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Small Ubiquitin-related Modifier (SUMO)-1 Promotes Glycolysis in Hypoxia*[S]

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Terence A. Agbor[‡§], Alex Cheong[‡¶], Katrina M. Comerford[‡], Carsten C. Scholz[‡], Ulrike Bruning[‡], Ambrose Clarke[‡], Eoin P. Cummins[‡], Gerard Cagney[‡], and Cormac T. Taylor[‡¶1]

From the [‡]UCD Conway Institute and [¶]Systems Biology Ireland, University College Dublin, Belfield, Dublin 4, Ireland and the [§]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Under conditions of hypoxia, most eukaryotic cells undergo a shift in metabolic strategy, which involves increased flux through the glycolytic pathway. Although this is critical for bioenergetic homeostasis, the underlying mechanisms have remained incompletely understood. Here, we report that the induction of hypoxia-induced glycolysis is retained in cells when gene transcription or protein synthesis are inhibited suggesting the involvement of additional post-translational mechanisms. Post-translational protein modification by the small ubiquitin related modifier-1 (SUMO-1) is induced in hypoxia and mass spectrometric analysis using yeast cells expressing tap-tagged Smt3 (the yeast homolog of mammalian SUMO) revealed hypoxia-dependent modification of a number of key glycolytic enzymes. Overexpression of SUMO-1 in mammalian cancer cells resulted in increased hypoxia-induced glycolysis and resistance to hypoxia-dependent ATP depletion. Supporting this, non-transformed cells also demonstrated increased glucose uptake upon SUMO-1 overexpression. Conversely, cells overexpressing the de-SUMOylating enzyme SENP-2 failed to demonstrate hypoxia-induced glycolysis. SUMO-1 overexpressing cells demonstrated focal clustering of glycolytic enzymes in response to hypoxia leading us to hypothesize a role for SUMOylation in promoting spatial re-organization of the glycolytic pathway. In summary, we hypothesize that SUMO modification of key metabolic enzymes plays an important role in shifting cellular metabolic strategies toward increased flux through the glycolytic pathway during periods of hypoxic stress.

In the steady state (when oxygen levels within a cell exceed bioenergetic requirements), activity of the glycolytic pathway, the TCA (tricarboxyic acid) cycle and electron transport chain combine to generate ~38 molecules of adenosine triphosphate (ATP) per molecule of glucose metabolized. Glycolysis and the TCA cycle each generate 2 molecules of ATP with the remaining 34 molecules being produced during oxidative phosphorylation. This process provides the ATP necessary to maintain physiologic function. However, under conditions where oxygen demand exceeds supply (hypoxia), most cells retain the capacity to fundamentally shift metabolic strategy to a state where mitochondrial activity is decreased and glycolysis becomes the primary pathway for ATP generation (1). Normally, this metabolic switch promotes cell survival during hypoxia and is thus adaptive in nature. In such cases, cells switch back to the predominantly oxidative metabolism when the oxygen balance is restored. This phenomenon is known as the Pasteur effect (3). Alternatively, in the hypoxic compartment of a growing tumor, which has outgrown the local blood supply, the ability of cells to develop enhanced glycolytic metabolism confers a survival advantage for the tumor. Furthermore, some cancer cells develop the ability to maintain enhanced glycolysis even when sufficient oxygen supply returns, a phenomenon known as the Warburg effect (35). Thus, the ability of cells to increase the rate of glycolysis in response to hypoxia has important implications in both health and disease. The molecular mechanisms underlying these important switches in metabolic function are an area of intense investigation.

One mechanism that underpins hypoxia-induced increase in flux through the glycolytic pathway involves transcriptional up-regulation of glycolytic enzymes by the hypoxia-inducible transcription factor, HIF (3). In the current study, we provide data that, in addition to this transcriptional pathway, a non-transcriptional mechanism involving post-translational modification of key glycolytic enzymes facilitates the rapid increase in glycolysis that occurs during hypoxia.

The small ubiquitin related modifier (SUMO)[2] protein family is a family of three small proteins of ~17 kDa in mammals and one member in the yeast *Saccharomyces cerevisiae,* which modify a wide range of target molecules (10, 11, 27). In *S. cerevisiae*, the SUMO homologue Smt3 has been shown to be essential for viability (10, 11). Recent work has demonstrated that HIF-1α itself is a target for SUMO modification (12, 15, 16, 18–20). The first identified targets for SUMO modification were mostly nuclear transcription factors. However, more recent work has identified a number of cytoplasmic SUMO targets (13). Extranuclear roles for protein SUMO-1 modification include regulation of G protein signaling, regulation of kinase/phosphatase activity, axonal mRNA trafficking,

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[1] To whom correspondence should be addressed: School of Medicine and Medical Science, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: 353-1-716-6732; E-mail: cormac.taylor@ucd.ie.

[2] The abbreviations used are: SUMO, small ubiquitin-related modifier; MEF, mouse embryonic fibroblasts.

mitochondrial fission/fusion, glucose transport, and glutamate receptor function as well as regulation of neuronal integrity and synaptic function (21, 27, 37). More recently, a growing number of studies have linked the SUMO modification pathway with tumor development and metastasis and as a response to DNA damage (28, 38).

We and others have shown that upon exposure of cells to hypoxia, there is an altered pattern of protein modification by SUMO-1 (2, 14–16). Furthermore, a number of studies have demonstrated enhanced protein SUMO modification in hibernation and transient ischemic episodes, both of which involve significant decreases in tissue perfusion and oxygenation (22–26). However, to date, the physiological implications for SUMO modification in hypoxia have remained largely unknown.

