normally present in blood. Our data indicate that these diseases should be considered as metabolite-repair deficiencies, an emerging new class of inborn errors of metabolism. Furthermore, understanding the underlying pathogenesis allowed us to devise a successful treatment strategy in G6PC3-deficient mice.

Results

G6PC3 Has a Wide Substrate Spectrum, but Only a Few Compounds Are Also Transported by G6PT. The role of G6PC1 as a glucose-6-phosphatase is well-established (23). In contrast, little is known about the substrate spectrum of G6PC3. Thus, we started by comparing the substrate preferences for G6PC3 and G6PC1. As G6PC3 and G6PC1 are integral membrane proteins, we studied their kinetic properties in membrane preparations from HEK293T cells that overexpressed the human recombinant proteins with a C-terminal 6xHis tag. To ensure that the observed phosphatase activities were genuinely contributed by G6PC1 or G6PC3, we subtracted baseline phosphatase activities observed in control cells expressing the respective catalytically dead mutants (H176A for G6PC1; H167A for G6PC3) where the histidine that transiently accepts a phosphoryl group during the catalytic cycle (24) is mutated. The abundance of the recombinant proteins was similar in the membrane preparations used (Fig. 1A), indicating that their activities could be readily compared without bias.

Phosphatase activity of the recombinant proteins with various phosphate esters (0.1 mM) was assayed by measuring the release of inorganic phosphate (Fig. 1B). This confirmed that G6PC1 acts best on glucose-6-P, mannose-6-P, and inorganic pyrophosphate and not, or almost not, on the other phosphate esters that we tested. By contrast, G6PC3 hydrolyzes a wider spectrum of substrates, and glucose-6-P was clearly not among its best substrates. Inorganic pyrophosphate, ribose-5-P, ribitol-5-P, and 1,5AG6P were indeed hydrolyzed four to eight times faster than glucose-6-P, whereas other phosphate esters were less well but still significantly hydrolyzed.

Detailed kinetic properties of the two enzymes were determined for the best substrates (SI Appendix, Table S1). Using apparent $V_{\text{max}}/K_M$ ratio as a criterion (a proxy for catalytic efficiency, which could not be determined because the proteins were impure), glucose-6-P and inorganic pyrophosphate were at least 10-fold better substrates for G6PC1 than for G6PC3, whereas 1,5AG6P was the best substrate for G6PC3 (Fig. 1C). The activity of skeletal muscle microsomes on 1,5AG6P was significantly inhibited by S3483, which provided complementary information. To test this hypothesis, we used a reversible and specific inhibitor of G6PT, S3483 (25), and assessed whether G6PT is required for the hydrolysis of the best substrates for G6PC3 by rat skeletal muscle microsomes, where G6PC3 is plentiful and G6PC1 is absent. The activity of skeletal muscle microsomes on glucose-6-P and 1,5AG6P was significantly inhibited by S3483, but this was not the case for the phosphatase activity on ribose-5-P, ribitol-5-P, and inorganic pyrophosphate (Fig. 1E).

1,5-Anhydroglucitol-6-Phosphate Accumulates in Human HAP1 Cell Lines Deficient in G6PC3 or G6PT. To investigate whether G6PC3 or G6PT deficiency leads to the accumulation of 1,5AG6P in cells, we used CRISPR-Cas9 to generate HAP1 cell lines deficient in either of these two proteins. Sequencing of the DNA allowed free entry into the ER. Together, this suggested that in skeletal muscle microsomes, entry of these metabolites into the ER is rate-limiting. In contrast, 1,5AG6P dephosphorylation was largely unaffected, likely because G6PT activity allowed free entry into the ER. Together, this suggested that 1,5AG6P could be the common metabolite that accumulates in G6PC3 and G6PT deficiency and accounts for the neutropenia that is found in these conditions.
In this particular experimental system, even at the highest concentration of 1,5AG (1 mM), the accumulation of 1,5AG6P only led to a modest decrease in glucose consumption of the three mutant cell lines (Fig. 2B). Interestingly, when we added 1,5-anhydrofructose, the precursor of 1,5AG, we observed a several-fold higher accumulation of 1,5AG6P in G6PC3- or G6PT-deficient cells (Fig. 2C), reaching ∼2 to 4 mM in the presence of 0.5 mM 1,5-anhydrofructose (SI Appendix, Fig. S3C) compared with only ∼0.1 to 0.3 mM when the same cell lines were cultured with 1 mM 1,5AG (SI Appendix, Fig. S3A), presumably due to faster entry of 1,5-anhydrofructose into these cells and its rapid conversion to 1,5AG (SI Appendix, Fig. S2).

