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Multitranscript analysis reveals an effect of 2-deoxy-D-glucose on gene expression linked to unfolded protein response and integrated stress response in primary human monocytes and monocyte-derived macrophages

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ABSTRACT

Background: Glycolytic inhibitor 2-deoxy-D-glucose (2-DG) binds to hexokinase in a non-competitive manner and phosphoglucose isomerase in a competitive manner, blocking the initial steps of the glycolytic pathway. Although 2-DG stimulates endoplasmic reticulum (ER) stress, activating the unfolded protein response to restore protein homeostasis, it is unclear which ER stress-related genes are modulated in response to 2-DG treatment in human primary cells. Here, we aimed to determine whether the treatment of monocytes and monocyte-derived macrophages (MDMs) with 2-DG leads to a transcriptional profile specific to ER stress.

Methods: We performed bioinformatics analysis to identify differentially expressed genes (DEGs) in previously reported RNA-seq datasets of 2-DG treated cells. RT-qPCR was performed to verify the sequencing data on cultured MDMs.

Results: A total of 95 common DEGs were found by transcriptional analysis of monocytes and MDMs treated with 2-DG. Among these, 74 were up-regulated and 21 were down-regulated. Multitranscript analysis showed that DEGs are linked to integrated stress response (GRP78/BiP, PERK, ATF4, CHOP, GADD34, IRE1α, XBP1, SESN2, ASNS, PHGDH), hexosamine biosynthetic pathway (GFAT1, GNA1, PGM3, UAP1), and mannose metabolism (GMPPA and GMPPB).

Conclusions: Results reveal that 2-DG triggers a gene expression program that might be involved in restoring protein homeostasis in primary cells.

General significance: 2-DG is known to inhibit glycolysis and induce ER stress; however, its effect on gene expression in primary cells is not well understood. This work shows that 2-DG is a stress inducer shifting the metabolic state of monocytes and macrophages.

1. Introduction

The endoplasmic reticulum (ER) is a membranous organelle responsible for one-third of the human proteome's folding, post-translational modifications, and trafficking [1]. The ER retained misfolded/unfolded proteins, and perturbations in post-translational modifications of proteins result in accumulation of misfolded proteins that cause ER stress [2]. The misfolding proteins are susceptible to proteasomal degradation through the ER-associated degradation (ERAD) system, which recognizes, ubiquitinates, and relocates proteins to the cytosol for degradation [3]. Specific signaling between ER and the nucleus, known as the Unfolded Protein Response (UPR), is activated upon detection of ER stress [4,5]. Three UPR sensors induce ER stress, protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1α). Under unstressed conditions, UPR sensors interact with glucose-regulated protein 78/binding immunoglobulin protein (GRP78/BiP) to keep receptors inactive on the ER membrane and inhibit downstream signaling events [6]. When GRP78/BiP interacts with misfolded proteins during ER stress, its inhibitory effects on UPR sensors are released. GRP78/BiP is generally accepted as a canonical ER stress marker [7].

The accumulation of unfolded proteins activates the Integrated Stress Response (ISR), an early protective response against stress, through the PERK sensor. The ISR activation leads to the

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phosphorylation of α subunit of eukaryotic translation initiation factor 2 (eIF2α) on serine 51 [8–11]. eIF2α phosphorylation leads to global attenuation of Cap-dependent protein synthesis while allowing preferential translation of selected transcripts, such as the basic leucine zipper transcription factor 4 (ATF4), linked to cell survival and recovery [12]. ATF4 promotes the transcription of CCAAT/enhancer-binding protein (C/EBP) homologous protein/DNA damage-inducible transcript 3 protein (CHOP/DDIT3), and growth arrest and DNA damage-inducible 34 (GADD34) [13]. Subsequently, GADD34 targets a stress-inducible regulatory subunit of protein phosphatase 1 (PP1) to dephosphorylate eIF2α restoring Cap-dependent translation [13,14].

The glucose analog, 2-deoxy-D-glucose (2-DG), is an effective inhibitor of the initial steps of glycolysis carried out by hexokinase (HK) and phosphoglucose isomerase (PGI) [15]. Glucose deprivation has been shown to cause ER stress and eIF2α phosphorylation [16]. Similarly, blocking glycolysis with 2-DG also leads to ER stress and UPR activation in cells [17]. Metabolic reprogramming using 2-DG has been shown to inhibit cancer cell growth in hypoxic conditions [18] and block of aerobic glycolysis [19]. However, under aerobic conditions, 2-DG does not mediate adenosine triphosphate (ATP) depletion; instead, it induces autophagy via ER stress [20]. Structurally, 2-DG is also an analog of D-mannose and, therefore, alters the oligosaccharide biosynthesis leading to abnormal N-linked glycosylation competing with D-mannose [21–23]. Thus, 2-DG not only blocks glycolysis, thereby reducing cellular ATP or increasing autophagy, but also interferes with N-linked glycosylation, which leads to ER stress.

Although 2-DG is known to interfere with N-linked glycosylation, resulting in ER stress, these studies were established mainly in cell lines [24]. However, little is known about the gene expression program linked to ER stress, including genes encoding by UPR and ISR, in 2-DG-treated human primary immune cells at transcriptome level. The overall aim of this study was to assess the effects of 2-DG on ER stress/metabolic reprogramming using available RNA-seq data from primary human cells, including non-adherent monocytes (n-Mon), adherent monocytes (a-Mon), and monocyte-derived macrophages (MDMs). We identify key marker genes associated with ER stress, ISR, and metabolic reprogramming, which should help understand the response of primary cells treated with stress inducers. We confirm the findings in cultured MDMs by RT-qPCR. We proposed a model for how 2-DG might disrupt N-glycosylation. Although 2-DG appeared to be a promising candidate to understand the link between metabolic reprogramming and the stress response, the crosstalk between these processes is not fully understood.