In the current study we found that even when gene transcription and translation were inhibited, hypoxia-induced flux through the glycolytic pathway, leading us to hypothesize a role for post-translational modifications in hypoxia-induced glycolysis. Because SUMO-1 modification has previously been shown to be increased in hypoxia in a range of models, we used a proteomic approach in *S. cerevisiae* to identify the proteins modified by Smt3 (the yeast homolog of mammalian SUMO) in hypoxia with an aim to identifying a possible general physiologic role for this modification. We found that a number of key metabolic enzymes were modified by Smt3 in hypoxic yeast. In mammalian cells, overexpression of SUMO-1 enhanced basal and hypoxia-induced glycolysis and glucose uptake. Conversely, overexpression of the SUMO isopeptidase SENP2 reversed hypoxia-induced glycolysis. We hypothesize that SUMO modification of metabolic enzymes is involved in the promotion of glycolysis in mammalian cells thus favoring a shift to glycolytic ATP generation. This compliments transcriptional up-regulation of glycolytic enzymes by HIF-dependent pathways and promotes metabolic adaptation to hypoxia, an event that may contribute to tumor cell survival.

MATERIALS AND METHODS

Reagents—Mouse anti-GMP-1 (SUMO-1) antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA), mouse anti-GAPDH from Calbiochem (Darmstadt, Germany), rabbit anti-GAPDH, goat biotin, anti-pyruvate kinase, and rabbit anti-HIF-1α antibodies were purchased from Abcam (Cambridge, United Kingdom), mouse anti-His_6 was obtained from Roche Applied Science (Basel, Switzerland). All secondary antibodies used for immunoblotting were obtained from Cell Signaling (Danvers, MA), whereas those used for immunofluoresence were obtained from the Jackson Laboratory (Westgrove, PA). The SENP2 adenovirus and His_6 SUMO-1 overexpressing HeLa cells were kind gifts from Professor Ron Hay of the University of Dundee.

Cell Culture—Human cervical adenocarcinoma cells (HeLa) were cultured in minimum essential medium containing glutamine supplemented with 10% FCS, 100 units/ml of penicillin/streptomycin. Cells were placed in one of two hypoxia chambers (Coy Laboratories, Grass Lake, MI) or a tissue culture incubator allowing the establishment of graded, humidified ambient atmospheric hypoxia of 21, 10, and 1% O_2 with 5% CO_2 and a balance of N_2 in all cases. Extracellular medium pO_2 measurements were monitored using fluorescence quenching oxymetry (Oxylite-2000; Oxford Optronix, Oxford, UK). This degree of hypoxic exposure used in these experiments did not induce cell death via apoptosis or necrosis (data not shown). Temperature was maintained at 37 °C in a humidified environment.

ATP Measurements—To measure cellular ATP levels, a commercial assay kit was used according to the manufacturer's instructions (Promega). Briefly, cells were maintained in 60-mm dishes in normoxia or hypoxia for the indicated periods. Whole cells lysates were generated using the luciferase reporter lysis buffer. Samples were protein normalized and the intracellular ATP levels were determined using a luciferin-luciferase assay with luminescence being measured on a desktop luminometer (Berthold Technologies Junior LB 9509). Measurements are expressed as micromolar ATP (relative light units).

Lactate Assay—Lactate levels in the medium were measured using a commercially available kit (Trinity Biotechnology). Briefly, 2.0×10^5 cells were maintained either in normoxia or hypoxia on 60-mm tissue culture plates for 24 h in phenol red-free medium. Lactate levels from the cells were measured on a 96-well plate using a lactate assay kit.

Immunoblotting—Protein samples were resolved on a 10% SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Protein patterns on the membranes were visualized by staining with Ponceau S prior to blocking with 5% nonfat skim milk in TBST for 1 h followed by overnight incubation with the primary antibody at 4 °C (mouse anti-His_6 or HIF-1α (1:500)). Membranes were washed (with TBST) for 1 h and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies at room temperature for a further hour (Santa Cruz). Specific bands were identified using enhanced chemiluminescence and development on an x-ray film. Band density was quantified by densitometry.

Tandem Affinity Purification—Cells were grown in normoxia or hypoxia for the indicated periods, scraped in ice-cold PBS, and centrifuged to generate a cell pellet. The cell pellet was washed twice in ice-cold PBS. The cells were lysed in 5 ml of lysis buffer (10% glycerol, 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 2 mM EDTA, 0.05% Nonidet P-40, and 0.25 mM sodium orthovanadate) for 30 min on ice, after which, two freeze-thaw cycles were used to improve protein recovery. The lysate was spun at $28{,}000 \times g$ for 1 h to remove any cell debris.

25 ml of IgG-Sepharose 6 fast flow beads were washed three times in lysis buffer before being added to the cleared lysate. The lysate was incubated on a rotor at 4 °C for 4 h. The lysate was then poured gently into a 1-ml column. The recovered beads were then washed three times with 1 ml of lysis buffer and three times with 1 ml of TEV buffer (10 mM HEPES-KOH, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM DTT). The beads were resuspended in 1 ml of TEV buffer and 100 units of TEV enzyme added. The slurry was incubated with gentle agitation overnight at 4 °C.

The column was drained but the supernatant was retained in a 15-ml tube. The beads were washed three times with calmodulin binding buffer (10 mM β-mercaptoethanol, 10 mM HEPES-KOH, pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% Nonidet P-40, and 2 mM $CaCl_2$), each time the wash supernatant was transfer to the 15-ml tube. 16 ml of a 1 M $CaCl_2$ solution was added to the protein mixture before 25 ml of washed calmodulin-Sepharose 4B beads were added. This solution was transferred to a new column and incubated at 4 °C for 90 min.

Again the column was drained and the beads were washed five times in calmodulin wash buffer (10 mM β-mercaptoethanol, 10 mM HEPES-KOH, pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.05% Nonidet P-40, and 2 mM $CaCl_2$). The beads were resuspended twice for 5 min and once for 15 min, in 340 ml of calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM HEPES-KOH, pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.05% Nonidet P-40, and 25 mM EGTA) to elute the resulting protein complex.