Of note, these high concentrations of 1,5AG6P in cells treated with 0.5 mM 1,5-anhydrofructose led to a 40 to 50% decrease in glucose consumption in G6PC3- and G6PT-deficient cells and no change in wild-type cells (Fig. 2D and SI Appendix, Fig. S3D). Consistent with the idea that 1,5AG6P might inhibit the first step in glycolysis (i.e., it was previously shown that in vitro 1,5AG6P inhibited HKI (27)), we observed a significant reduction of glycolytic intermediates (glucose-6-P, fructose-6-P, fructose-1,6-bisphosphate, 6-phosphogluconate, and ribose-5-P; Fig. 2E–H and SI Appendix, Fig. S3 H–J and N) while the concentration of the Krebs cycle intermediates malate (Fig. 2H), α-ketoglutarate, and succinate (SI Appendix, Fig. S3 O and P) were largely unaffected. We also observed that cell viability was decreased when G6PC3- or G6PT-deficient cells were cultured with 0.5 mM 1,5-anhydrofructose (Fig. 2F), while little or no toxicity was observed in wild-type cells or when 1,5AG was used, presumably because the latter caused a much lower accumulation of 1,5AG6P (Fig. 2A and C and SI Appendix, Fig. S3 A and C) and smaller effects on glucose consumption (Fig. 2B and D and SI Appendix, Fig. S3 B and D).

Thus, our data demonstrate that G6PC3 and G6PT collaborate to dephosphorylate 1,5AG6P, and that accumulation of high concentrations of this metabolite (∼3 mM) lead to inhibition of glycolysis and reduced cell viability.

**Toxicity of Physiological Concentrations of 1,5-Anhydroglucitol in an Immortalized Mouse Neutrophil Precursor Cell Line Deficient in G6PC3.** Even though 1,5AG6P accumulated in G6PC3- or G6PT-deficient HAP1 cells when 1,5AG was added to the culture media, 1,5AG barely affected glucose metabolism and cell viability, regardless of the use of concentrations that far exceeded the physiological concentration of this polyol in plasma. We hypothesized that neutrophils might be more sensitive to 1,5AG and more prone to accumulate 1,5AG6P because of differences in their hexose transporters and hexose-phosphorylating enzymes. To investigate this question, we studied Hox8b-immortalized neutrophil progenitor cell lines from wild-type or G6PC3-deficient mice (28).

Remarkably, in G6PC3-deficient neutrophils, 1,5AG prompted cell death and was about as effective as 1,5-anhydrofructose in this respect (Fig. 3A). Addition of a physiological concentration of 1,5AG (0.2 mM) to the culture media resulted in a significant decrease in the rate of glucose consumption in G6PC3-deficient cells but not in wild-type cells (Fig. 3B). Targeted
metabolomic analysis by LC-MS showed that the presence of 1,5AG resulted in a time-dependent increase in 1,5AG6P, which reached ~3 mM after 22 h (Fig. 3C). In parallel, we observed a progressive decrease in the concentration of glycolytic metabolites such as glucose-6-P, fructose-1,6-bisphosphate, and triose-phosphates in G6PC3-deficient cells (Fig. 3D–G) but no significant change in the concentration of Krebs cycle intermediates, as illustrated for fumarate (Fig. 3H). In contrast, no changes were observed when wild-type cells were analyzed.

These observations indicated that physiological concentrations of 1,5AG lead to the accumulation of 1,5AG6P in G6PC3-deficient neutrophils to concentrations that are sufficient to inhibit phosphorylation of glucose and reduce cell viability.

Phosphorylation of 1,5-Anhydroglucitol by Human Hexokinases and ADPGK and Their Inhibition by 1,5-Anhydroglucitol-6-Phosphate. Phosphorylation of 1,5AG likely results from a side activity of a hexose-phosphorylating enzyme. To investigate this problem, we produced human recombinant low-KM hexokinases (HK1, HK2, and HK3) and ADP-glucokinase (ADPGK), and compared their capacity to phosphorylate glucose and 1,5AG. The kinetic constants (Table 1) show that low-KM hexokinases have a low capacity to phosphorylate 1,5AG compared with glucose. In vitro, catalytic efficiency values were indeed 10^2- to 10^3-fold higher for glucose than they were for 1,5AG, consistent with previous data obtained on brain hexokinase (HK1) (27). ADPGK, on the other hand, phosphorylated 1,5AG with a catalytic efficiency that was about 1/20 of that observed with glucose (Table 1).

Together, these findings suggest that in vivo, one cannot exclude that the side activity of several enzymes may contribute to the phosphorylation of 1,5AG.

To assess which enzymes could be affected by the accumulation of 1,5AG6P, we measured the activity of recombinant hexokinases in the presence of different concentrations of 1,5AG6P (Table 1). While ADPGK was not inhibited even at the highest concentration of 1,5AG6P tested (1 mM), low-KM hexokinases showed inhibition constants well below the intracellular concentrations measured in HAP1 cells (SI Appendix, Fig. S3 A and C) and in mouse neutrophil progenitors (Fig. 3C). Thus, the concentrations of 1,5AG6P that accumulated in these model systems are expected to inhibit glycolytic flux.

Modulating the Concentration of 1,5-Anhydroglucitol in Blood Impacts Neutrophils in G6PC3-Deficient Mice. If 1,5AG6P is responsible for the toxicity observed in neutrophils, then 1,5AG administration to G6PC3-deficient mice should exacerbate their neutropenia. On the other hand, depletion of 1,5AG in the blood by treatment with an inhibitor of the renal glucose transporter SGLT2 (29, 30) should raise neutrophil numbers in these mice.