2. Material and methods

2.1. Ethical statement

The protocols for individual enrollment and sample collection were approved by the Bioethics Research Committee of Sede de Investigación Universitaria - Universidad de Antioquia's (Medellín, Colombia). Prior to inclusion in the study, all participants provided a signed informed consent form in accordance with the principles expressed in the Declaration of Helsinki. The study involved the participation of 3 healthy donors from Medellín, Colombia.

2.2. Transcriptomic analysis of previously published RNA-Seq studies

To explore the gene expression patterns in human primary cells treated with 2-DG, we reanalyzed RNA-seq data downloaded from the gene expression omnibus (GEO) database. Essential information about the included datasets is shown in Table S1. The first dataset, GSE161839, was performed in non-adherent and adherent monocytes (n-Mon and a-Mon, respectively; number of donors (n) = 5). We selected samples that were untreated (in glucose medium) and treated with 2-DG (2.0 mM) for 24 h [25]. This was because we did not find significant differences in ER-stress gene expression between cells cultured in glucose-deprivation medium and those cultured in glucose medium. The second dataset, GSE74508, was performed in MDMs ($n = 3$) untreated or treated with 2-DG (20 mM) for 4 h [26].

2.3. Data annotation and batch effect correction

The raw counts of GSE161839 and GSE74508 were subject to the following workflow, executed on R software (version 4.2.0) [27]. Initially, we annotated gene rows with its respective genotype (non-coding gene, pseudogene, and protein-coding gene), symbols, and entrezID. Based on the genotype, we selected only those genes that are protein-coding and then generate a list with its lengths using the Homo. sapiens library [28]. Batch effect correction on raw count data for each transcriptome was performed using ComBat seq [29].

2.4. Analysis of differentially expressed genes (DEGs)

To determine the top DEGs, we selected genes with a false discovery rate (FDR) < 0.05 and $|$Log2 Fold Change (FC) (treated/untreated) > 0.6, using DESeq2 library [30]. The multidimensional scaling (MDS) analysis plot was performed using Glimma [31]. Common differentially expressed genes between the transcriptomes were identified using Venny (version 2.1) [32], drawing a Venn diagram.

2.5. Gene set enrichment analysis and transcription factor prediction

Common genes were uploaded to Cytoscape (version 3.9.1) [33]. ClueGO plugin (version 2.5.9) [34] carries out enrichment analysis with default parameters, GO term fusion, and with $p \leq 0.05$. Subsequently, iRegulon plugin (version 1.3) [35] was used to analyze transcription factors (TFs) that potentially regulate common DEGs (regulons); genomic regions for TF-binding motif search were limited to 500 upstream and 10 kb around the respective transcriptional start sites. At higher the scores, the more reliable the results. TF and target gene pairs with normalized enrichment scores (NES) >3 were selected.

2.6. Multitranscript analysis

The raw counts' data were normalized to transcript per million (TPM) using R statistical software. We selected the genes linked to ER stress, ISR, HBP, and Man-M with the entrezID using Kyoto Encyclopedia of Genes and Genomes (KEGG) and plotted the data using bar plots with GraphPad Prism for Windows (GraphPad Software version 8.0.1, San Diego, CA, USA; www.graphpad.com).

2.7. Culture of primary human monocytes and differentiation into monocyte-derived macrophages

Human peripheral blood mononuclear cells (PBMCs) from leukocyte-enriched blood units from healthy donors were isolated through density gradient with Lymphoprep (STEMCELL Technologies Inc., Vancouver, Canada), as previously described [36]. Platelet depletion was performed by washing with PBS 1× (Sigma-Aldrich) three times at 250 x g for 10 min and using flow cytometry the percentage of CD14 positive cells was determined. To obtain primary monocytes, 24-well plastic plates were scratched with a 1000 μL pipette tip, and 5×10^5 CD14 positive cells per well were seeded to allow for adherence during 2 h in RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.5% plasma, 4 mM L-glutamine, and 0.3% NaCO3 and cultured at 37 °C and 5% CO_2. Non-adherent cells were removed by washing twice with PBS 1×, and monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 0.3% NaCO3, and 1% antibiotic-antimycotic solution 100× (complete medium) and incubated at 37 °C and 5% CO2 for 6 days to obtain MDMs, as previously described [37].

2.8. Treatment of monocyte-derived macrophages with 2-deoxy-D-glucose

2-DG (Sigma-Aldrich), at a concentration of 100 mM was prepared in the same medium used to maintain MDMs, i.e., RPMI-1640 supplemented with 10% FBS, 4 mM L-glutamine, 0.3% NaCO3, and 1% antibiotic-antimycotic solution 100×. The MDMs were treated with increasing concentrations of 2-DG (1, 2, 4, 8, 16, 20, 40, 80, 100 nM) and cultured at 37 °C with 5% CO_2 for 24 h. After incubation, cell viability was determined by flow cytometry using the LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (Invitrogen, Molecular probes, USA), following the manufacturer's instructions. The flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Life Sciences). mRNA expression of ER stress biomarkers was measured by quantitative real-time PCR (RT-qPCR) at 4 h post-treatment.