Eluted protein complexes were precipitated with trichloroacetic acid and resuspended in 2× sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.1% (w/v) bromphenol blue, 20% (w/v) glycerol) by boiling at 100 °C for 5 min. Samples were resolved on a 4–20% pre-cast gel and stained with Coomassie stain. The lanes containing the resolved proteins were cut into gel slices from the top to the bottom of the gel. The gel slices were then subjected to trypsin digestion.

For LC-MS/MS analysis, protein digests were injected using an autosampler onto an LC Packing PepSep column (75 mm inner diameter, 150 mm). Nano-HPLC was used to separate peptides. Two solvents, A and B (A: 0.1% formic acid; B: 0.1% formic acid in 100% MeCN) were used to generate a gradient from 5% B to 55% B over 30 min, then up to 90% B over 5 min and introduced to a LTQ ion trap mass spectrometer (ThemoFinnegan) using electrospray ionization from a nanoflow probe at 300 nl/min. Full MS scans were recorded on the eluting peptides over the 600–2000 m/z range. Tandem MS (MS/MS) spectra were acquired at 35% collision energy in a data-dependent manner, sequentially on the first to 10th most intense ion selected from the full MS scan. MS/MS data were analyzed using X-tandem and searched against a Uniprot data base. All positive proteins identified were only accepted if they had a X-tandem score below −5.

GAPDH Activity Assay—To measure GAPDH enzymatic activity, the KDalert™ GAPDH Assay kit (Ambion Inc.) was used according to the manufacturer's instructions. Briefly, cells exposed to hypoxia for different periods of time were washed with ice-cold PBS and then 1 ml of lysis buffer (supplied with the kit) was added and agitated at 4 °C for 20 min. Cells were then scraped and transferred into labeled microcentrifuge tubes. 20 μl of the lysates were mixed with 180 μl of the master mixture, left for 15 min at room temperature, and the absorbance measured at 615 nm. Each experiment was preceded by a GAPDH standard curve using recombinant GAPDH enzyme from where the concentrations of GAPDH for the samples were obtained and expressed in units/ml.

Glucose Uptake Assay—Mouse embryonic fibroblasts (MEF) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Cells were transfected using lipofection (FuGENE, Roche Applied Science) with 0.5 μg of either plasmid DNA encoding the His_6-SUMO-1 protein (Ron Hay, University of Dundee, UK) or pcDNA3 empty vector. A colorimetric glucose assay (Biovision) was used to determine the effect of hypoxia on glucose uptake. Briefly, conditioned medium from the cell cultures were collected and the glucose level was measured according to the manufacturer's protocol.

Infection of Cells with SENP2 Adenovirus—Cells were infected with SENP2 adenovirus, exposed to hypoxia, and their metabolic activity analyzed. Briefly, 25,000 HeLa cells per well were seeded in 24-well plates and maintained for 24 h. 900 μl of the medium was removed from the cells leaving 100 μl on the cells onto which 2 μl of SENP2 adenovirus or 1 μl of GFP adenovirus control were added and left for 2 h before adding 900 μl of medium. Infected cells were left in the incubator for 48 h, medium was replaced with phenol red-free medium before being exposed to 1% O_2 or left in normoxia for a further 24 h.

Immunofluorescence—1×10^4 cells were seeded on 10-mm coverslips in four-well plates and grown overnight. Cells were maintained in normoxia or hypoxia (1% O_2) for 18–24 h before immunofluorescence studies were carried out. Unless otherwise indicted all washes were 5 min. Briefly, cells were washed twice with ice-cold PBS before fixation with 5% paraformaldehyde/PBS for 15 min. Following two washes in PBS, cells were washed with 50 mM ammonium chloride in PBS for 15 min to quench the free aldehyde groups. Coverslips were washed with PBS twice before being transferred to a humidified chamber where 50 μl of the appropriate dilutions of the primary antibodies diluted in 5% FCS/PBS (anti-mouse GAPDH, 1:200) were added and incubated for 1 h at room temperature. This is followed by two PBS washes and then incubated with 50 μl of a 1:200 dilution (diluted in 5% FCS/PBS) of the appropriate species-labeled secondary antibodies (FITC for anti-mouse or Texas Red for anti-goat) for 45 min at room temperature covered with aluminium foil. After 45 min, the coverslips were washed twice with PBS, rinsed in distilled water, and mounted on ethanol-cleaned glass slide fluorescent mounting medium and left overnight. Slides were examined and images were acquired on an upright fluorescent microscope (Zeiss) using the Axiovision image analysis program.

Statistical Analysis—All data are presented as mean ± S.E. for n independent experiments. Statistical significance was evaluated by one-tailed paired Student's t test (asterisk corresponds to $p < 0.05$ ($n = 3–6$ for each individual experiment)).

