CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Testosterone and Follicle Stimulating Hormone—Dependent Glyoxalase 1 Up-Regulation Sustains the Viability of Porcine Sertoli Cells through the Control of Hydroimidazolone— and Argpyrimidine-Mediated NF- κ B Pathway

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Because Sertoli cells (SCs) play a central role in germ cell survival, their death may result in marked germ cell loss and infertility. SCs are the only somatic cells within the seminiferous tubules and are essential for regulating spermatogenesis. Factors that enhance or diminish the viability of SCs may have profound effects on spermatogenesis. Yet the mechanisms underlying the maintenance of SC viability remain largely unknown. Glyoxalase 1 (Glo1) detoxifies methylglyoxal (MG), a highly reactive carbonyl species mainly formed during glycolysis, which is a potent precursor of cytotoxic advanced glycation end products (AGEs). Hydroimidazolone (MG-H1) and argpyrimidine (ArgPyr) are AGEs resulting from MG-mediated post-translational modification of arginine residues in various proteins. The role of Glo1 and MG-derived AGEs in regulating the fate of SCs has never been investigated. By using gene silencing and the specific MG scavenger, aminoguanidine, the authors demonstrate that Glo1, under testosterone and follicle-stimulating hormone control, sustains viability of porcine neonatal SCs through a mechanism involving the NF- κ B pathway. *Glo1* knockdown induces a mitochondrial apoptotic pathway driven by the intracellular accumulation of MG-H1 and ArgPyr that desensitizes NF- κ B signaling by modifying the inhibitor of NF- κ B kinase, IKK β . This is the first report describing a role for Glo1 and MG-derived AGEs in SC biology, providing valuable new insights into the potential involvement of this metabolic axis into spermatogenesis. (*Am J Pathol* 2018, 188: 2553–2563; <https://doi.org/10.1016/j.ajpath.2018.07.013>)

Sertoli cells (SCs) are the only somatic cells within the seminiferous tubules and play a pivotal role in the maintenance and control of spermatogenesis.¹ They create tight junctions that make up the blood–testis barrier, forming a peculiar morphological and chemical microenvironment to provide nutrients and essential regulatory factors for developing germ cells while protecting seminiferous tubules from autoantigens and invading pathogens.² For all these functions, SCs, traditionally considered merely “sustentacular cells”,^{3,pp.38} have been recently re-evaluated and elevated to the rank of a “sentinel”^{3,pp.1} in spermatogenesis.⁴

In line with this pivotal role of SCs in spermatogenesis is the fact that the number of SCs present at puberty sets the upper limit for potential sperm production in adulthood, each SC being able to support a fixed number of germ cells,⁵ consequently controlling male reproductive capacity.^{6–8} In addition to the number of SCs, defects in their quality can lead to the imperfect formation of spermatozoa.⁷ In this

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ambit, SCs metabolic performance, especially glucose metabolism, which is under a tight endocrine control in these cells, is essential for the success of spermatogenesis and fertility.⁹ In fact, a close metabolic cooperation between SCs and developing germ cells has long been postulated. In particular, developing germ cells are highly dependent on the lactate produced by SCs as metabolic fuel,^{10,11} whereas high lactate production in SCs is in turn underpinned by their unusual carbohydrate metabolism; they present a high glycolytic flux where the majority of glucose is converted into lactate and not oxidized via the citric acid cycle.¹¹ This metabolic behavior of SCs is consistent with Otto Warburg's observations in cancer cells.^{6,11,12} Any factor that impairs or supports SC viability (and hence, SC number), and/or functionality, may have profound effects on spermatogenesis.¹³