2.9. RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions, and the concentration was determined using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). To synthesize complementary DNA (cDNA) from RNA, the commercial iScript™ cDNA Synthesis Kit (Bio-Rad, USA) was used, following the manufacturer's instructions. RT-qPCR amplifications were conducted using the SsoAdvanced™ Universal SYBR® Green Supermax, (Bio-Rad, USA), where the gene-specific primer pairs were used (Table S2). The Bio-Rad CFX manager was used to obtain the cycle thresholds (Ct), which were determined for each sample using a regression fit in the linear phase of the PCR amplification curve. The relative expressions of genes were determined using the 2(−delta delta Ct) method, taking GAPDH as a reference gene.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism for Windows (GraphPad Software version 8.0.1, San Diego, CA, USA; www.graphpad.com). The normality of data was evaluated using Shapiro-Wilks test. The data are represented as mean ± SEM. The statistical tests are indicated in the figure legends. The threshold for statistical significance in multiple *t*-tests for the multitranscript analysis was set at $p < 0.05$ (*). In the validation experiments, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant for unpaired t-test.

3. Results

3.1. 2-DG treatment induces transcription of ER stress genes: IRS-related protein-coding genes in human primary cells

Principal component analysis (PCA) showed a good dispersion between the groups, i.e., n-Mon, a-Mon, MDMs, and their controls (Fig. S1). The transcriptional profiles of human primary cells (n-Mon, a-Mon, and MDMs) treated with 2-DG were examined to establish the relationship between 2-DG treatment and the expression of genes implicated in ER stress. A total of 4833, 4667, and 251 DEGs were identified in n-Mon, a-Mon, and MDMs, respectively. Next, we focused on common genes using a Venn diagram. As shown in Fig. 1A, out of 95 common genes that were differentially expressed, 74 were significantly up-regulated, and 21 significantly down-regulated, in human primary cells after 2-DG treatment. The plugin ClueGO was used to assess the enrichment of these genes in functionally grouped biological processes. Most of the commonly modulated genes in 2-DG-treated n-Mon, a-Mon, and MDMs align with regulation of ER stress (39.6%), ISR pathway (18.8%), response to acid chemical (8.4%), glutamine metabolism (8.4%), protein targeting to ER (6.2%), and ATP-dependent protein folding (6.2%), among others (Fig. 1B). To note, the results indicate that 2-DG treatment induces genes primarily associated with ER stress, ISR and metabolic reprogramming.

Next, we generated a network of TFs and their target genes using iRegulon to determine the relationship between ER stress genes that are highly enriched in response to 2-DG. As shown in Fig. 2A, among the three main TFs of the UPR pathway -ATF6 (which also acts as stress sensor), ATF4, and XBP1, that are deliberately activated to alleviate ER stress and maintain proteostasis [38], were shown to be enriched for regulons in 2-DG-treated n-Mon, a-Mon, and MDMs, demonstrating that common transcripts expressed in human primary cells are connected to ER stress and ISR (Fig. 2A). Although ATF6 activity is required for ER stress induction, this TF was not predicted in the regulons of 2-DG-treated cells.

3.2. 2-DG treatment induces the expression of ER stress biomarkers in human primary cells

As the results show, 2-DG treatment enriches transcripts linked with ER stress (Figs. 1 and 2), and it had been suggested that n-Mon, a-Mon, and MDMs treated with 2-DG cause ER stress due to the synthesis of truncated N-glycans that limit glycoprotein production (Fig. 3A) [21–23]. To test the hypothesis, we used a multitranscript approach to identify genes involved in ER stress and N-glycan production.

First, we examined the transcript levels of ER-membrane-resident heat shock protein 40 (Hsp40) family genes such as DNAJB9 and DNAJB11, both of which are involved in UPR. We found that its mRNA expression was significantly increased in 2-DG-treated cells as compared to untreated cells (Fig. 3B). To further establish a potential relationship between ER stress and 2-DG treatment, we determined the transcript levels of ER stress markers. Results showed significant increase in mRNA expression of GRP78/BiP and glucose-related protein 94 (GRP94) (Fig. 3B), as well as mRNA of ISR components, including PERK, ATF4, CHOP/DDIT3, and GADD34 (Fig. 3C). Additionally, mRNA expression of protein-coding genes involved in the ERAD system, such as IRE1α, X-box binding protein 1 (XBP1), homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) and hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (HRD1) were modulated (Fig. 3D).

Next, we asked whether 2-DG treatment increases the transcript levels of ISR target genes. The mRNA expression of asparagine synthetase (ASNS), phosphoglycerate dehydrogenase (PHGDH), sestrin 2 (SESN2), and vascular endothelial growth factor A (VEGFA) were increased in n-Mon, a-Mon, and MDMs cells treated with 2-DG (Fig. 3E). Thus, 2-DG promotes alternative biological pathways associated with amino acid metabolism, autophagy, and angiogenesis to alleviate ER stress.

To confirm the multitranscript results, we carried out an RT-qPCR analysis on MDMs. We chose a sample of DEGs using gene-specific primers (Table S2). First, we examined if the treatment of MDMs with increasing concentrations of 2-DG influences cell viability. As observed in Fig. S2, the viability of MDMs was not significantly affected after treatment with 1, 2, 4, 8, 16, and 20 mM of 2-DG compared to control. However, the viability was significantly decreased with 40, 80, and 100 mM of 2-DG. To note, the concentration at which the transcriptome of MDMs was performed with 20 mM of 2-DG had no significant influence on the viability of MDMs, compared to control (Fig. 3F).

For the validation of the gene expression levels obtained by RNA-seq, we reproduce the experimental conditions reported in [26] (GSE74508), wherein MDMs were treated with 2-DG for 4 h (Table S1). We carried out validation by RT-qPCR for a selected group of genes. As shown in Fig. 3G, 2-DG-treated MDMs showed significantly higher levels of mRNA expression for GRP78/BiP, PERK, ATF4, CHOP, GADD34, total XBP1 (tXBP1), spliced XBP1 (sXBP1), and HERPUD1 as compared to untreated MDMs. These findings indicate that the gene expression data obtained by RNA-seq (multitranscript) are consistent with the expression data determined by real-time PCR, indicating upregulation of genes involved in response to ER stress and ISR in MDMs treated with 2-DG.