RESULTS

Hypoxia Increases Glycolysis in Mammalian Cells—Hypoxia induces a shift in cellular metabolism from oxidative phosphorylation to glycolysis in most eukaryotic cell types (1, 3, 9). To confirm this response in a transformed tumor cell line, HeLa cells were exposed to graded decreases in atmospheric oxygen (from 21 to 1% O_2) for 24 h and ATP levels were determined. Graded decreases in oxygen resulted in an initial decrease in ATP between 21 and 5% atmospheric oxy-

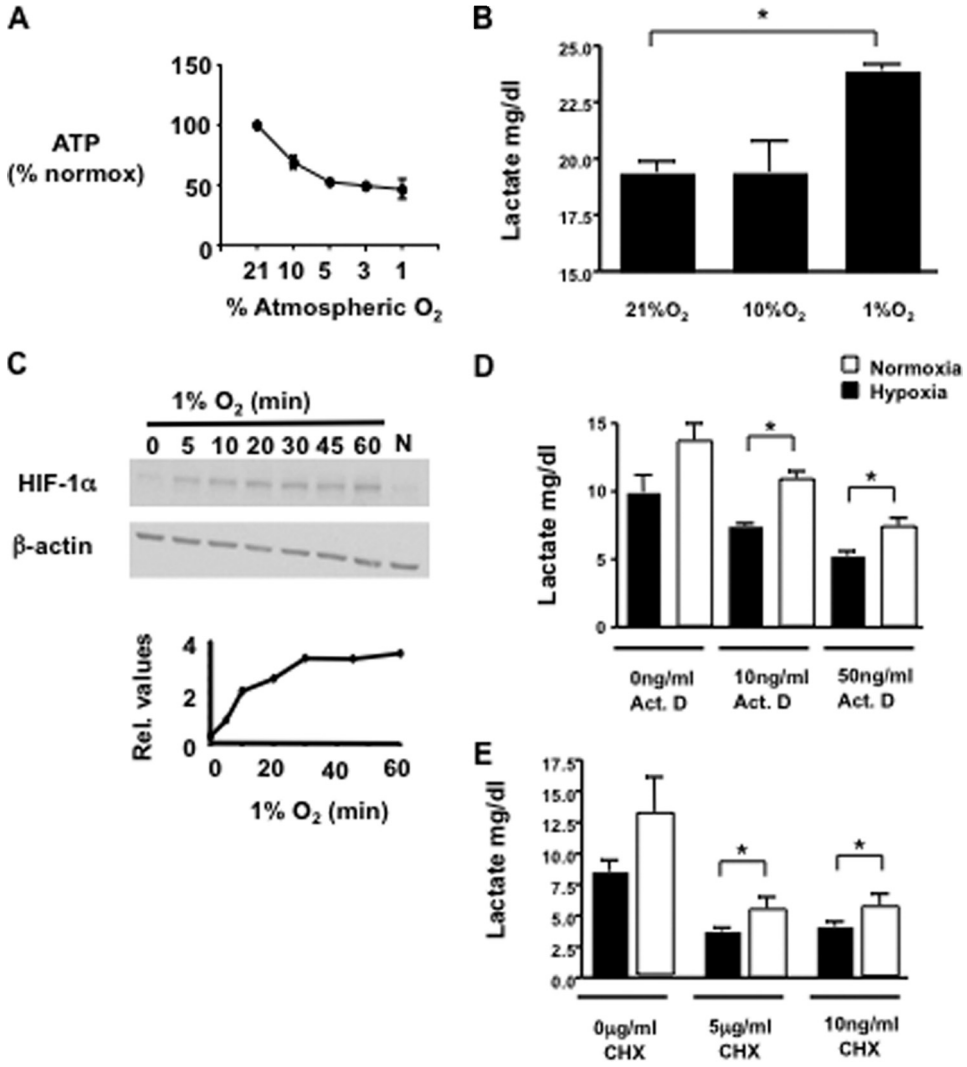


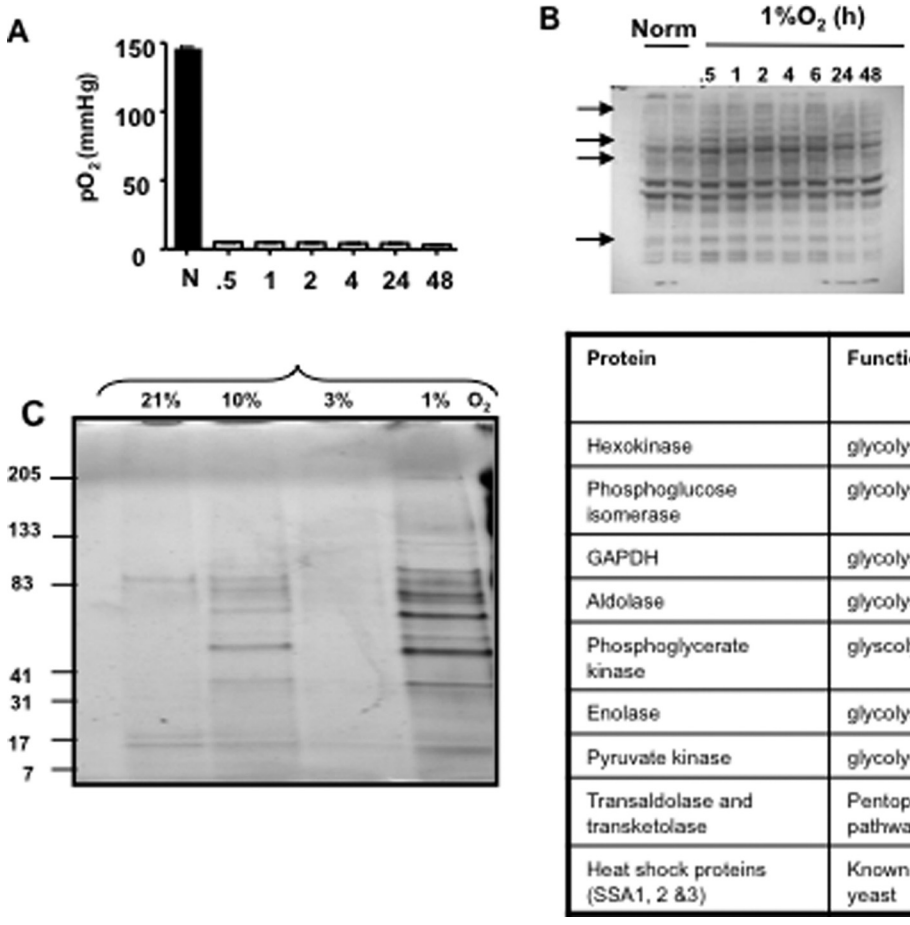
FIGURE 1. **Hypoxia increases cellular glycolysis and decreases ATP generation.** *A*, HeLa cells were exposed to graded hypoxia (21, 10, and 1% for 24 h) and ATP levels were determined. *B*, HeLa cells were exposed to graded hypoxia and the rate of glycolysis was determined by measuring lactate generation. *C*, HeLa cells were exposed to 1% oxygen for the indicated time points and whole cell lysates were immunoblotted for HIF-1α. HIF-1α levels were quantified by densitometry (*lower panel*). *D*, cells were treated with 0–50 ng/ml of actinomycin D prior to exposure to hypoxia and lactate production was measured. *E*, cells were exposed to 5–10 μg/ml of cycloheximide (*CHX*) before being exposed to hypoxia for 24 h and lactate levels were determined ($n = 3$; *, $p < 0.05$).