To test these predictions, we turned to a mouse model of G6PC3 deficiency. When we administered empagliflozin to mice, the blood concentration of 1,5AG decreased by approximately fivefold in 5 d, while oral administration of 1,5AG increased the plasma

![Fig. 3.](image-url)
The rapid decrease in blood 1,5AG, as a result of empagliflozin treatment, was independent of the mouse genotype (Fig. 4 A and B). Flow cytometric analysis of Mac-1/Gr-1–positive leukocytes revealed that G6PC3-deficient mice were neutropenic and had on average twofold fewer granulocytes than their heterozygous littermates (Fig. 4 C).

In a remarkable way, administration of empagliflozin achieved an almost complete normalization of neutrophil numbers in G6PC3-deficient mice. Since this change just failed to reach statistical significance in the experiment shown in Fig. 4, we performed a second experiment with an independent cohort of G6PC3-deficient mice. Here, treatment with empagliflozin led to a highly significant increase in neutrophil numbers, confirming that depletion of 1,5AG can be used to treat neutropenia in these mice (SI Appendix, Fig. S4). Conversely, when we orally administered 1,5AG to G6PC3-deficient mice, Mac-1/Gr-1–positive granulocytes became barely detectable, while this treatment had no impact in the heterozygous littermates (Fig. 4 C), LC-MS analysis of leukocytes revealed that those from G6PC3-deficient mice accumulated about 200-fold more 1,5AG6P compared with the heterozygous littermates. As expected, 1,5AG6P levels were further increased by the administration of 1,5AG and reduced by empagliflozin (Fig. 4 D). Of note, glucose in blood from G6PC3-deficient mice was neither significantly affected by empagliflozin nor by 1,5AG (107 ± 11 or 84 ± 9% compared with the untreated group, respectively). This indicates that variations in glucose concentration did not play a role in the changes shown in Fig. 4.

The mechanism that we propose to explain the neutropenia/neutrophil dysfunction seen in G6PT and G6PC3 deficiency is illustrated in Fig. 6. 1,5AG, a polyol normally present in blood, is slowly phosphorylated by a side activity of several glucose-phosphorylating enzymes to 1,5AG6P. This compound is normally dephosphorylated by the combined action of G6PC3 and G6PT. If not, it accumulates to concentrations that inhibit hexokinases.

**Table 1.** Catalytic constants showing the specificity of human recombinant hexokinases 1, 2, and 3 and ADP-glucokinase for glucose versus 1,5-anhydroglucitol and the inhibition constants for 1,5-anhydroglucitol-6-phosphate

| Enzyme          | \( K_M, \mu M \) | \( V_{max}, \mu mol \cdot min^{-1} \cdot mg^{-1} \) | \( K_{cat}/K_M, s^{-1} \cdot \mu M^{-1} \) | \( K_{ass}, \mu M \) | \( V_{max}, \mu mol \cdot min^{-1} \cdot mg^{-1} \) | \( K_{cat}/K_M, s^{-1} \cdot \mu M^{-1} \) | Inhibitor: 1,5AG6P* | Substrate: Glucose | Ratio \( K_{cat}/K_M \) | Glucose vs. 1,5AG | \( K_a, \mu M \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|------------------|-----------------|-----------------|-----------------|
| \( \Delta 21-hHK1\) | 50 ± 2          | 40 ± 0.5        | 1.36            | 57.9 ± 2.9      | 0.89 ± 0.03     | 2.6 × 10^{-5}   | 52,308         | 53 ± 2           |                 |                 |                 |
| \( \Delta 28-hHK2\) | 250 ± 9         | 35.4 ± 0.4      | 0.25            | 126 ± 52        | 0.19 ± 0.06     | 0.26 × 10^{-5}  | 96,153         | 96 ± 3           |                 |                 |                 |
| hHK3\) | 12.2 ± 1        | 10 ± 0.2        | 1.35            | 22.1 ± 3.9      | 1.2 ± 0.1       | 9.6 × 10^{-5}   | 27,000         | 134 ± 9          |                 |                 |                 |
| \( \Delta 50-hADPGK\) | 4.3 ± 0.5  | 12.3 ± 1.3      | 5.2 ± 0.2       | 0.01            | 506 ± 36        | 7.0 ± 0.3       | 19 ± 0.4        | 96 ± 3           |                 |                 |                 |

Data indicate means ± SEM (n ≥ 3).

*Noncompetitive inhibition versus glucose.

†Spectrophotometric assay: 0.5 mM ATP-Mg\(^{2+}\) and increasing concentrations of glucose or 1,5AG6P.

‡For optimal production, recombinant HK1, HK2, and ADPGK were expressed without hydrophobic N-terminal 21 (HK1), 28 (HK2), or 50 (ADPGK) amino acids (see SI Appendix, Materials, Methods, and Patient Information for details).

†Radiochemical assay: 1 mM ATP-Mg\(^{2+}\) and increasing concentrations of glucose or 1,5AG6P.

No inhibition of hADPGK detected in the presence of 1 mM 1,5AG6P.

1,5-Anhydroglucitol-6-Phosphate Accumulates in Neutrophils from Patients Deficient in G6PT or G6PC3. To assess whether comparable pathogenic mechanisms are at play in neutrophils of human patients, we analyzed blood samples from one patient with a mutation in G6PC3 (PT3) and from two GSDib patients with mutations in G6PT (PT1 and PT2). All three patients were neutropenic. PT2 was liver-transplanted ~8 years ago and is currently taking G-CSF. Further information concerning the patients’ pathology can be found in SI Appendix, Patient Information.