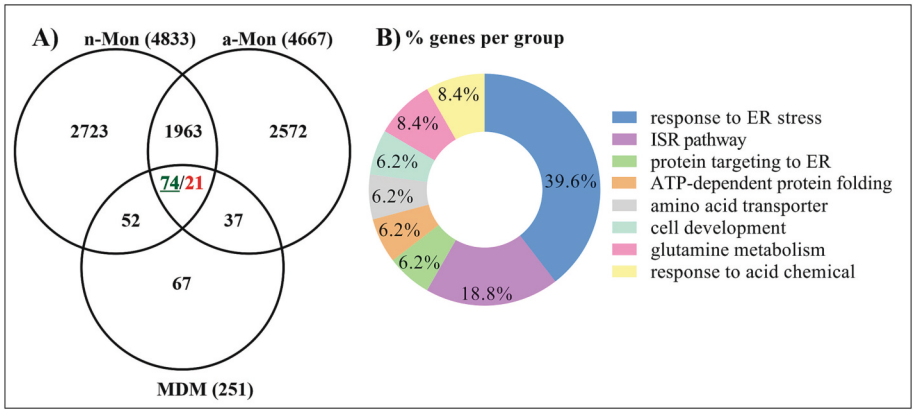

Fig. 1. 2-DG treatment promotes the expression of UPR and ISR genes. Common genes in 2-DG treated human primary cells (A). Pie chart of the ontology processes associated with the 95 common genes (B).

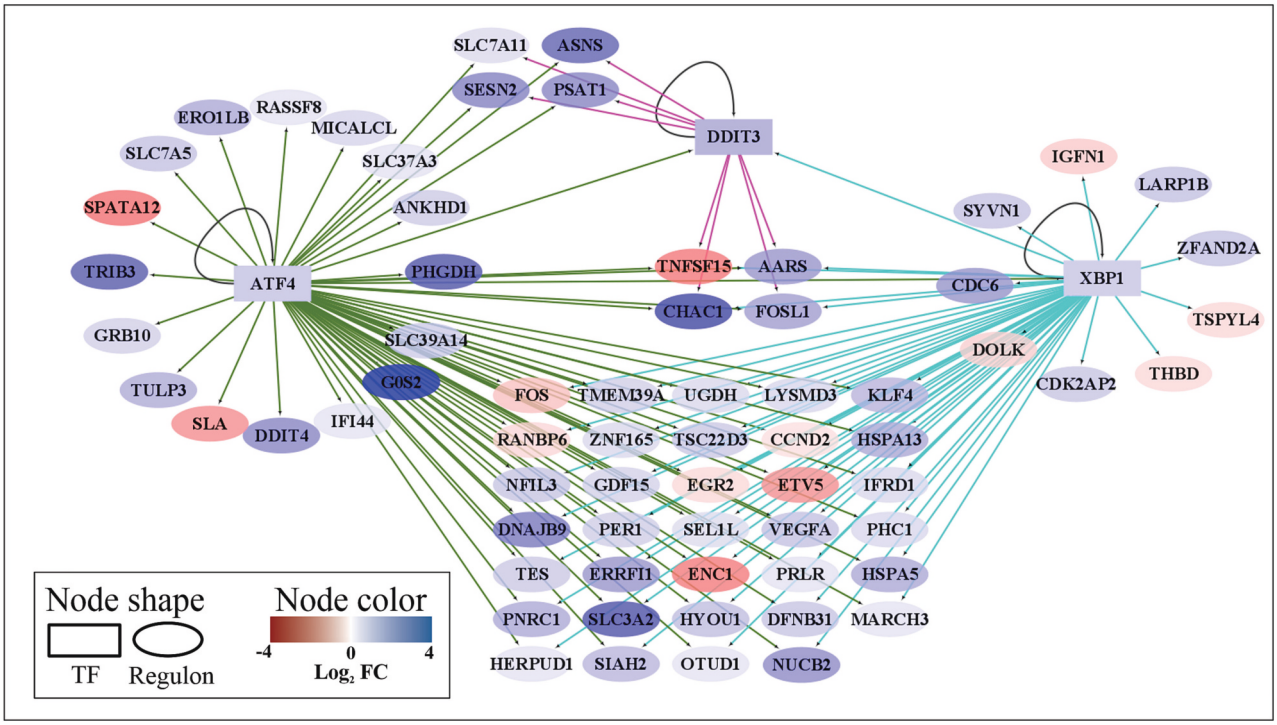

Fig. 2. 2-DG treatment induces the gene expression of ISR-associated genes. Predicted transcription factors in 2-DG treated primary human cells. The motif/track was obtained from iRegulon. Primary transcription factors are shown in the form of a rectangle and their regulons in the form of an ellipse. Node colors represent the mean of $\log_2$ fold change between cells.

3.3. 2-DG treatment induces the gene expression linked to hexosamine biosynthetic and mannose metabolism pathways

Glycolysis consists of two main phases: the preparatory and the payoff phase. To investigate whether 2-DG has a role in the glycolytic preparatory phase, we focused on the multitranscript analysis to determine whether 2-DG treatment increases the expression of protein-coding genes involved in the hexosamine biosynthetic pathway (HBP) and mannose metabolism (Man-M).