Glyoxalase 1 (Glo1) is the major detoxifying enzyme of methylglyoxal (MG),¹⁴ a metabolic byproduct of glycolysis with a potent ability to modify primary amine-containing amino acid side chains (such as lysine and arginine residues) of key cellular proteins, generating advanced glycation end products (AGEs), whose intracellular accumulation leads to so-called carbonyl stress.¹⁵ The modification of protein AGEs can affect their biological activity^{16,17} or stability/degradation,¹⁸ leading to impaired cell function.¹⁹ Hydroimidazolone (MG-H1) and argpyrimidine (ArgPyr) are two such AGEs derived from the spontaneous reaction of MG with protein arginine residues.^{15,20,21} Several studies have demonstrated that MG-induced carbonyl stress can affect many biological processes in human cells,^{22–24} including apoptosis.^{25–32} By using the cofactor glutathione (GSH), Glo1 prevents the accumulation of MG, thereby suppressing dicarbonyl-mediated glycation reactions. More importantly, *Glo1* overexpression decreases accumulation of AGEs in *Caenorhabditis elegans*,³³ diabetic rats,³⁴ and human cells,^{29,32} and *Glo1* knockdown induces the intracellular accumulation of MG-derived AGEs.^{25–27,32,35} Therefore, Glo1, by preventing the onset of MG-dependent carbonyl stress leading to apoptosis, is a key antiglycation defense that sustains cell viability. Despite this, the role of Glo1 in the context of infertility remains underinvestigated, and those studies that have been done have been mainly limited to female infertility.^{36–38} However, one proteomic study, more than 10 years ago, identified Glo1 among the antigens expressed in prostasomes of infertile men, suggesting Glo1 as a target for naturally occurring sperm agglutinating antibodies, a well-known cause of infertility.³⁹ In the present study, the authors attempted to shed further light on the role of Glo1 in male infertility, focusing on SCs because their biology remains poorly understood and because they have a primary role in spermatogenesis.^{3,4} In particular, whether and how Glo1 might be involved in sustaining the viability of SCs isolated from porcine neonatal pre-pubertal pig testes cotreated with testosterone (T) and follicle-stimulating hormone (FSH) was investigated, focusing on MG-derived AGEs and NF- κ B signaling.

Materials and Methods

Materials

The reagents included: T (Sigma-Aldrich, St. Louis, MO); FSH (GONAL-f; Merck Serono, Rome, Italy); Laemmli buffer (Invitrogen, Milan, Italy); Roti-Block (Carl Roth, Karlsruhe, Germany); BCA Assay kit (Pierce; Thermo Fisher Scientific, Waltham, MA), and aminoguanidine bicarbonate (AG) (Sigma-Aldrich, Milan, Italy). The antibodies used in this study included: mouse anti-Glo1 (D6), rabbit anti-Bcl-XL, rabbit anti-Bax (N20), mouse anti- β -actin (DBA Italia, Milan, Italy); mouse anti-ArgPyr (Antibodies-online, Aachen, Germany); mouse anti-MG-H1 (STA-011; Cell Biolabs, DBA Italia); rabbit anti-caspase-3, rabbit anti-phospho-Ik β (Ser32) (14D4) (Cell Signaling Technology, Milan, Italy); mouse anti-Bcl-2 (Dako, Milan, Italy); mouse anti-cytochrome c (Cyt c) (BD Pharmingen, Milan, Italy); mouse anti-Cyt c oxidase subunit IV (Cox IV) (Molecular Probes, Monza, Italy).

Primary Cultures of Neonatal Porcine SCs

Studies with animals were conducted in agreement with national (Italian Approved Animal Welfare Assurance A-3143-01) and local (University of Perugia Animal Care and Use Committee) guidelines. Large white neonatal pigs (15 to 20 days old) underwent bilateral orchidectomy after general anesthesia with ketamine (Ketavet 100; Intervet, Milan, Italy), at a dose of 40 mg/kg, and dexmedetomidine (Dexdomitor, Orion Corporation, Finland), at a dose of 40 g/kg, and were used as SC donors. Specifically, pure porcine pre-pubertal SCs were isolated, exhaustively characterized, and tested for functional competence according to previously established methods.^{40–42} SCs were stimulated with FSH (1 mg/mL of α -folitropin) for 48 hours; thereafter, 0.2 mg/mL of testosterone enanthate was added for 8 hours, as previously described.^{41,42} Exposure of SCs to FSH and T was deemed necessary to mimic the physiological condition whereby both hormones are essential for adequate spermatogenesis.^{11,42} Untreated cells, as well cells treated with FSH or T singly, were used as controls.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time TaqMan PCR Analysis