Polymorphonuclear cells (PMNs; mostly neutrophils) were separated from peripheral blood mononuclear cells (PBMCs; mostly lymphocytes), and the concentration of 1,5AG6P was quantified. Leukocytes from all three patients had a concentration of 1,5AG6P that was more than 500-fold higher than that measured in controls (Fig. 5A), while the average concentration of 1,5AG in plasma was not significantly different between patients and healthy controls (Fig. 5B).

Taken together, these results support the idea that G6PT and G6PC3 are involved in the breakdown of 1,5AG6P and that, in their absence, 1,5AG6P can reach concentrations that are likely to inhibit hexokinases.

**Discussion**

The mechanism that we propose to explain the neutropenia/neutrophil dysfunction seen in G6PT and G6PC3 deficiency is illustrated in Fig. 6. 1,5AG, a polyol normally present in blood, is slowly phosphorylated by a side activity of several glucose-phosphorylating enzymes to 1,5AG6P. This compound is normally dephosphorylated by the combined action of G6PC3 and G6PT. If not, it accumulates to concentrations that inhibit glucose phosphorylation by low-\( K_M \) hexokinases. Since mature neutrophils hardly have any functional mitochondria able to synthesize ATP and mainly rely on glucose metabolism for energy production (31, 32), it is likely that their maturation and function will be greatly affected by the accumulation of 1,5AG6P.

**Origin and Homeostasis of 1,5-Anhydroglucitol.** 1,5AG, the major polyol present in humans (33, 34) and rodents, essentially comes from food, with little (<10%) endogenous production (35). It results from the enzymatic reduction of 1,5-anhydrofructose, which is produced by a minor side activity of glucosidases (36). 1,5AG is present in most foods at concentrations ranging from 0.3 to 3 \( \mu g \cdot g^{-1} \) (35). The calculated daily intake in humans is

| Enzyme          | \( K_M, \mu M \) | \( V_{max}, \mu mol \cdot min^{-1} \cdot mg^{-1} \) | \( K_{cat}/K_M, s^{-1} \cdot \mu M^{-1} \) | \( K_{ass}, \mu M \) | \( V_{max}, \mu mol \cdot min^{-1} \cdot mg^{-1} \) | \( K_{cat}/K_M, s^{-1} \cdot \mu M^{-1} \) | Inhibitor: 1,5AG6P* | Substrate: Glucose | Ratio \( K_{cat}/K_M \) | Glucose vs. 1,5AG | \( K_a, \mu M \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|------------------|-----------------|-----------------|-----------------|
| \( \Delta 21-hHK1\) | 50 ± 2          | 40 ± 0.5        | 1.36            | 57.9 ± 2.9      | 0.89 ± 0.03     | 2.6 × 10^{-5}   | 52,308         | 53 ± 2           |                 |                 |                 |
| \( \Delta 28-hHK2\) | 250 ± 9         | 35.4 ± 0.4      | 0.25            | 126 ± 52        | 0.19 ± 0.06     | 0.26 × 10^{-5}  | 96,153         | 96 ± 3           |                 |                 |                 |
| hHK3\) | 12.2 ± 1        | 10 ± 0.2        | 1.35            | 22.1 ± 3.9      | 1.2 ± 0.1       | 9.6 × 10^{-5}   | 27,000         | 134 ± 9          |                 |                 |                 |
| \( \Delta 50-hADPGK\) | 4.3 ± 0.5  | 12.3 ± 1.3      | 5.2 ± 0.2       | 0.01            | 506 ± 36        | 7.0 ± 0.3       | 19 ± 0.4        | 96 ± 3           |                 |                 |                 |

Data indicate means ± SEM (n ≥ 3).

*Noncompetitive inhibition versus glucose.

†Spectrophotometric assay: 0.5 mM ATP-Mg\(^{2+}\) and increasing concentrations of glucose or 1,5AG6P.

‡For optimal production, recombinant HK1, HK2, and ADPGK were expressed without hydrophobic N-terminal 21 (HK1), 28 (HK2), or 50 (ADPGK) amino acids (see SI Appendix, Materials, Methods, and Patient Information for details).

†Radiochemical assay: 1 mM ATP-Mg\(^{2+}\) and increasing concentrations of glucose or 1,5AG6P.

No inhibition of hADPGK detected in the presence of 1 mM 1,5AG6P.
about 4 mg, and the total body pool amounts to 500 to 1,000 mg. There is extremely little metabolism of 1,5AG, and there is no
evidence that it has any physiological role (37). Its long half-life (more than 3 mo) is due to its renal reabsorption at least in part by
SGLT4/SLC5A9 (38), a sodium-dependent sugar transporter
present in the kidney tubules, which is mainly a D-mannose
transporter but which also carries glucose, fructose, and 1,5AG.
Glucose competes with the kidney reuptake of 1,5AG, and this
explains that 1,5AG is eliminated in urine in diabetic patients who
have a blood glucose level above the renal threshold, or if they are
treated with gliflozins (39), which cause glucosuria by inhibiting
SGLT2, the main glucose transporter in the kidney (40).