The HBP regulates metabolic events to link nutrient availability to numerous signaling networks. Through a series of enzymatic steps, it leads to the formation of uridine diphosphate (UDP)-N-Acetyl glucosamine (UDP-GlcNAc), which is used for the synthesis of N-glycans (Fig. 4A). Lowering glucose concentration in cell culture media decreases cellular UDP-GlcNAc levels [39] and the UDP-GlcNAc shortage results in altered N-linked glycosylation [40]. Here, we aimed to investigate whether 2-DG treatment induces N-glycans biosynthesis in a way dependent on gene expression linked to the HBP pathway. The treatment of n-Mon, a-Mon and MDMs with 2-DG promotes the mRNA expression of glutamine-fructose-6-phosphate transaminase 1 (GFAT1), glucosamine-phosphate N-Acetyltransferase 1 (GNA1), phosphoglucomutase 3 (PGM3), and UDP-*N*-acetylglucosamine pyrophosphorylase 1 (UAP1), except GNA1, which was significantly decreased n-Mon treated with 2-DG, as compared to the control (Fig. 4B).

The Man-M is an ample supply of guanosine diphosphate (GDP)-Man required for N-glycans synthesis. In this pathway, the ratio of mannose-6-phosphate isomerase (MPI) and phosphomannomutase 2 (PMM2) determines the source of mannose (glucose-derived or extracellular mannose), because both compete for Man-6-Phosphate [41]. Lowering glucose concentration in cell culture media increases the use of exogenous mannose [42]. We investigated whether 2-DG treatment, as a D-mannose analog, increases the expression of key enzyme-coding genes