gen, which then leveled off indicating that anaerobic ATP production predominates at lower oxygen concentrations (Fig. 1*A*). The maintenance of ATP levels at low O_2 was accompanied by an increase in lactate production reflecting an increased glycolysis in hypoxia (Fig. 1*B*). Enhanced lactate production in HeLa cells exposed to hypoxia is also demonstrated in time course studies (supplemental Fig. S1). Thus, although highly transformed and glycolytic, HeLa cells exposed to severe hypoxia undergo a shift in metabolism to a higher degree of flux through the glycolytic pathway.

Increased glycolysis during hypoxia has been attributed to the induction of transcription factor HIF with subsequent expression of glycolytic genes (3). Consistent with this, we found that hypoxia rapidly and reversibly induces HIF-1α expression within 5 min (Fig. 1*C*). This is reflected by increased expression of glycolytic enzymes including phosphoglycerate kinase and GAPDH (supplementary Fig. S2). However, in HeLa cells treated with either actinomycin D (an inhibitor of gene transcription) or cycloheximide (an inhibitor of protein synthesis) in concentrations that inhibit increased glycolytic enzyme expression, increased lactate production was still detectable in hypoxia (Fig. 1, *D* and *E*, and supplemental Fig. S1). Thus, a degree of hypoxia-induced glycolysis occurs even when new gene or protein synthesis is blocked. These data lead us to the hypothesis that post-translational mechanisms may play a role in the switch to increased glycolysis in hypoxia.

One such post-translational modification that we have previously shown to be enhanced in hypoxic and ischemic conditions is by SUMO-1 (2), leading us to the hypothesis that post-translational protein modification by members of the SUMO family may contribute to the regulation of cellular metabolism during hypoxia.

Hypoxia Increases Protein Sumoylation—We next confirmed previous reports (2, 14–16) that hypoxia increases global protein modification by SUMO-1, utilizing HeLa cells overexpressing His_6-SUMO-1. Cells exposed to a time course of hypoxia (1% atmospheric oxygen) demonstrated decreased



Protein	Function	mass spectrometry score
Hexokinase	glycolysis	-91
Phosphoglucose isomerase	glycolysis	34.6
GAPDH	glycolysis	-122.5
Aldolase	glycolysis	-16.7
Phosphoglycerate kinase	glyscolysis	-136
Enolase	glycolysis	-207
Pyruvate kinase	glycolysis	-139.4
Transaldolase and transketolase	Pentophosphate pathway	-16.8
Heat shock proteins (SSA1, 2 &3)	Known Smt3 target in yeast	-34.7, -33.1 & -8.7

FIGURE 2. **Hypoxia increases global protein sumoylation.** *A*, HeLa cells overexpressing His_6-tagged SUMO-1 were exposed to normoxia or hypoxia (1% O_2; 0–48 h). Extracellular pO_2 values were measured by fluorescence quenching oxymetry. *B*, Western blot analysis for SUMO-1-modified proteins in whole cell extracts from His_6-tagged SUMO-1 overexpressing HeLa cells using an anti-His antibody reveals a time-dependent increase in sumoylation patterns in hypoxia. *C*, yeast cells overexpressing tap-tagged Smt3 were exposed to graded hypoxia (21 to 1% O_2) for 48 h. Samples were separated by gel electrophoresis following tap-tag pulldown, the silver stain demonstrates increased Smt3 protein modification. Smt3-modified proteins that were identified by mass spectrometry included multiple metabolic enzymes.

cellular oxygen levels as determined by oxygen-quenching fluorimetry (Fig. 2*A*). Consistent with previous studies, this was matched by global patterns of increased protein SUMOylation (Fig. 2*B*). We next used an unbiased proteomic approach to identify possible targets of modification with the aim of identifying a possible physiologic role. To do this we used *S. cerevisiae* expressing tap-tagged Smt3. Although mammalian cells express three isoforms of SUMO, Smt3 is the only homologue in *S. cerevisiae* (10, 11). Consistent with mammalian cells, yeast cells expressing tap-tagged Smt3 exposed to graded hypoxia (10, 3, and 1% O_2 levels) for 48 h demonstrated a global increase in protein modification by Smt3 in response to hypoxia. The bands were excised from the gels, trypsin digested, and those proteins modified by Smt3 in hypoxia were identified by mass spectrometry. This analysis identified a set of proteins involved in cellular metabolism of which a subset were glycolytic enzymes, suggesting that increased protein modification by Smt3 in yeast may play a role in regulating cellular glycolysis in hypoxia (Fig. 2*C, right*).