Fig. 4. Modulating the concentration of 1,5-anhydroglucitol in blood impacts neutrophils in G6PC3-deficient mice. G6PC3 KO or heterozygous littermates (8 to 11 wk old; 20 to 24 g) were left untreated or treated with 100 μL of an empagliflozin (EMPA) suspension (10 mg kg⁻¹, 10 times over 14 d) or 1,5AG (100 μL of a 50 mM solution; five times from day 7) until euthanasia on day 14 (n = 6 to 8 for each group). (A and B) 1,5AG in serum was measured at the indicated time points by LC-MS analysis (A) or from plasma recovered from EDTA-blood collected after euthanasia (B). (C) Flow cytometry analysis with anti-
Mac-1 (CD11b-PE) and anti-Gr-1 (Ly-6G-FITC) antibodies was performed to quantify granulocytes. (D) LC-MS analysis of 1,5AG6P in extracts from isolated white blood cells (WBCs). (E–H) Bone marrow smears from 1,5AG-treated G6PC3 heterozygous (E and G) or knockout (F and H) mice were stained with May-
Grünwald (E and F) or peroxidase (G and H), showing a maturation arrest with accumulation of apparent promyelocytes (“P”) in G6PC3 KO mice (F and H) and normal maturation with plenty of mature neutrophils (“N”) in heterozygous mice (E and G), and confirming the myeloid nature of these cells (peroxidase-positive in G and H). (Scale bar, 20 μm.) Statistical analysis: (A–D) P values were determined with one-way ANOVA followed by multiple t tests using the Bonferroni correction, with alpha = 0.05. Data are means ± SD (n = 6 to 8). ns, not significant, P > 0.05; ∗∗P ≤ 0.01; ∗∗∗P ≤ 0.001; ∗∗∗P ≤ 0.0001. (Magnification: G, 100×.)

Origin of 1,5-Anhydroglucitol-6-Phosphate and Its Impact on Glucose Metabolism. Exploration of the kinetic properties of G6PC3 led
us to find that this enzyme dephosphorylates several phosphate esters better than glucose-6-P. Studies on intact microsomes in-
volving the G6PT inhibitor S3483 indicated that one of these phosphate esters, 1,5AG6P, requires the glucose-6-P transporter
G6PT/SLC37A4 to be hydrolyzed by G6PC3. Not unexpectedly,
1,5AG6P is structurally closer to glucose-6-P than are other
phosphate esters that were apparently not transported by G6PT.
Involvement of both G6PC3 and G6PT in the hydrolysis of
1,5AG6P was further supported by the fact that 1,5AG6P ac-
cumulates in G6PC3- or G6PT-deficient cells incubated with
1,5AG or 1,5-anhydrofructose, a compound that is readily con-
verted in the cells to 1,5AG.

Accumulation of 1,5AG6P under these conditions indicates the presence of enzymes capable of phosphorylating the polyol
on its sixth carbon. Our studies with recombinant enzymes indicate that several enzymes that normally phosphorylate glucose
can also phosphorylate 1,5AG. In vitro, these include low-K_M
hexokinases and mainly ADPGK, which is an ADP-dependent
glucose kinase with an enigmatic function and which was until
hexokinases phosphorylated

K

hexokinases are physiologically inhibited by their

Veiga-da-Cunha et al.

1,5-Anhydroglucitol-6-phosphate accumulates in neutrophils from mouse ER-Hox8b G6PC3-deficient neutrophil progenitor cell

estimate that the intracellular concentration reaches phosphorylation. From the data obtained with neutrophils, we

glucose-6-P). It is therefore not possible at pre-
tive contribution of these kinases to 1,5AG6P formation in cells

tpe enzyme involved in 1,5AG phosphorylation. However, the rela-
tivity that this on its own could reduce glucose phosphorylation and
glucose uptake. Thus, all published data are consistent with an in-
h glucose phosphorylation rather than of glucose entry.

now considered to be very specific for glucose (41). Intriguingly,
this enzyme is associated with the endoplasmic reticulum, but
appears to have its catalytic site oriented toward the cytosol (42).
Hexokinase 1, hexokinase 3, and ADPGK are present in neu-

not considered to be very specific for glucose (41). Intriguingly,
this enzyme is associated with the endoplasmic reticulum, but
appears to have its catalytic site oriented toward the cytosol (42).
Hexokinase 1, hexokinase 3, and ADPGK are present in neu-

neutrophils (43) and in agreement with its K_i for
1,5AG6P (150 μM), the accumulation of 1,5AG6P that is seen in
G6PC3 or G6PT deficiencies is the finding that neutrophil toxicity is eli-
ited by the addition of 1,5AG6P to cultures of neutrophil pre-
cursors and that the administration of 1,5AG to mice further
opens perspectives for the treatment of the neutropenias caused
by G6PT or G6PC3 deficiencies. 1,5AG is widely distributed in
food and has a long half-life (more than 3 mo) (49); therefore,
limiting the food intake of this polyol is difficult to achieve and in