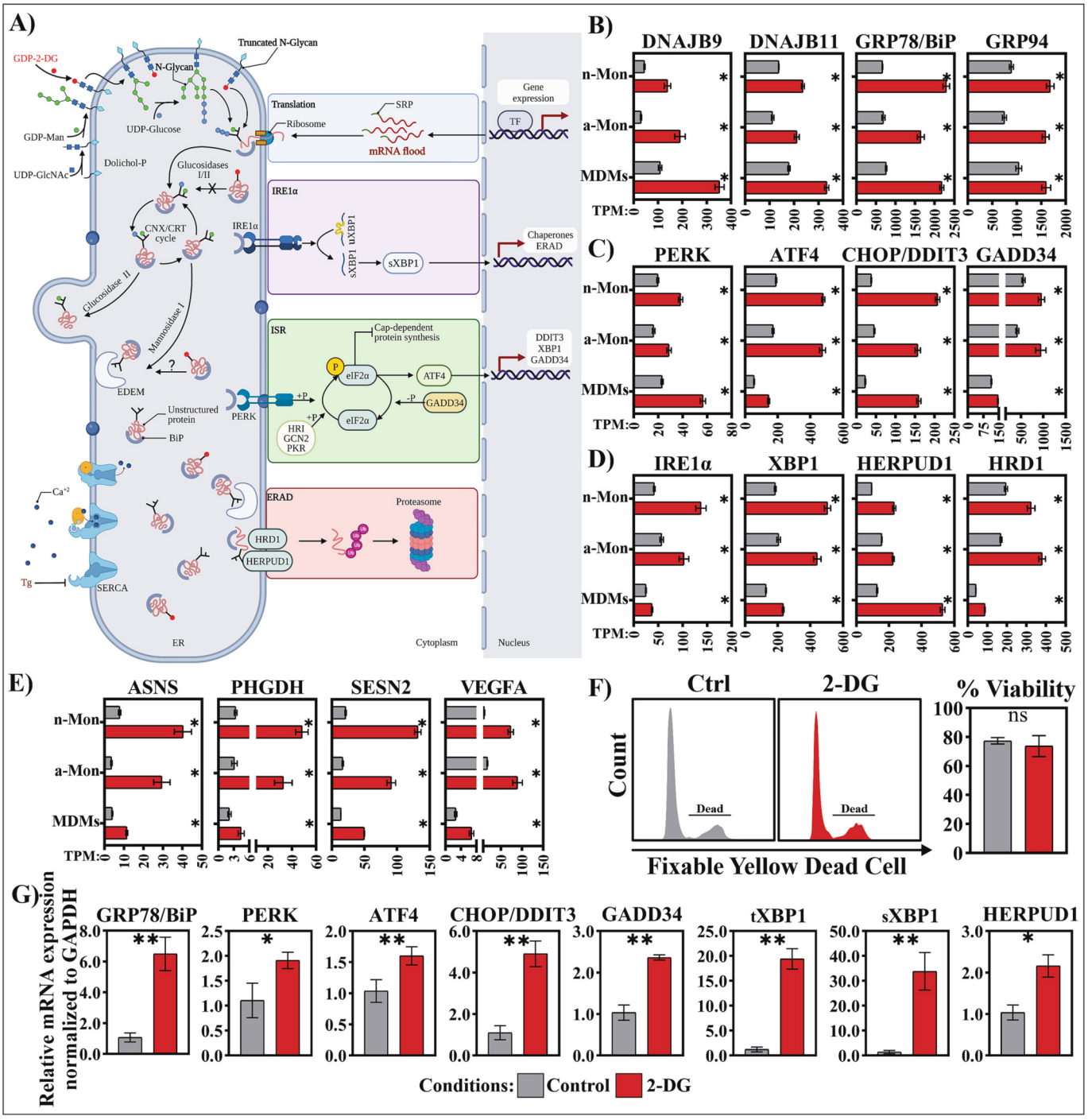

Fig. 3. 2-DG treatment promotes the gene expression of the UPR and ISR pathways. Gene expression increases the mRNA that is co-translationally translocated to the ER lumen. In the lumen, the N-glycan ($Glc_3Man_9GlcNAc_2$) is added to the protein, and the glucosidases I/II produces the N-glycan ($GlcMan_9GlcNAc_2$), which is recognized by the CNX/CRT cycle. If the glycoprotein has the proper structure, it is transported outside of the ER to Golgi. If the glycoprotein did not acquire the proper structure, the ERAD pathway is activated. 2-DG truncates the formation of the N-glycans, leading to altered N-glycosylation that prevents the proper folding of glycoproteins. The recognition of the truncated N-glycan as an error in the ER is still unknown but may involve the same components of the ERAD system. Created with BioRender.com (A). Protein-coding genes markers of ER stress in Mon and MDMs are presented as transcript per million (TPM) (B, C, D and E). Viability of 2-DG-treated MDMs for 24 h is shown. The left image shows the flow cytometry representative of one donor. The right image shows the mean percentage of viability representative of tree donors. $n = 3$ (F). Validation experiments were performed by RT-qPCR of main genes involved in UPR and ISR activation in MDMs treated with 2-DG for 4 h. n = 3 (G). Multiple *t*-tests and unpaired t-tests were performed. Significant codes: '***' 0.001; '**' 0.01; '*' 0.05; 'ns' no significance.

implicated in the Man-M pathway in human primary cells. To note, MPI mRNA expression was significantly decreased in n-Mon and a-Mon treated with 2-DG, while its expression was not altered in MDMs, as compared to the untreated cells (Fig. 4C). Furthermore, PMM2 mRNA expression was significantly decreased in n-Mon, but significantly increased in a-Mon. However, 2-DG treatment had no significant effect on the PMM2 mRNA expression level in MDMs compared to the untreated cells (Fig. 4C). In addition, while guanosine diphosphate (GDP)-mannose pyrophosphorylase A (GMPPA) and GMPPB mRNA expression was significantly increased in both n-Mon and a-Mon, its expression was