SUMO-1 Overexpression Enhances Glycolysis in Normoxia and Hypoxia—The data presented thus far led us to hypothesize a role for Smt3 modification in hypoxia-induced glycolysis in yeast. To test whether SUMOylation plays a role in hypoxia-induced glycolysis in mammalian cells, we measured the activity of the glycolytic enzyme GAPDH in SUMO-1 overexpressing HeLa cells exposed to hypoxia relative to wild-type cells. Exposure to hypoxia increased GAPDH activity in wild-type cells (Fig. 3*A*). Furthermore, GAPDH activity in SUMO-1 overexpressing cells was significantly higher than that in wild-type cells exposed to hypoxia. Also, the basal activity of GAPDH in SUMO-1 overexpressing cells was higher than in HeLa cells. We further demonstrated that SUMO-1 overexpressing cells generate significantly higher amounts of lactate in hypoxia compared with control cells (Fig. 3*B*) suggesting that SUMO-1 modification increases overall glycolytic flux in hypoxia. These results demonstrate that SUMO-1 overexpression increases hypoxia-induced GAPDH activity and lactate production.

To test whether the increased hypoxia-induced glycolysis observed in SUMO-1 overexpressing cells is a function of protein sumoylation, HeLa cells growing on 24-well plates were infected with SENP2 adenovirus (which results in global desumoylation) or GFP control adenovirus for 48 h before exposure to 1% O_2 for 24 h and the lactate levels in the culture medium were measured. SENP2 is a SUMO isopeptidase enzyme

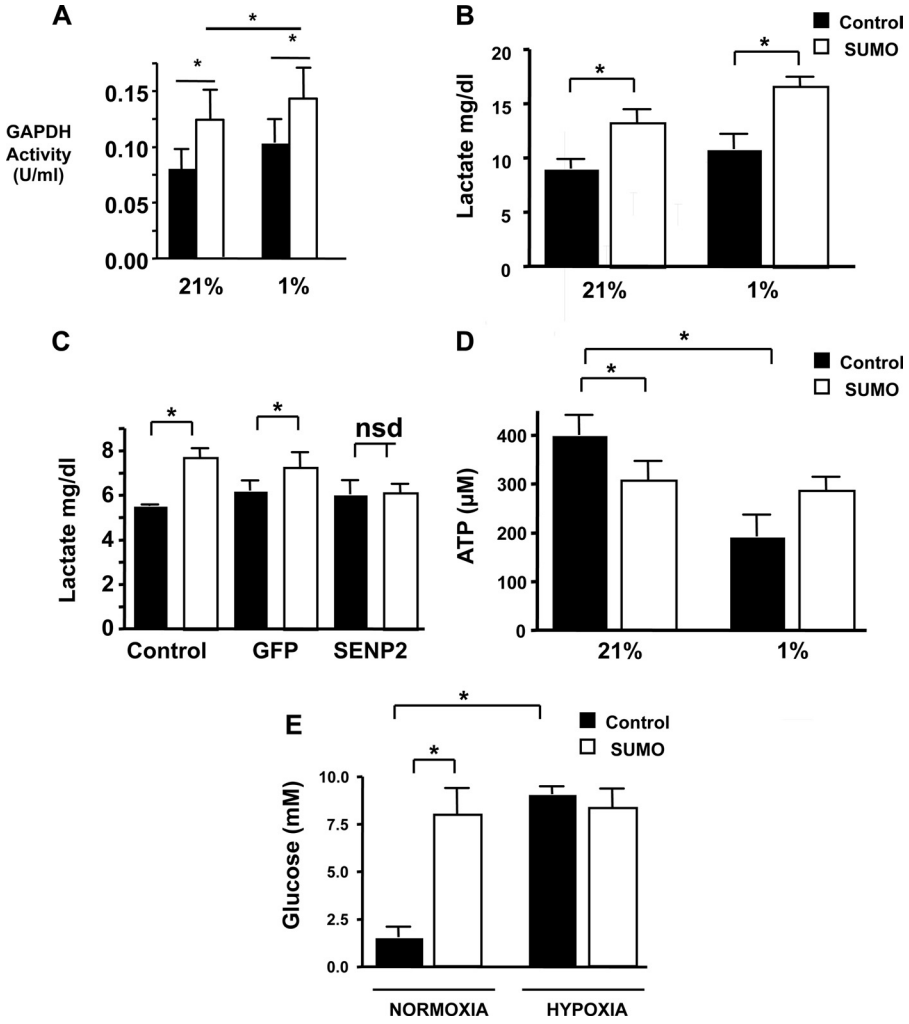


FIGURE 3. **SUMO-1 overexpressing cells demonstrate an enhanced cellular glycolytic capacity.** *A*, measurement of GAPDH enzymatic activity in cells demonstrates that overexpression of SUMO-1 leads to increased GAPDH activity both in normoxia and hypoxia. *B*, cells overexpressing SUMO-1 (*white bars*) demonstrate increased lactate production in both normoxia and hypoxia when compared with control cells (*black bars*) reflecting increased glycolytic activity. *C*, infection of HeLa cells with adenovirus overexpressing SENP2 results in a reversal of hypoxia-induced increase in lactate generation. *D*, total cell ATP levels decrease with hypoxia in wild-type cells but not in SUMO-1 overexpressing cells consistent with a role for SUMO-1 in the positive regulation of glycolytic metabolism. *E*, glucose uptake in MEF cells transfected with His-SUMO-1 or pCDNA plasmid control. MEF cells transfected with 0.5 μg of SUMO-1 plasmid show increased glucose uptake in normoxia compared with control cells. Data are presented as mean $\pm$ S.E. for at least $n = 3–6$ independent experiments; *, $p < 0.05$, unpaired Student's *t* test.

that efficiently removes SUMO-1 from all modified proteins. As shown in Fig. 3*C*, SENP2 overexpression resulted in a significant decrease in lactate levels in hypoxia compared with uninfected cells or GFP adenovirus-infected cells. These data demonstrate that general protein desumoylation reverses the increased lactate production normally observed in hypoxia lending further evidence to the hypothesis that protein sumoylation is in part required for the increased glycolytic flux that occurs in hypoxia.

