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

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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-K\textsubscript{M} 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-K\textsubscript{M} hexokinases. Granulocytes from patients deficient in G6PC3 or G6PT accumulate 1,5AG6P to concentrations (∼3 mM) that strongly inhibit hexokinase 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.

Neutrophils play an important role in our defense against infections, and neutropenias represent major health threats. Among the genetic causes of neutropenia, two are due to mutations inactivating G6PC3 (1, 2) and G6PT/SLC37A4 (3), proteins thought to be involved in glucose-6-P metabolism. G6PC3 is homologous to G6PC1, the well-known glucose-6-phosphatase that produces glucose from glucose-6-P in the liver and the kidneys and plays an essential role in glucose homeostasis. Like G6PC1, G6PC3 is an integral membrane protein of the endoplasmic reticulum whose catalytic site faces the lumen of this organelle. Unlike G6PC1, which has a narrow tissue distribution limited to the glucose-producing organs, G6PC3 is expressed in virtually all tissues. It is therefore sometimes referred to as “ubiquitous glucose-6-phosphatase,” although its glucose-6-phosphatase activity, if detectable, is low (4–7). G6PC3 deficiency causes a syndrome (severe congenital neutropenia type 4) associating neutropenia and neutrophil dysfunction with malformations, but has no impact on glucose homeostasis. In contrast, G6PC1 deficiency leads to a dramatic impairment of glucose metabolism known as glycogen storage disease type Ia, but not to problems with neutrophils (8).

Neutropenia may also be caused by a deficiency in G6PT. This ubiquitously expressed protein is encoded by the SLC37A4 gene and transports glucose-6-P from the cytosol to the lumen of the endoplasmic reticulum, where it can be hydrolyzed by G6PC1 (9) and possibly G6PC3. Deficiency in G6PT leads to glycogen storage disease type Ib (GSDIb), which associates all of the metabolic symptoms of G6PC1 deficiency with neutropenia and neutrophil dysfunction. While the origin of the metabolic symptoms is easily understandable, the cause of the decrease in neutrophil numbers and of their impaired function remains a puzzle.

**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.


The authors declare no conflict of interest.

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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

Fig. 1. Substrate specificity of G6PC1, G6PC3, and the glucose-6-phosphate transporter. (A) Wild-type (WT) and catalytically inactive (H176A or H167A) human G6PC1 and G6PC3 were produced as recombinant proteins with a C-terminal 6×His tag in HEK293T cells. Equal amounts of the membrane fraction were analyzed by Western blotting with anti-6×His antibody. (B) The membrane preparations were used at appropriate concentrations to assay the phosphatase activity on the indicated substrates (all tested at 100 μM) through the release of inorganic phosphate. Phosphatase activity observed in preparations from cells expressing the catalytically dead mutants was assessed under identical conditions and subtracted to account for baseline phosphatase activity. (C) Ribose-5-P and glucose-6-P phosphatase activities were assayed with a radiochemical assay (10 μM) in rat microsomes from the indicated tissues. (D) Ratios of the ribose-5-P and glucose-6-P phosphatase activities measured in C are compared with those measured with membrane preparations from HEK293T cells overexpressing recombinant G6PC3 or G6PC1. (E) Hydrolysis of the best substrates (100 μM) for G6PC3 was assayed in rat skeletal muscle microsomes in the presence or absence of the G6PT inhibitor S3483 (100 μM) and of 7.5 mM octyl glucoside (OG). For B and C, data are means and error bars are ±SEM (n = 3). For E, data are means and error bars are ±SD (n ≥ 4). P values (ns, not significant, P > 0.05; *P ≤ 0.05; $P ≤ 0.0001) were determined with unpaired t tests using the two-stage linear step-up procedure of Benjamini et al. (51), with Q = 1%.
products (15–19) and in some cases cause metabolic diseases, like 1,2-hydroxyglutaric aciduria (20, 21) and NAD(P)H dehydrogenase 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 6×His tag. To ensure that the observed phosphatase activities were genuinely contributed by G6PC1 or G6PC3, we subtracted basal 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, while the 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, while on the contrary it was 13- and 4.6-fold higher for G6PC3 than for G6PC1 in the case of ribose-5-P and 1,5AG6P, respectively. Comparable results were obtained with proteins lacking or carrying an N-terminal 6×His tag (SI Appendix, Fig. S1), ensuring that the presence of a tag and its location do not affect the kinetic properties of G6PC3 and G6PC1. Taken together, our data confirmed the preference of G6PC1 for glucose 6-P, and revealed that G6PC3 is much more efficient on other substrates such as ribose 5-P and 1,5AG6P.