line grown in the presence of physiological concentrations of
1,5AG (Fig. 3C) as well as in human neutrophils isolated from
blood samples of G6PC3-deficient or GSDIb patients (Fig. 5A).
Taking into account that this inhibition is competitive with re-
spect to ATP (44), we calculate that this concentration is suf-
cient to cause more than 80% inhibition of hexokinase activity at
a physiological concentration of 2.5 mM ATP. This is consistent
with data obtained on leukocytes from GSDIb patients, which
indicated that the defect found in these cells is due to a reduction
in glucose phosphorylation, while glucose entry, as assessed with the
nonphosphorylatable glucose analog 3-O-methyl glucose, is unaf-
ected (45). These observations might seem at odds with the pre-
vious claim that G6PC3-deficient neutrophils show a decreased
take of glucose (11). However, in the latter study, 2-deoxyglucose
was used to assess glucose uptake, despite the fact that it measures
a combination of hexeose entry and phosphorylation (46). Further-
more, another confounding factor in this study might have been
that the G6PC3-deficient neutrophils have decreased viability and
that this on its own could reduce glucose phosphorylation and
glucose uptake. Thus, all published data are consistent with an in-
h glucose phosphorylation rather than of glucose entry.

**Figure 5. 1,5-Anhydroglucitol-6-phosphate Leads to Neutropenia.**

Neutrophils are extremely sensitive to a reduction of glucose
metabolism. This is believed to be due to the fact that they have a
limited number of mitochondria, which furthermore hardly dis-
play any marker of mitochondrial enzymatic activity and do not
synthesize much ATP (31, 32). They therefore mainly rely on
glycosis for their metabolism and energy supply (31). A re-
duction in glucose phosphorylation, due to a defect in the de-
phosphorylation of 1,5AG6P, may therefore explain several of the
deficiencies that have been observed in G6PC3- or G6PT-
deficient neutrophils. Indeed, decreased neutrophil motility and
defects in respiratory burst (likely due to a reduction in NADPH
production) that lead to an increased susceptibility to bacterial
infection (1, 2) can be explained by the inhibition of glucose
phosphorylation due to the accumulation of 1,5AG6P in pa-
tients’ neutrophils. Furthermore, reduced levels of glucose-6-P
are expected to compromise synthesis of NDP-sugars, leading to the
observed defects in protein glycosylation in G6PC3- and
G6PT-deficient neutrophils (10). Of note, accumulation of
1,5AG6P might also explain the functional defects in granu-
lactate, and ATP compared with wild-type mice (47).

Patients with G6PT or G6PC3 deficiency show an arrest in
neutrophil maturation at the myelocytic and promyelocytic stage
(48). When we treated G6PC3-deficient mice with 1,5AG, we
observed evidence for an arrest at this stage in the bone marrow.
In contrast, treatment with the SGLT2 inhibitor empagliflozin
released the blockage and allowed neutrophil maturation (**SI
Appendix, Fig. S5**). This suggests that accumulation of 1,5AG6P
is responsible for the maturation arrest.

The best proof that the effect of 1,5AG6P is the main, if not
the sole, explanation for the neutrophil defect in G6PC3 and
G6PT deficiencies is the finding that neutrophil toxicity is eli-
cited by the addition of 1,5AG to cultures of neutrophil pre-
cursors and that the administration of 1,5AG to mice further
decreases their neutrophil counts, while reducing the concen-
tration of 1,5AG considerably increases their number.

**Lowering of Blood 1,5-Anhydroglucitol Can Treat Neutropenia.**

Our observation that modulating the concentration of blood 1,5AG
in G6PC3-deficient mice strongly impacted neutrophil counts
opens perspectives for the treatment of the neutropenias caused
by G6PT or G6PC3 deficiencies. 1,5AG is widely distributed in
food and has a long half-life (more than 3 mo) (49); therefore,
limiting the food intake of this polyol is difficult to achieve and in

| Leukocytes | [1,5-AG6P] (μM) |
|-----------|----------------|
| Neutrophils (%) | Lymphocytes (%) | PMN (granulocytes) | PBMC (monocytes) |
| PT1 - GSD1b | 20.6 | 65 | 3.18 | 0.24 |
| CT1        | (<0.001) | | | |
| PT2 - GSD1b | 36 | 51 | 4.9 | 0.66 |
| CT2        | (0.01) | | | |
| PT4 - G6PC3 | 6 | 73 | 3.2 | 0.74 |
| CT4        | (0.002) | | | |
Illustration of the role played by 1,5-anhydroglucitol-6-phosphate accumulation in the neutropenia found in G6PC3 and G6PT deficiency. 1,5AG (also called 1-deoxyglucose) is a polyol that resembles glucose and is normally present in blood. It is transported into neutrophils and slowly phosphorylated by hexokinases (HK1, HK2, and HK3) and ADP-dependent glucokinase to 1,5AG6P. The glucose-6-P transporter of the endoplasmic reticulum (G6PT) transports 1,5AG6P into the ER, where it is dephosphorylated by G6PC3. When patients are deficient in G6PT or G6PC3, 1,5AG6P accumulates in neutrophils to concentrations that strongly inhibit low-K_M hexokinases, the enzymes catalyzing the first step of glycolysis. This depletes the intracellular pool of glucose-6-P in granulocytes, which likely decreases ATP production in glycolysis, as well as likely NADPH production in the pentose-P pathway and also UDP-glucose availability for protein glycosylation, explaining neutrophil dysfunction and neutropenia described in these patients.