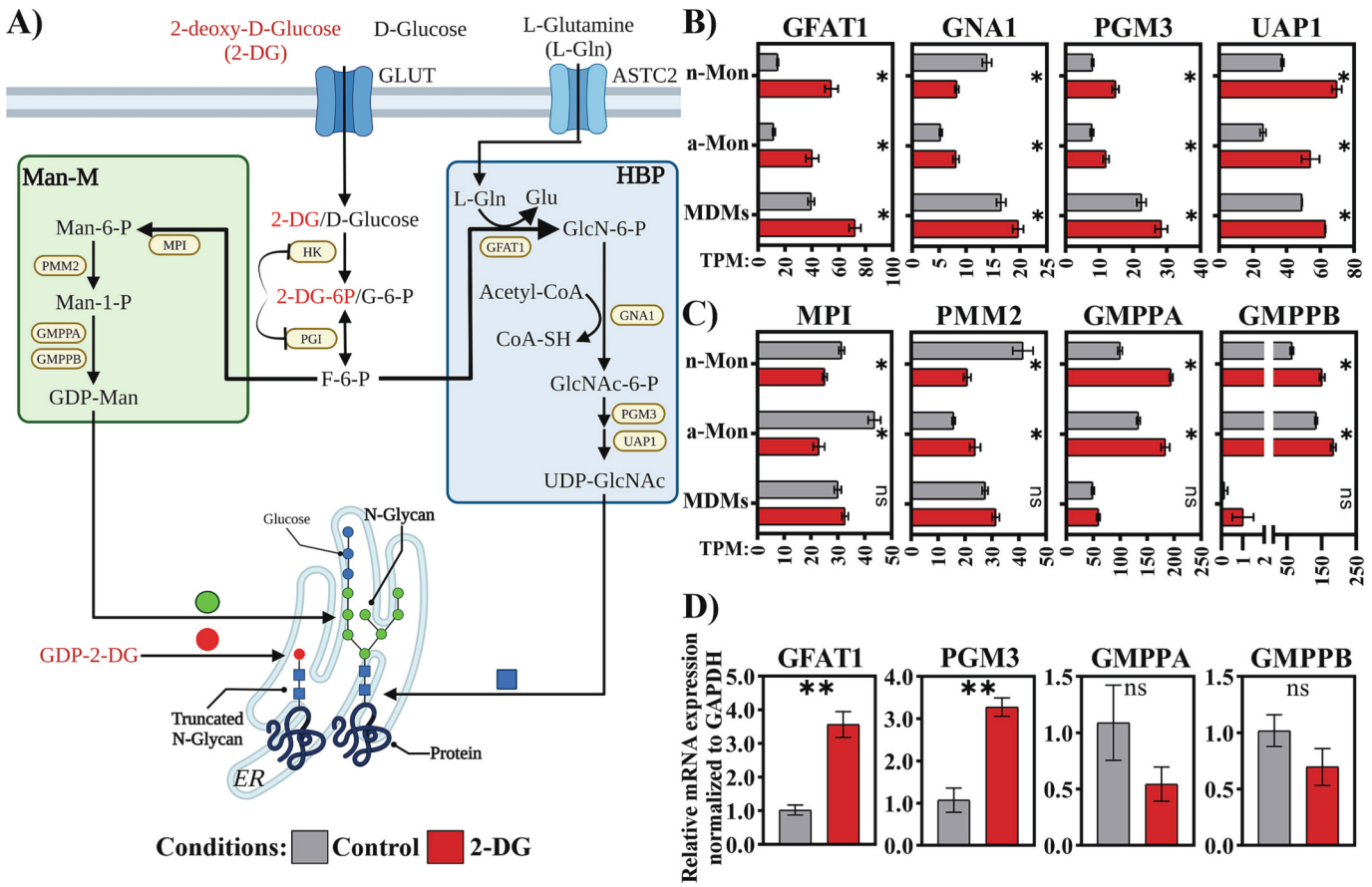

Fig. 4. 2-DG treatment enhance mannose metabolism and hexosamine biosynthetic pathway. Cells utilize glucose, which is the major carbon source for synthesizing nucleotides, N-glycans, lipids, and producing energy. Under 2-DG treatment, cells take up 2-DG and inhibit the enzymes HK and PGI, leading to an inhibition of the glycolytic pathway. Cells can synthesize GDP-2-DG from 2-DG, which affects the formation of N-glycans. Created with BioRender.com **(A)**. The expression of enzymes involved in the Mannose metabolism (Man-M) pathway linked to GDP-Man production, and the expression of enzymes involved in the hexosamine biosynthetic pathway (HBP) in the production of UDP-GlcNAc are shown as transcript per million (TPM) **(B** and **C)**. Validation of main genes involved in Man-M and HBP activation in MDMs treated with 2-DG for 4 h was carried out by RT-qPCR. $n = 3$ **(D)**. The blue square stands for GlcNAc, the green circle for mannose, the blue circle for glucose, and the red circle for 2-DG incorporation. Multiple *t*-test and unpaired *t*-tests were performed. Significant codes: '***' 0.001; '**' 0.01; '*' 0.05; 'ns' no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not significantly affected in MDMs (Fig. 4C). Thus, GMPPA and GMPPB mRNA expression was modulated in both n-Mon and a-Mon after 2-DG treatment.

We carried out validation by RT-qPCR for a selected gene linked with both HBP and Man-M in 2-DG-treated MDMs to validate our multi-transcript results. The results confirmed the increased expression level of GFAT1 and PGM3 mRNA, as compared to the controls. Treatment of MDMs with 2-DG did not show an effect on the GMPPA and GMPPB mRNA expression as compared to the controls (Fig. 4D).

4. Discussion

Previous studies have shown that 2-DG treatment induces ATP depletion, ER stress, autophagy, cell cycle arrest, and apoptosis in several cell lines [43]. In this study, we analyzed multitranscript data and found that 2-DG treatment up-regulate the expression of protein-coding genes involved in ER stress, ISR, HBP, and Man-M in human primary cells. Our results showed significant enrichment of genes in 2-DG-treated human primary cells associated with biological processes linked to ER stress, ISR pathway, protein targeting to ER, and ATP-dependent protein folding (Fig. 1). Although the induction of ER stress and ISR by 2-DG has been studied before [44–46], we demonstrate for the first time, the expression of specific genes in MDMs treated with 2-DG. Our multitranscript analysis indicated that 2-DG treatment increased the transcript levels of protein-coding genes, including DNAJB9, DNAJB11, GRP78/BiP, GRP94, PERK, ATF4, CHOP/DDIT3, GADD34, IRE1α, XBP1, HERPUD1, and HRD1, all known ER stress-related molecules, in monocytes (adherent and non-adherent) and MDMs (Figs. 2 and 3), suggesting the activation of ISR and ERAD pathways. In human epidermoid carcinoma A431 and A549 cells, GRP78/BiP mRNA was induced under a chemical stress condition with 2-DG [47]. Increased induction/overexpression of co-chaperones (DNAJB9 and DNAJB11) and chaperones (GRP78/BiP and GRP94) are interesting since they are integral components of ER stress [48]. We also showed that 2-DG increased ISR components in MDMs without compromising their viability (Fig. 3), as was reported in rabbit articular chondrocytes [49]. In general, our results show that the induction of ER stress by 2-DG leads to the activation of two of the three major mediators of UPR (PERK-ATF4 and IRE1α-XBP1).

In addition, we observed an increase in the mRNA expression of ASNS, PHGDH, SESN2, and VEGFA, in Mon and MDMs treated with 2-DG (Fig. 3E). SESN2, a stress-inducible protein, has been widely reported to be involved in multiple biological processes in response to cellular stress, including a role in cell survival under various stress stimuli, and is a critical effector of the PERK-mediated cell survival response to ER stress [50–56]. ASNS expression is driven by an intricate feedback network within the ISR induced under amino acid deprivation (reviewed in [57]). ASNS and PHGDH, which enable human cells to proliferate respectively under asparagine or serine deprivation, are the major targets of ATF4 [58–60]. Additionally, XBP1, a key ER stress-

inducible TF, increased VEGF-A expression, while its silencing inhibited VEGF-A expression in cardiomyocytes [61]. Based on our results, we propose that 2-DG treatment leads to protein misfolding and ER stress through ISR activation (Fig. 3A). However, more detailed studies are needed to elucidate how.

Eukaryotic cells utilize glycosylation to regulate protein folding and quality control, i.e., add carbohydrates primarily to the nascent unfolded protein in the ER [62,63]. 2-DG can compete with D-mannose to participate in N-glycan synthesis, leading to the production of misfolded glycoproteins and ultimately causing ER stress [17]. The amino-sugar metabolic enzymes, GFAT1, GNA1, PGM3 and, UAP1, classified as HBP-related enzymes, were also up-regulated upon 2-DG treatment (Fig. 4). Although these results are at the transcriptomic level, it has been reported that 2-DG-treated pancreatic cancer cells increase GFAT1 mRNA expression and protein phosphorylation in a dose-dependent manner [64]. The author also reported that 2-DG treatment reduces protein N-glycosylation, but it does not change the expression levels of glycolytic enzymes. GFAT1 is the first rate-limiting enzyme in the formation of hexosamine products (Fig. 4A) and a key regulator of HBP [65]. These results are consistent with previous reports showing that the enzymes of HBP are up-regulated under ER stress in a sXBP1-dependent manner and highlight the importance of this link in the biosynthesis and architecture of mature N-glycans [66,67]. Therefore, in response to 2-DG, human primary cells increase the expression of genes related to the N-glycans biosynthesis.