RNA isolation, reverse transcription, and real-time quantitative PCR analyses were performed as previously described.^{25–29} Briefly, total cellular RNA was isolated using TRIzol Reagent (Life Technologies Italia, Milan, Italy). Using 1 μ g of total RNA, cDNA was then synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies Italia). The expression of target genes relative to a reference gene (*Actb*) was evaluated by RT-qPCR, using SYBR Green biochemistry, on a MX3000P Real-Time PCR System

(Agilent Technology, Milan, Italy). The sequences of oligonucleotide primers used were as follows: *Glo1*: 5'-CTCTCCAGAAAAGCTACACTTTGAG-3' (sense, 400 nmol/L), 5'-CGAGGGTCTGAATTGCCATTG-3' (antisense, 400 nmol/L); *Actb*: 5'-CACTCTTCCAGCCTTCCTTCC-3' (sense, 600 nmol/L), 5'-ACAGCACTGTGTTGGCGTAC-3' (antisense, 600 nmol/L). The thermal cycling conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 45 cycles at 95°C for 20 seconds and 60°C for 1 minute. Data for comparative analysis of gene expression were obtained using the $2^{(-\Delta\Delta C_T)}$ method.⁴³

Protein Extraction and Western Blot Analysis

Total protein extracts were prepared by lysing the cells (4°C) in radioimmunoprecipitation assay lysis buffer.²⁹ For subcellular fractionation, cells were resuspended in Mito-buffer.²⁹ Protein concentration in cell extracts was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), by reference to a standard curve prepared using dilutions of bovine serum albumin.

For Western blot analysis, samples (containing 30 µg total protein), in Laemmli buffer, were boiled for 5 minutes, resolved on 10%, 12%, or 15% SDS-PAGE, and then transferred (iBlot Dry Blotting System; Invitrogen) to nitrocellulose membranes. Membranes were blocked in Roti-Block for 1 hour at room temperature, incubated overnight at 4°C with an appropriate dilution of the indicated primary antibody, washed with Tris-buffered saline/Tween, before being incubated (1 hour at room temperature) with the appropriate horseradish peroxidase-conjugated secondary antibody and visualized using the ECL system (Microtech, Naples, Italy). As internal loading controls, all membranes were subsequently stripped of the first antibody in a stripping buffer [100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.8)] and reprobed with the appropriate housekeeping antibody.

Glo1 Enzyme Activity

The activity of Glo1 was assessed as previously described in lysates from cell lines.^{28,29} Glo1 activity was assayed according to Mannervik et al.⁴⁴ Briefly, the assay solution contained 0.1 mol/L sodium phosphate buffer (pH 7.2), 2 mmol/L MG, and 1 mmol/L GSH. The reaction was monitored spectrophotometrically by following the increase in absorbance at 240 nm and 25°C. One unit of activity was defined as 1 µmol of S-D-lactoylglylthione produced per minute.

MG-H1 Detection

OxiSelect Methylglyoxal Competitive ELISA kit (DBA Italia) was used to detect MG-H1 levels according to the manufacturer's instructions.^{45,46} Results were also confirmed by a specific MG-H1 antibody (mouse anti-methylglyoxal, STA-011; DBA Italia, Milan, Italy).

Apoptosis Detection

Apoptosis was detected by evaluating caspase 3 activation, using an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Milan, Italy), specific for activated human Caspase-3 following the manufacturer's instructions, as well as by DNA fragmentation by agarose gel electrophoresis.^{28,29}

Gene Silencing

Glo1 silencing was performed as previously described.^{23,27} Briefly, cells were transiently transfected with a pool of four siRNA oligonucleotides targeting *Glo1* (ON-TARGET plus SMART pool siRNA) or with a pool of nontargeting siRNA oligonucleotides (siCtrl) (ON-TARGET plus siCONTROL) to exclude off-target effects (all from Dharmacon RNA Technologies, Carlo Erba, Milan, Italy), using DharmaFECT 2 transfection reagent (Dharmacon RNA

Figure 1 Expression levels of Glo1 in prepubertal porcine SCs treated with T and FSH, alone or in combination or untreated. The bar graphs show Glo1 transcript level, measured by quantitative RT-PCR (A), Glo1 protein level, assessed by densitometric analysis from Western blot detection (B), and Glo1-specific enzyme activity, measured by spectrophotometric methods (C). Western blots were obtained by using the appropriate antibodies. The blots were stripped of the bound antibodies and reprobed with anti-β-actin to confirm equal loading. The Western blots shown are representative of three separate experiments. Data are expressed as means ± SD. $n = 3$ cultures in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus Ctrl. Ctrl, control untreated cells.