As the principal objective of increased glycolysis in hypoxia is increased anaerobic generation of ATP to support cell survival, we next examined whether the increased glycolytic capacity of the SUMO-1 overexpressing cells is coupled to ATP generation. Wild-type and SUMO-1 overexpressing HeLa cells were exposed to 1% oxygen for 24 h following which the ATP levels in the cells were measured. As shown in Fig. 3*D*, wild-type cells exposed to hypoxia demonstrated a significant decrease in ATP levels compared with normoxic cells. Basal ATP levels were lower in SUMO-1 overexpressing cells, however, the resulting hypoxia did not cause a decrease in cellular ATP consistent with the hypothesis that the increased glycolysis in these cells is accompanied by glycolytic ATP production and is independent of oxygen.

To test whether SUMO overexpression increases glucose metabolism in a non-transformed cell type, we used MEF. Overexpression of SUMO in MEF resulted in a dramatic increase in glucose uptake in normoxia (Fig. 3*E*) supporting a role for sumoylation in the promotion of glucose metabolism. Hypoxia also greatly enhanced glucose uptake but this was not effected by SUMO overexpression, indicating that glucose transporters may be operating at maximum activity in hypoxia and that overexpression of SUMO cannot further increase this activity.

Hypoxia Alters Spatial Organization of Glycolytic Enzymes—We next sought to gain mechanistic insights into how SUMOylation may be impacting upon the glycolytic pathway.

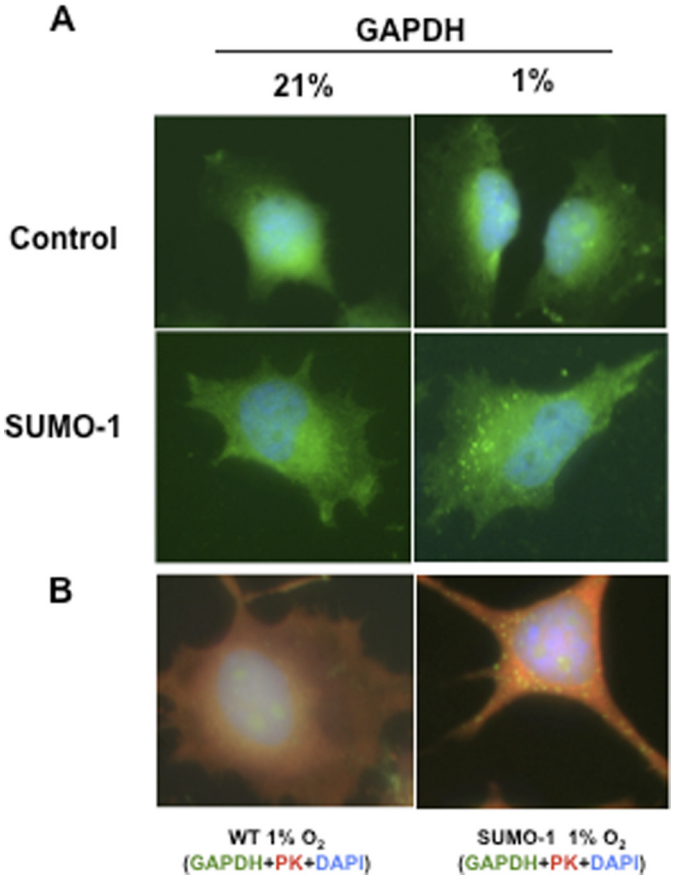


FIGURE 4. **Hypoxia alters spatial organization of GAPDH into speckles in SUMO-1 overexpressing cells.** *A*, HeLa cells (*control*) and SUMO-1 overexpressing cells were maintained in normoxia or exposed to hypoxia and GAPDH was localized by immunofluorescence (*green*). *B*, immunofluorescence co-staining of GAPDH (*green*) and pyruvate kinase (*red*) in wild-type and SUMO-1 overexpressing HeLa cells exposed to hypoxia.

Post-translational modification of proteins by SUMO-1 has been reported to modulate protein-protein interactions, nucleocytoplasmic transport, as well as subcellular localization and stability of the modified proteins (8, 25).

Based on these known effects of SUMO modification of target proteins, we investigated the effect of sumoylation on expression, localization, and spatial organization of GAPDH, a key glycolytic enzyme. HeLa cells and His_6-SUMO-1 overexpressing cells were maintained in normoxia (21% O_2) or exposed to 1% O_2 for 24 h and immunostained with antibodies for GAPDH. As shown in Fig. 4*A*, GAPDH is expressed in both the cytosol and nucleus. Hypoxia increases both the expression and focal speckling of GAPDH in HeLa cells exposed to hypoxia. This effect is significantly enhanced in SUMO-1 overexpressing cells as the punctuate staining and the speckles become more prominent (Fig. 4*A*). Co-immunostaining for GAPDH and pyruvate kinase (another glycolytic enzyme) demonstrates that in SUMO-1 overexpressing cells hypoxia results in co-localization of the two enzymes (Fig. 4*A*, *lower panel*) suggesting that protein sumoylation may promote the interaction or spatial organization of glycolytic enzymes. These results lead us to hypothesize that SUMO-1 modification of glycolytic enzymes promotes their co-localization and facilitates substrate channeling and flux through the glycolytic pathway during hypoxia.