The Man-M greatly contributes to the incorporation of D-mannose into N-glycans in approximately 25–30% of mannose influx under physiological conditions in human fibroblast [68]. In earlier studies with crude membrane preparations from chick embryos cells, which glycosylate lipids upon addition of UPD-GlcNAc, the standard N-glycan ($Glc_3Man_9GlcNAc_2$-PP-Dol) was formed using $GlcNAc_2$-PP-Dol and GDP-mannose as a substrate [21]. When the authors substituted GDP-mannose for GDP-2-DG (Processes illustrated in Fig. 4A), an abnormal N-glycan was formed (2-DGGlcNAc2-PP-Dol), in which subsequent additions of D-mannose or 2-DG were no longer possible. In a recent study, using MDA-MB-231 breast cancer cells treated with 2-DG, the N-glycans were altered, resulting in abnormal N-glycans ($Man_7GlcNAc_2$-PP-Dol) compared to untreated cells [23]. Overall, in both studies, the effect of 2-DG was reversed by D-mannose.

We investigated the effects of 2-DG treatment on mRNA expression of Man-M enzymes in human primary cells. Our findings indicated that GMPPB mRNA was significantly increased in n-Mon, a-Mon, and MDMs after 2-DG treatment, while GMPPA showed increased expression in n-Mon specifically. The upregulation of GMPPA and GMPPB, which are responsible for GDP-Man synthesis, suggests that 2-DG-6P may serve as a substrate for Man-M enzymes, as shown in Fig. 4A. GMPPB is essential for N- and O-mannosylations processes (as reviewed in [69]) and previous research has demonstrated that 2-DG, can enhance mannosylation in a dose-dependent manner [70]. Furthermore, it has been reported that GMPPA and GMPPB function together to maintain GDP-Man homeostasis and support normal organismal development [71]. GMPPB catalyzes the production of GDP-mannose from mannose-1-phosphate and GTP [72], and GMPPA defects cause a neuromuscular disorder with α-dystroglycan hyperglycosylation [73]. In agreement with our results, these studies support the hypothesis that cells treated with 2-DG undergo modulation of HBP and Man-M enzymes, leading to the production of truncated N-glycans and the activation of the ISR and ERAD system.

We conducted a comprehensive analysis of gene expression profiles using a multitranscript approach, utilizing previous transcriptome data from human primary cells treated with 2-DG. Although the results revealed significant changes in the expression of specific genes in response to treatment, it is crucial to further investigate and understand the underlying mechanism by which 2-DG directly or indirectly regulates gene expression. Previously, it was reported that 2-DG can have a direct impact on both chromatin structure and gene expression [74,75]. For instance, in a study involving 2-DG-treated rats, genes containing the neuron-restrictive silencing element exhibit a notable decrease in H3-k9 acetylation compared to control samples [75]. Furthermore, the authors demonstrated that 2-DG treatment results in transcriptional changes in metabolic genes. Although the precise mechanism of action of 2-DG remains controversial, we suggest that it may alter the expression of genes such as GMPPB through significant changes in the chromatin structure. Further investigation is warranted to elucidate the specific molecular mechanisms through which 2-DG influence gene expression, including the potential impact on chromatin structure.

In summary, we show that our identified key makers allow us to discriminate ER-stress sensitivity in primary cells under 2-DG treatment. We suggest that the mechanism of 2-DG-induced ER stress is activation-dependent of GRP78/BiP-PERK-ATF4-CHOP/DDIT3-GADD34 (ISR pathway) and consequently, the induction of tXBP1 and its spliced form sXBP1, which mediates the expression of target genes associated with the ERAD system, HBP and Man-M. Therefore, 2-DG, commonly considered a glycolytic inhibitor, is also an important stress inducer in human primary monocytes and monocyte-derived macrophages with mechanisms and consequences yet to be investigated.

5. Limitations of the study

A major limitation of this work was that the results were obtained by comparing transcriptome data sets with different treatments that are not homogeneous in concentrations and times. Nevertheless, our results allowed us to suggest that 2-DG-mediated changes to Mon and MDMs transcriptome are highly indicative of ER stress, particularly genes relating to IRS and protein translational capacity, on two primary cell types under different experimental conditions, which is a robust comparison to the capacity of 2-DG-induced ER stress. Further development and validation may better analyze the molecular mechanism and perform conformational experiments to clarify how 2-DG regulates gene expression. Another limitation of this study is that no real protein change was explored. However, other studies had been evaluated protein expression under 2-DG treatment and the results are according to our multitranscript analysis.

6. Conclusion

In conclusion, the multitranscript analysis reveals that 2-DG treatment alters the transcriptomic profile of Mon and MDMs resulting in the expression of protein-coding genes related to ER stress and IRS, as well as HBP and Man-M signaling pathways. Taken together, this research gives a deeper understanding of the regulated transcripts of human primary cells in response to 2-DG.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2023.130397.

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CRediT authorship contribution statement

Y.S. Tamayo-Molina: Conceptualization, Investigation, Software, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Paula A. Velilla:** Funding acquisition, Project administration, Writing – review & editing. **Lady Johana Hernández-Sarmiento:** Investigation, Methodology, Validation. **Silvio Urcuqui-Inchima:** Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing - original draft, Writing - review &

editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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