To confirm that the specificity observed for the human recombinant proteins corresponded to that of the endogenous G6PC1 and G6PC3 found in tissues, we prepared microsomes from rat tissues that express G6PC3 but not G6PC1 (skeletal muscle, heart, and spleen) or mainly G6PC1 (liver). In these preparations, we then assayed the capacity to hydrolyze radio-labeled glucose-6-P and ribose-5-P (Fig. 1C) as surrogate measures for G6PC1 and G6PC3 activity, respectively. These assays were performed in the presence of a mild detergent to prevent any limitation in the entry of substrate into the microsomes. The ratio of the phosphatase activity on ribose-5-P and glucose-6-P was between 2.5 and 4 in the case of the microsomes prepared from skeletal muscle, heart, and spleen but only 0.05 in the case of the liver microsomes (Fig. 1D). These values were in excellent agreement with those observed for human recombinant G6PC3 (4.6) and G6PC1 (0.02) tested under the same conditions. These results indicated that the physiological substrate of G6PC3, unlike G6PC1, is not glucose-6-phosphate but rather one of the good substrates that we found.

Neutropenia is found in patients with G6PC3 or G6PT deficiency. In liver and kidney, G6PT transports glucose-6-P into the endoplasmic reticulum (ER), where it is dephosphorylated by G6PC1 (23). We therefore reasoned that in other tissues, the role of G6PT could be to transport a phosphoric ester, a substrate of G6PC3, into the ER. Thus, deficiency of either of these proteins would lead to the accumulation of this substrate, which might be toxic to neutrophils and explain neutropenia.

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). This indicated that G6PT transports glucose-6-P and its close structural analog 1,5AG6P but not ribose-5-P, ribitol-5-P, and inorganic pyrophosphate. Of note, treatment of microsomes with the detergent octyl glucoside (7.5 mM) led to a significant increase in the phosphatase activity toward ribose-5-P, ribitol-5-P, inorganic pyrophosphate, and, to a lesser degree, glucose-6-P, suggesting 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.

**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 demonstrated 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 HK1 (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. S3H–J and N) while the concentration of the Krebs cycle intermediates malate (Fig. 2F), α-ketoglutarate, and succinate (SI Appendix, Fig. S3O 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. 2I), 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. S3A and C) and smaller effects on glucose consumption (Fig. 2B and D and SI Appendix, Fig. S3B 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-Anhydrogulitol 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 promptly caused 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
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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_M$, $\mu$M</th>
<th>$V_{\text{max}}$, mol min$^{-1}$ mg$^{-1}$</th>
<th>$K_{\text{cat}}/K_M$, s$^{-1}$</th>
<th>$K_{\text{sp}}$, mM</th>
<th>$V_{\text{max}}$, mol min$^{-1}$ mg$^{-1}$</th>
<th>$K_{\text{cat}}/K_M$, s$^{-1}$</th>
<th>Ratio $K_{\text{cat}}/K_M$: Glucose vs. 1,5AG</th>
<th>Inhibitor: 1,5AG6P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta 21$-hHK1$^{\text{b,c}}$</td>
<td>50 ± 2</td>
<td>40 ± 0.5</td>
<td>1.36</td>
<td>57.9 ± 2.9</td>
<td>0.89 ± 0.03</td>
<td>2.6 × 10$^{-5}$</td>
<td>52,308</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>$\Delta 28$-hHK2$^{\text{b,c}}$</td>
<td>250 ± 9</td>
<td>35.4 ± 0.4</td>
<td>0.25</td>
<td>126 ± 52</td>
<td>0.19 ± 0.06</td>
<td>0.26 × 10$^{-5}$</td>
<td>96,153</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>hHK3$^{\text{a}}$</td>
<td>12.2 ± 1</td>
<td>10 ± 0.2</td>
<td>1.35</td>
<td>22.1 ± 3.9</td>
<td>1.2 ± 0.1</td>
<td>5.0 × 10$^{-5}$</td>
<td>27,000</td>
<td>134 ± 9</td>
</tr>
<tr>
<td>$\Delta 50$-hADPGK$^{\text{c,d}}$</td>
<td>12.3 ± 1.3</td>
<td>5.2 ± 0.2</td>
<td>0.41</td>
<td>306 ± 36</td>
<td>7.0 ± 0.3</td>
<td>2.2 × 10$^{-2}$</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