Figure 2 Intracellular content of MG-derived AGEs MG-H1 and ArgPyr in prepubertal porcine SCs treated with T and FSH, alone or in combination, or untreated. MG-H1 intracellular levels, measured by a specific ELISA kit (**A**), MG-H1 and ArgPyr intracellular levels, evaluated by Western blot analysis. Western blot analysis for both MG-H1 and ArgPyr show a major band with an approximate molecular weight of 80 kDa (arrows; **B**). The blots were generated by the appropriate antibodies. The blots were stripped of the bound antibodies and reprobed with anti-β-actin to confirm equal loading. The Western blots shown are representative of three independent experiments. Data are expressed as means ± SD. $n = 3$ cultures in triplicate (**A**). ** $P < 0.01$, *** $P < 0.001$ versus Ctrl. Ctrl, control untreated cells.

Technologies, Carlo Erba, Milan, Italy), according to the manufacturer's instructions and as previously described.^{25,47} Knockdown of *Glo1* was verified biochemically (protein and enzymatic activity) and at the molecular level (transcript) by Western blotting and spectrophotometric assay, or RT-qPCR, respectively.²³ Mock transfection was performed to control for nonspecific effects of the transfection reagent. Because siCtrl- or mock-treated and nontransfected cells were indistinguishable in the assays of biological phenomena examined here (data not shown), the observed changes were shown with respect only to siCtrl-exposed cells. The cells were vital throughout the course of all experiments as determined by trypan blue exclusion assay.

Affinity Purification of MG-Modified Proteins and Amino Acid Sequence Analysis

Affinity purification of MG-modified proteins and amino acid sequence analysis was performed essentially according to Sakamoto et al.⁴⁸ Briefly, cells were lysed in buffer A [50 mmol/L HEPES-KOH (pH 7.0), 0.1% CHAPS, 2 mmol/L

EDTA, 10 mmol/L dithiothreitol, 10% glycerol] by tissue grinder homogenization. The lysate was clarified through successive centrifugation steps (a $1000 \times g$ spin followed by a $10,000 \times g$ spin) at 4°C . Mouse monoclonal anti-ArgPyr antibody (Antibodies-online) was coupled with Affi Gel-HZ (BioRad Laboratories, Hercules, CA), according to the supplier's instructions. The cell lysate was applied to an immunoaffinity column equilibrated with buffer A. The column was washed with buffer A containing 0.5 mol/L NaCl to remove unbound proteins. Bound proteins were eluted with 0.2 mol/L glycine-HCl (pH 2.5), resolved on SDS-PAGE, and visualized using Coomassie Blue staining. After digestion with lysyl endopeptidase (Wako Chemicals, Neuss, Germany) in 50 mmol/L Tris/HCl (pH 8.5), at 37°C for 16 hours, peptide fragments from a purified 80-KDa protein were separated by reversed-phase high-performance liquid chromatography (HPLC). Peptide peaks were monitored by absorbance at 220 nm, and peptide fragments were collected manually. The amino acid sequence of the peptide fragments fractionated by HPLC was defined with a PPSQ-21 gas-phase sequencer (Shimadzu, Kyoto, Japan).

Figure 3 Apoptosis in prepubertal porcine SCs treated with T and FSH, alone or in combination. Apoptosis was evaluated by caspase-3 activation by ELISA (**A**) and DNA fragmentation by electrophoresis (**B**). Electrophoresis is representative of three independent experiments providing the same result. Data are expressed as means ± SD. $n = 3$ cultures in triplicate (**A**). ** $P < 0.01$, *** $P < 0.001$ versus Ctrl. Ctrl, control untreated cells.