DISCUSSION

Under steady state conditions, the primary method of ATP generation for most eukaryotic cells is aerobic respiration involving the combined activities of the glycolytic pathway, the TCA cycle, and the electron transport chain (1, 9). Collectively, these processes allow the efficient generation of ~38 molecules of ATP per molecule of glucose completely metabolized. When oxygen is limited, cells must change their metabolic strategy to sustain ATP generation. Because glycolysis alone generates a small net amount of ATP (2 molecules per molecule of glucose metabolized) without the requirement of oxygen, one metabolic option for cells is to enhance the rate of glycolysis such that this pathway becomes the primary source of cellular ATP generation, whereas actively decreasing oxidative phosphorylation. For this to occur, a significant elevation in the rate of glycolytic activity is required. One method by which this may be facilitated is through transcriptional up-regulation of glycolytic enzymes and glucose transporters, an event governed by the master regulator of hypoxia-dependent gene expression, HIF-1α (3). Supporting this, HIF-1α-dependent alterations in critical regulators of mitochondrial respiration favor a decrease in activity of the TCA cycle and the mitochondrial electron transport chain, respectively, leading to decreased oxidative stress (4–9). We hypothesized that these transcriptional events are supported by posttranslational modification of metabolic enzymes that facilitate a rapid shift in the metabolic strategy of a cell when challenged with an acute hypoxic insult.

Modification of proteins by the small ubiquitin-related modifier SUMO is critical in a range of cellular physiological processes. We and others have previously demonstrated that hypoxia alters the profile of SUMO-modified proteins (2, 14–16). In the current study, we have demonstrated that hypoxia increases SUMO-1 modification of metabolic enzymes, which is accompanied by enhancement of the glycolytic pathway leading us to the hypothesis that such a modification may positively regulate glycolysis and promote the generation of ATP in the absence of oxygen. Three key questions remain in the development of our understanding of the implications of this finding.

First, what is the hypoxia-sensing mechanism underlying SUMO-1 modification of metabolic proteins? Although the functional importance of protein sumoylation has been described, the regulation of sumoylation is currently poorly understood. A recent publication has provided evidence for an inverse relationship between oxidative stress and activation of the cellular sumoylation machinery (17). In these studies, it is proposed that decreased oxidative stress is related to enhanced protein sumoylation. This effect is thought to be due to reactive oxygen species sensitivity of desumoylating enzymes. Because the production of reactive oxygen species is related to aerobic respiration in cells through the proton leak, it is possible that decreased reactive oxygen species production in hypoxia as a result of decreased activity of the electron

transport chain leads to a subcellular microenvironment favorable to increased sumoylation of glycolytic enzymes.

Second, a critical question relates to the source of SUMO-1 for protein modification in hypoxia. In the steady state, the majority of SUMO-1 is conjugated to proteins with little free SUMO-1 available (27). This leads to two possibilities: first, that *de novo* production of SUMO-1 in hypoxia provides the pool with which to modify the metabolic enzymes. We and others have previously demonstrated that hypoxia up-regulates expression of SUMO-1 mRNA and protein expression (2, 14–16). An alternative explanation for our observation of increased SUMO-1 modification in hypoxia may be a redistribution of SUMO-1 in hypoxia from substrates preferentially modified in normoxia to those preferentially modified in hypoxia. In support of this, several studies have recently reported changes in the pattern of SUMO modification in other cellular stresses including heat shock, electrophil and oxidative stress (17, 30, 31), consistent with the "preferential redistribution" hypothesis.

A third key question relates to how SUMO-1 modification increases the overall cellular glycolytic activity. One possibility here is that SUMO modification of glycolytic enzymes as well as other metabolic proteins in hypoxia may directly increase enzyme activity. How SUMO-1 modification may function to increase enzyme activity remains unknown. One mechanism may be change in enzyme conformation as a result of the modification that may influence its activity as in the case with ubiquitination of the deiodinase enzyme where its modification by ubiquitin results in reversible deactivation of the enzyme (29). Whether this is the case with GAPDH or other glycolytic enzymes remains unknown.

Alternatively spatial rearrangement of SUMO-1-modified glycolytic enzymes in the cytoplasm into discreet complexes with other glycolytic enzymes may allow the generation of complexes that favor substrate channeling in the glycolytic pathway thus enhancing overall efficiency of this pathway. The relative importance of these processes remains to be determined but has been shown to be essential in the glycolytic pathways of some human protozoan parasites, trypanosomes (34). Furthermore, a recent study demonstrated the interaction of SUMO-1, the SUMO E3 ligase PIAS3, and the rate-limiting glycolytic enzyme, pyruvate kinase (36). Whether this interaction of pyruvate kinase with SUMO has any effects on its enzyme activity or its interaction with other glycolytic enzymes is yet to be investigated.

Finally, the physiological significance of this response remains to be clearly elucidated. We propose that this mechanism may have significance in the rapid adaptation to hypoxic/ischemic stress during which cells must switch to a state of enhanced flux through the glycolytic pathway for their survival as occurs during physiological processes such as hibernation and ischemia. Tumor cells on the other hand may exploit the same strategy to survive as a maladaptive mechanism. The SUMO conjugation pathway has recently been implicated in cancer development and metastasis (28). Several reports of functional alterations and changes in expression pattern of components of the SUMO conjugation machinery in different cancers including prostate cancers and thyroid adenomas exist (32, 33). A recent study demonstrated a significant increase in protein sumoylation in two leukemic cell lines (HL60 and HL60RV) but more abundant in the vincristine-resistant HL60RV cancer cell line (39). Taken together such tumors may exploit the SUMO pathway as a metabolic advantage for their development and survival. In summary, we have demonstrated that SUMO-1 modification is associated with enhanced glycolysis indicating a possible role for this modification in mediating the fundamental shift in metabolic strategy observed when a eukaryotic cell encounters a hypoxic microenvironment.

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Terence A. Agbor, Alex Cheong, Katrina M. Comerford, Carsten C. Scholz, Ulrike Bruning, Ambrose Clarke, Eoin P. Cummins, Gerard Cagney and Cormac T. Taylor

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