Data indicate means ± SEM (n ≥ 3).

* $K_i$: noncompetitive inhibition versus glucose.

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

$^c$ 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).

$^d$ 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). Furthermore, the concentration of 1,5AG6P was always higher in PMN cells compared with PBMCs in the same patient (4.3- to 13-fold higher depending on the sample) (Fig. 5A). Taken together, these results support the idea that G6PT and G6PC3 are involved in the breakdown of 1,5AG6P in human neutrophils and that, in their absence, 1,5AG6P reaches 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 glycosidases (36). 1,5AG is present in most foods at concentrations ranging from 0.3 to 3 μg·g$^{-1}$ (35). The calculated daily intake in humans is...
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).

**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 involving 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 accumulates in G6PC3- or G6PT-deficient cells incubated with 1,5AG or 1,5-anhydrofructose, a compound that is readily converted 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
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 neutrophils. In vitro, the human low-K_m hexokinases phosphorylated 1,5AG with a much lower catalytic efficiency than ADPGK (Table 1), which suggested that the latter might be the main enzyme involved in 1,5AG phosphorylation. However, the relative contribution of these kinases to 1,5AG6P formation in cells also depends on their relative abundance, which is not known, and on the presence of competing substrates (mainly glucose) and inhibitors (glucose-6-P). It is therefore not possible at present to know which kinase contributes the most to the production of 1,5AG6P in vivo.

Low-K_m hexokinases are physiologically inhibited by their product glucose-6-P. Previous studies performed with glucose-6-P analogs on brain hexokinase (hexokinase 1) (27) showed that 1,5AG6P is also a good inhibitor, while mannose-6-P and 2-deoxyglucose-6-P are not. This points to the importance of a hydroxyl group in the appropriate orientation on carbon 2, as present in glucose-6-P and 1,5AG6P, for the inhibitory effect to take place. Here, we showed with human recombinant enzymes that the inhibition by 1,5AG6P is also found with hexokinases 2 and 3. As hexokinase 3 is the main glucose-phosphorylating enzyme in neutrophils (43) and in agreement with its K_m for 1,5AG6P (150 μM), the accumulation of 1,5AG6P that is seen in G6PC3 or G6PT-deficient neutrophils is expected to inhibit glucose phosphorylation. From the data obtained with neutrophils, we estimate that the intracellular concentration reaches ~3 mM in a mouse ER-Hox8b G6PC3-deficient neutrophil progenitor cell 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 GSD1b patients (Fig. S4). Taking into account that this inhibition is competitive with respect to ATP (44), we calculate that this concentration is sufficient 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 GSD1b 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 unaffected (45). These observations might seem at odds with the previous claim that G6PC3-deficient neutrophils show a decreased uptake of glucose (11). However, in the latter study, 2-deoxyglucose was used to assess glucose uptake, despite the fact that it measures inhibition of glucose phosphorylation rather than of glucose entry.

**Fig. 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 display any marker of mitochondrial enzymatic activity and do not synthesize much ATP (31, 32). They therefore mainly rely on glycolysis for their metabolism and energy supply (31). A reduction in glucose phosphorylation, due to a defect in the dephosphorylation 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 patients’ 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 granulocytes and monocytes of G6PC3-deficient mice, as these cells also show a reduction in glucose uptake and lower levels of glucose-6-P, 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 elicited by the addition of 1,5AG to cultures of neutrophil precursors and that the administration of 1,5AG to mice further decreases their neutrophil counts, while reducing the concentration 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
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 culturing, 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 and 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.

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