Fig. 6. Illustration of the role played by 1,5-anhydroglucitol-6-phosphate accumulation in the neutropenia found in G6PC3 and G6PT deficiency. 1,5AG (also called 1-deoxyglucose) is a polyol that resembles glucose and is normally present in blood. It is transported into neutrophils and slowly phosphorylated by hexokinases (HK1, HK2, and HK3) and ADP-dependent glucokinase to 1,5AG6P. The glucose-6-P transporter of the endoplasmic reticulum (G6PT) transports 1,5AG6P into the ER, where it is dephosphorylated by G6PC3. When patients are deficient in G6PT or G6PC3, 1,5AG6P accumulates in neutrophils to concentrations that strongly inhibit low-K_M hexokinases, the enzymes catalyzing the first step of glycolysis. This depletes the intracellular pool of glucose-6-P in granulocytes, which likely decreases ATP production in glycolysis, as well as likely NADPH production in the pentose-P pathway and also UDP-glucose availability for protein glycosylation, explaining neutrophil dysfunction and neutropenia described in these patients.

any case would be effective only after several weeks. Therapies aimed at enhancing the urinary excretion of 1,5AG should therefore be much more effective. Indeed, administration of the SGLT2 inhibitor empagliflozin to mice led in a few days to a >80% decrease in the plasma concentration of 1,5AG. Of note, given the slow turnover of the 1,5AG pool, it is likely that an intermittent therapy with this drug might suffice to treat neutropenia.

The potential drawback of such treatment is that it causes glucosuria, which may therefore lead to hypoglycemia, particularly in G6PT-deficient patients, because of their reduced capacity of producing glucose (unless they have been liver-transplanted). This drawback has to be kept in balance with the side effects of the G-CSF therapy, which is used in the most severely affected patients. G-CSF administration may indeed be painful and result in an increased risk of development of myelodysplastic syndrome or acute myeloid leukemia (50).

Can Other Symptoms in Patients with G6PC3 Deficiency Also Be Explained by the Accumulation of 1,5AG6P? Patients with G6PC3 deficiency do not only present with neutropenia but also with cardiac, urogenital, and venous malformations (2), which are not observed in patients with G6PT deficiency. These symptoms might be caused by the accumulation of 1,5AG6P in the endoplasmic reticulum of specific cell types, which is not expected to occur in G6PT deficiency. Alternatively, it is possible that the accumulation of a G6PC3 substrate, other than 1,5AG6P, is responsible for these changes. The role of this accumulation and the identification of additional G6PC3 substrates deserves further studies.

Concluding Remarks
In conclusion, G6PC3, in collaboration with G6PT, catalyzes a new metabolite-repair reaction, and their deficiencies qualify therefore as diseases of metabolite repair. This new example adds to the growing list of metabolite-repair reactions, which is probably still far from complete. In the present case, the identification of the nonclassical metabolite that exerts toxic effects (1,5AG6P) leads to therapeutic perspectives. This should further motivate the search for other metabolite-repair reactions and their involvement in unclear metabolic diseases.

Materials and Methods
Detailed procedures involving the creation of knockout cell lines and cell culture, production, and purification of recombinant proteins, enzymatic assays, LC-MS and GC-MS metabolite analysis, establishment of G6PC3-deficient mouse models and experiments involving the effect of modulating the blood concentration of 1,5AG on neutropenia in these mice, and isolation of leukocytes from human G6PC3- and G6PT-deficient patients and their metabolite analysis are described in SI Appendix, Materials, Methods, and Patient Information. All procedures involving mice were performed following protocols accepted by the UCLouvain Animal Care Ethics Committee. Every effort was made to minimize mouse suffering. The blood samples in the experiments involving human blood were deidentified before use in experiments, informed consent was obtained, and followed protocols accepted by the Human Ethics Committee from Cliniques Universitaires Saint-Luc, UCLouvain.

ACKNOWLEDGMENTS. We thank Susanne Kirschnek and Georg Häcker (Institute for Medical Microbiology and Hygiene, University Medical Center, Freiburg) for kindly providing the estrogen-dependent immortalized mouse wild-type and G6PC3-deficient neurtrophil progenitors (ER-Hoxb8) and advising on cell culture conditions; Feng Zhang (Massachusetts Institute of Technology) for providing the plasmids used to clone the guide RNAs in the CRISPR-Cas9 experiments; and Maude Minsart and Nathalie Greisch for helping with G6PC3 and hexokinase experiments. Funding was provided by WELBIO (CR-2015A-09), FNRS (Fonds National de la Recherche Scientifique) (J.0104.18 to M.V.-d.-C.; J.0139.18 to E.V.S.; and F-4509.17 to G.T.B.), Fondation Contre le Cancer (2016-075 to G.T.B.), and Fonds Joseph Veiga-da-Cunha et al.
Maison, European Research Council (NOMEPACA 771704 to G.T.B.), M.V.-d.-C. and G.T.B. are “Chercheur qualifié,” and J.P.D. is an “Aspirant” of the FNSR. We thank the Association Luxembougeoise des Amis de la Fondation Louvain for their donation to this research. N.P. was supported by a CORE junior grant (C16/BM/11339953) of the Fonds National de la Recherche Luxembourg.