Figure 4 Glo1 silencing demonstrates the causative prosurvival role of this enzyme in prepubertal porcine SCs cotreated with T and FSH. Effect of Glo1 silencing (siGlo1) was evaluated on Glo1 transcript (**A**), protein (**B**), and functional (**C**) levels or MG-H1 and ArgPyr intracellular amounts (**D**; only the 80-kDa band is shown) and apoptosis, by caspase-3 activation (**E**). Western blot was obtained by using the appropriate antibodies. The blots were stripped of the bound antibodies and reprobed with anti- β -actin, to confirm equal loading. The Western blots shown are representative of three separate experiments. Experiments performed in the presence of siCtr plus (T + FSH) yielded comparable results to those obtained with (T + FSH) alone (data not shown). Data are expressed as means $\pm$ SD. $n = 3$ (cultures in triplicate). $**P < 0.01$, $***P < 0.001$ versus untreated cells. –, untreated cells; +, treated cells; siCtr, silencing negative control.

Immunoprecipitation

Immunoprecipitation (IP) was performed using Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) according

to the manufacturer's instructions. Briefly, cells were lysed in ice-cold lysis buffer containing PBS, 1% Triton X100, 12 mmol/L sodiumdeoxycholate, 0.1% SDS, and a cocktail of protease inhibitors. The lysate was incubated with

Figure 5 Effect of AG on MG-H1 and ArgPyr intracellular levels and apoptosis under T and FSH cotreatment in porcine prepubertal SCs. **A**: MG-H1 intracellular levels, measured by a specific ELISA kit, and MG-H1 and ArgPyr intracellular levels, evaluated by Western blot (only the 80-kDa band is shown). **B**: Apoptosis, evaluated by caspase-3 activation by ELISA. The blots were generated by the appropriate antibodies. The blots were stripped of the bound antibodies and reprobed with anti- β -actin to confirm equal loading. The Western blots shown are representative of three independent experiments. Data are expressed as means $\pm$ SD. $n = 3$ cultures in triplicate. $**P < 0.01$, $***P < 0.001$. –, untreated cells; +, treated cells.

Figure 6 The apoptosis induced by Glo1 silencing and MG-H1/ArgPyr accumulation occurs via an intrinsic apoptotic pathway involving NF-κB signaling in SCs. Antiapoptotic Bcl-2 and Bcl-XL or proapoptotic Bax proteins, cytochrome c (Cyt c), caspase-3 (**A** and **C**), phospho-IkBα (p-IkBα) protein expression (**B** and **D**) in Glo1-silenced (siGlo1) (**A** and **B**) or AG (**C** and **D**) untreated (–) and treated (+) SCs. Serine 32-phosphorylated IkBα monoclonal antibody was used as a marker of NF-κB. Western blot analysis of β-actin or Cox IV expression is provided to show equal loading of the samples. Blots are representative of three different experiments performed independently, which gave the same results. Experiments performed in the presence of siCtr plus [T + follicle-stimulating hormone (FSH)] yielded comparable results to those obtained with (T + FSH) alone (data not shown). *n* = 3 experiments. siCtr, silencing negative control.

Dynabeads protein G conjugated with mouse anti-IKKβ monoclonal antibody (10AG2; Life Technologies Italia, Monza MB, Italy). Immune complexes were analyzed by Western blot as described above, using the anti-MG-H1 and anti-ArgPyr antibodies.

Statistical Analysis

Data were analyzed using SPSS software version 11.0 (IBM, Armonk, NY). Differences between control and test groups were statistically analyzed using either *t*-test or analysis of variance followed by Bonferroni’s test for

multiple comparisons as necessary. Correlation analyses were performed using Spearman’s correlation test. *P* < 0.05 was considered statistically significant.

Results

Expression of Glo1 in SCs

A significant up-regulation of *Glo1* transcript levels was observed in cells exposed to T and FSH, either alone or in combination, compared with controls (**Figure 1A**). Western blot analysis revealed a significant increase of Glo1 at the

protein level in cells exposed to T and FSH compared with control cells (Figure 1B). In line with the trend of *Glo1* mRNA and protein levels, Glo1 enzymatic activity was also markedly increased in cells treated with T and FSH, compared with untreated control cells (Figure 1C). In all cases, a synergic effect on Glo1 expression was observed when cells were cotreated with both hormones rather than individually.

Intracellular Content of MG-Derived Advanced Glycation End Product MG-H1 and ArgPyr in SCs

Immunodetection of MG-H1 and ArgPyr, AGEs generated from the spontaneous modification of arginine residues by MG, the substrate of Glo1,²³ was performed in total protein extracts from SCs unexposed (control) or exposed to T and FSH. These hormones, singly or in combination, induced a significant decrease in MG-H1 (Figure 2) and ArgPyr (Figure 2) intracellular levels compared with untreated cells. In particular, Western blot analysis for both MG-H1 and ArgPyr showed a major band with an approximate molecular weight of 80 kDa, whose intensity was markedly decreased upon treatments (Figure 2). Again, a synergic effect on MG-H1 and ArgPyr intracellular levels was observed when cells were cotreated with both hormones rather than individually.

T and FSH-Mediated Glo1 Up-Regulation, Paralleled by MG-H1/ArgPyr Intracellular Depletion, Correlates with SC Viability

T, FSH, and T/FSH treatment significantly decreased caspase-3 activation compared with untreated cells, suggesting a role for Glo1 in sustaining SC viability under hormonal exposure (Figure 3A). DNA fragmentation into oligonucleosomes, a morphological hallmark of apoptosis, confirmed the apoptotic responses as evidenced by the decrease in the staining of the typical DNA laddering (Figure 3B).

Glo1/MG-H1—ArgPyr Axis Sustains the Viability of SCs on T/FSH Cotreatment

Glo1 silencing, successfully obtained in SCs at all its expression levels (Figure 4, A–C), was significantly effective in inducing MG-H1 and ArgPyr accumulation (Figure 4D) and apoptosis (Figure 4E). Moreover, pretreatment with AG, a scavenger of free MG^{23,27} (1 mmol/L for 3 hours), and consequently of MG-derived MG-H1 and ArgPyr (Figure 5A), potentiated the apoptotic level observed under T/FSH coexposure (Figure 5B). Altogether, these data showed a role for Glo1/MG-H1—ArgPyr axis in sustaining the viability of SCs upon T and FSH cotreatment.

Figure 7 Identification of MG-H1— and ArgPyr-modified 80-KDa protein. **A:** MG-H1— and ArgPyr-modified 80-KDa protein was purified from the lysate of Glo1-silenced and T/FSH-cotreated SCs by immunoaffinity chromatography with an anti-MG-H1 or anti-ArgPyr monoclonal antibody. Chromatography fractions containing the eluted 80-kDa MG-H1— or ArgPyr-modified proteins were identified by SDS-PAGE and staining with Coomassie blue. The isolated 80-kDa MG-H1— or ArgPyr-modified protein was then digested and resolved as individual peptides by HPLC. **B:** The respective internal peptide was identified as *Sus scrofa* IKKβ upon comparison with standard sequencing databases in the public domain (BLAST). **C:** Lysate from FSH/T-cotreated and Glo1-silenced (siGlo1) SCs was immunoprecipitated with protein G agarose-coupled anti-IKKβ (IP: IKKβ) and subjected to immunoblot (IB) with anti-MG-H1 or anti-ArgPyr antibodies. Mouse IgG was used as negative control for immunoprecipitation. **D:** Supernatant (Sup) and precipitated pellet (Ppt) of IKKβ-IP reactions were run along with whole lysate (Input) and probed with anti-IKKβ antibody. The Western blots shown are representative of three independent experiments.