1. Boz tug K, et al. (2009) A syndrome with congenital neutropenia and mutations in G6PC3. N Engl J Med 360:32-43.
2. Cheung YY, et al. (2007) Impaired neutrophil activity and increased susceptibility to bacterial infection in mice lacking glucose-6-phosphatase-beta. J Clin Invest 119:4047-4057.
3. Veiga-da-Cunha M, et al. (1998) A gene on chromosome 11q23 coding for a putative glucose-6-phosphate translocase is mutated in glycogen-storage disease type Ib and type IIa. Am J Hum Genet 63:976-983.
4. Martin CC, et al. (2002) Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. Mol Endocrinol 26:205-222.
5. Boutillet JN, et al. (2004) Identification and characterization of a cDNA and the gene encoding the mouse ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. Mol Endocrinol 18:32-53.
6. Shieh JI, Pan CJ, Mansfield BC, Chou JY (2003) A glucose-6-phosphate hydrolase, widely expressed outside the liver, can explain age-dependent resolution of hypoglycemia in glycogen storage disease type Ia. J Biol Chem 278:47098-47103.
7. Shieh JI, Pan CJ, Mansfield BC, Chou JY (2004) A potential new role for muscle in blood glucose homeostasis. J Biol Chem 279:26215-26219.
8. Bali DS, Chen YT, Austin S, Goldstein JL (2006) Glycogen storage disease type I. J Mol Endocrinol 36:32-40.
9. Veiga-da-Cunha M, Chevalier N, Stroobant V, Vertommen D, Van Schaftingen E (1997) Sequence of a putative glucose-6-phosphate translocase, mutated in glycogen storage disease type Ib. FEBS Lett 419:235-238.
10. Hayeie B, et al. (2011) G6PC3 mutations are associated with a major defect of glycosylation: A novel mechanism for neutrophil dysfunction. Glycobiology 21:914-924.
11. Jun HS, et al. (2010) Lack of glucose recycling between endoplasmic reticulum and cytoplasm underlies cellular dysfunction in glucose-6-phosphatase-beta-deficient neutrophils in a congenital neutropenia syndrome. Blood 116:2785-2792.
12. Marcolongo P, et al. (2013) Multiple roles of glucose-6-phosphatases in reticuloendothelial: State of the art and future trends. Biochim Biophys Acta 1830:2608-2618.
13. Linster CL, Van Schaftingen E, Hanson AD (2013) Metabolite damage and its repair or pre-emption. Nat Chem Biol 9:72-80.
14. Van Schaftingen E, et al. (2013) Metabolite proofreading, a neglected aspect of intermediary metabolism. J Inherit Metab Dis 36:427-434.
15. Linster CL, et al. (2011) Ethylmalonyl-CoA decarboxylase, a new enzyme involved in metabolite proofreading. J Biol Chem 286:4296-43003.
16. Adler LN, Gomez TA, Clarke SG, Linster CL (2011) A novel GDP-D-glucose phosphorylase involved in quality control of the nucleoside diphosphate sugar pool in Caenorhabditis elegans and mammals. J Biol Chem 286:21511-21523.
17. Collard F, et al. (2016) A conserved phosphatase destroys toxic glycolytic side products in mammals and yeast. Nat Chem Biol 12:601-607.
18. Peracchi A, et al. (2017) Nf1t is a metabolite repair enzyme that hydrolyzes de-aminated glutathione. Proc Natl Acad Sci USA 114:E3223-E3242.
19. Veiga-da-Cunha M, Chevalier N, Stroobant V, Vertommen D, Van Schaftingen E (2014) Metabolite proofreading in carnosine and homocarnosine: Isolation of the enzyme involved of PM20D2 as α-l-lysine lydeptidase. J Biol Chem 289:19726-19736.
20. Rzemi R, et al. (2015) A mouse model of L-2-hydroxyglutaric aciduria: a disorder of metabolite repair. PLoS One 10:e0119540.
21. Rzemi R, Vincent MF, Van Schaftingen E, Veiga-da-Cunha M (2007) L-2-hydroxyglutaric aciduria, a defect of metabolite repair. J Inherit Metab Dis 30:681-689.
22. Kremer LS, et al. (2016) NAXE mutations disrupt the cellular NAD(P)HX repair system and cause a lethal neutrophilic neutropathy of early childhood. Am J Hum Genet 99:894-902.
23. Van Schaftingen E, Gerin I (2002) The glucose-6-phosphatase system. Biochem J 362:513-532.
24. Hemrika W, Wever R (1997) A new model for the membrane topology of glucose-6-phosphate: The enzyme involved in von Gierke disease. FEBS Lett 409:317-319.
25. Arion WJ, et al. (1998) Chlorogenic acid analogue S 3483: A potent competitive inhibitor of the hepatic and renal glucose-6-phosphatase systems. Arch Biochem Biophys 351:279-285.
26. Sakuma M, Kametani S, Akanuma H (1998) Purification and some properties of a hepatic NADPH-dependent reductase that specifically acts on 1,5-anhydro-D-fructose. J Biochem 123:189-193.