The Apoptosis Induced by Glo1 Silencing and MG-H1/ArgPyr Accumulation Occurs via an Intrinsic Apoptotic Pathway Involving NF-κB Signaling in SCs

Figure 6A shows the levels of some of the major proteins typically activated in a mitochondrial intrinsic apoptotic pathway, namely the antiapoptotic Bcl-2 or Bcl-XL, the proapoptotic Bax proteins and Cyt c, and the final executioner of apoptosis, caspase-3 (active fragments). All these markers confirmed activation of apoptosis in T/FSH cotreated compared with control cells, being that Bcl-2 or Bcl-XL decreased, Bax increased, Cyt c was released into the cytosol, and the activated caspase 3 level increased

Figure 8 T/FSH-dependent glyoxalase 1 up-regulation sustains the viability of porcine SCs through the control of MG-H1- and ArgPyr-mediated NF- κ B pathway. T and FSH up-regulate (arrows) Glo1 that limits (bracket) intracellular MG-H1 and ArgPyr levels. The low intracellular concentration of MG-H1 and ArgPyr prevents IKK β inhibition, which, in turn, keeps an active NF- κ B signaling, thus preventing apoptosis.

(Figure 6A). More importantly, under hormone coexposure, *Glo1* knockdown reactivated the intrinsic apoptotic mechanism (Figure 6A), showing that *Glo1*, by keeping low the intracellular levels of MG-H1/ArgPyr, was essential to sustain SC viability through the negative control of a mitochondrial apoptotic pathway. A marked reduction in NF- κ B activation, as indicated by the decrease in serine 32-phosphorylated I κ B α , was observed in T/FSH-cotreated cells under *Glo1* silencing, compared with T/FSH-exposed cells (Figure 6B). The use of the monoclonal antibody that detects endogenous levels of serine 32-phosphorylated I κ B α , is an excellent marker of NF- κ B activation,⁴⁹ being that phosphorylation of I κ B α at Ser32 is essential for the release of active NF- κ B.^{25–29,32} Pretreatment with AG confirmed the involvement of MG-H1 and ArgPyr in the induction of the intrinsic apoptotic mechanism involving NF- κ B signaling (Figure 6, C and D).

Identification of MG-H1- and ArgPyr-Modified 80-KDa Protein

MG-H1- and ArgPyr-modified proteins were purified from the lysates of SCs upon *Glo1* silencing and T/FSH cotreatment by immunoaffinity chromatography with the same antibodies used for MG-H1 and ArgPyr immunodetection. The column chromatography fraction containing the eluted 80-kDa MG-H1- or ArgPyr-modified protein was identified by SDS-PAGE and stained with Coomassie Blue (Figure 7A). To determine their identity, the purified 80-kDa MG-H1- and ArgPyr-modified proteins were then digested and resolved as individual peptides by HPLC. As a

result, the internal peptides identified as I κ B kinase β (IKK β) upon comparison with standard sequencing databases in the public domain (Basic Local Alignment Search Tool, BLAST, NCBI; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Figure 7B), suggesting that *Glo1* silencing, upon T/FSH cotreatment, leads to the accumulation of MG-H1- and AP-modified IKK β protein. To further confirm that IKK β was a target of MG-mediated carbonylation, IP was performed. Lysates from SCs cotreated with T and FSH upon *Glo1* silencing were used for IP with beads-coupled anti-IKK β . MG-H1 and ArgPyr were detectable in the immunoprecipitate of IKK β antibody, but not of mouse IgG (control of IP), thus indicating that IKK β was specifically modified by MG to form MG-H1 and ArgPyr (Figure 7C). Supernatant and precipitated pellet of IKK β IP reactions together with whole lysate were probed with anti-IKK β antibody as internal control of IP (Figure 7D).

Discussion

Very little information is known about the determinants that act as prosurvival factors in SCs. *Glo1* is a frontline defense against dicarbonyl glycation in physiological systems, limiting carbonyl stress, a condition occurring under the overload of reactive carbonyl compounds,¹⁵ especially MG, the substrate of *Glo1*.¹⁴ AGEs derived from spontaneous MG adduction of arginine residues are, primarily, hydroimidazolone (MG-H1),^{15,20} and, secondly, ArgPyr.¹⁵ It has been described that both MG and MG-derived AGEs can trigger apoptosis in human cells.^{26–31} In the present work, by performing *Glo1* silencing and using the specific MG

scavenger, AG, we demonstrated, for the first time to our knowledge, that Glo1 sustains viability of porcine prepubertal SCs costimulated with T and FSH, by limiting the intracellular accumulation of MG-H1 and ArgPyr that otherwise would induce a mitochondrial apoptotic pathway (Figure 8). SCs from porcine neonatal prepubertal pig testes were chosen because the pig represents an experimental animal model sharing significant physiological similarity with humans. SCs were cotreated with T and FSH, being a well-established and reliable *in vitro* experimental model mimicking the pituitary–hypothalamic axis–derived endocrine signals associated with puberty.^{8,40,41} The importance of these hormones (FSH and T) in driving spermatogenesis is well documented.^{6,11,42} Augmented hormonal levels trigger the division and differentiation of germ cells, leading to the initiation of spermatogenesis at puberty.⁵⁰ Moreover, both FSH and T promote the supportive role of SCs in this process by regulating SC physiology^{6,11} and specifically metabolism⁶ through stimulating glycolysis by increasing glucose uptake and/or modulating glycolysis-related enzymes.^{6,51,52} Our results on the role of Glo1 in protecting cells from apoptosis through the control of MG-derived AGEs levels are in agreement with the existing literature in other cell models,^{26–31,53–55} including oocytes.^{36–38} More importantly, our findings extend our knowledge on Glo1's role in the male reproductive system, which has never been done before.

NF- κ B is a transcription factor that plays a pivotal role in a number of biological processes, including cell survival. For example, NF- κ B acts as a prosurvival factor by up-regulating the antiapoptotic genes *Bcl-2* and *Bcl-XL*.⁵⁶ The transcriptionally active Rel subunits of NF- κ B are maintained in an inactive form in the cytoplasm via sequestration by inhibitory κ B (I κ B) proteins. NF- κ B activation stimuli induce the phosphorylation of I κ B as a prelude to its proteosomal degradation, unmasking the nuclear localization signal of NF- κ B, allowing its nuclear translocation.⁵⁷ The enzyme complex responsible for phosphorylation of I κ B on specific serine residues is IKK, which consists of at least three subunits: IKK α , IKK β , and IKK γ . Although IKK α and IKK β are both catalytically active, studies in knockout mice have demonstrated that IKK β is responsible for degradation of I κ B.⁵⁷ Hence, the activation of NF- κ B can be blocked by inhibitors of IKK β . Here, it is also first demonstrated that, under T/FSH and *Glo1* knockdown or AG treatment, the intracellular accumulation of MG-H1 and ArgPyr induces a mitochondrial apoptotic pathway driven by the desensitization of NF- κ B signaling, constitutively expressed in SCs,^{58,59} through the modification of MG-H1/ArgPyr-mediated IKK β (Figure 8), thus extending our knowledge of the mechanisms through which MG-derived AGEs mediate desensitization of NF- κ B signaling to induce apoptosis.^{26,29,32,60,61} It is assumed that both MG-H1 and ArgPyr may inhibit IKK β activation in SCs because these modifications have been shown to modify both biological activity^{16,17} and stability^{18,62} of other proteins.

SCs mainly support spermatogenesis under the regulation of T and FSH, whose receptor, among testicular cells, is located exclusively on SCs. Here, a novel mechanism is proposed through which both hormones sustain SC viability and, thus, spermatogenesis, which is mediated by Glo1 up-regulation through the above-described mechanism. Although the presence of regulatory sequences responsive to testosterone in the *Glo1* gene have been previously described,⁶³ no data on the responsiveness of Glo1 to FSH are currently available. Thus, this study extends the limited understanding of the molecular control of Glo1 regulation.¹⁵

The idea that SCs are essential for successful spermatogenesis has now gained wide acceptance. Indeed, the mechanism underpinning this effect is now also largely understood; SCs take up extracellular glucose and convert it into lactate, the preferred fuel of developing germ cells.^{6,9,64} To fulfill this provisioning role, SCs produce high quantities of lactate through glycolysis⁶; indeed, in their high glycolytic flux and lactate production, the metabolism of SCs is often compared with the “Warburg-like”^{12,pp.127,128} metabolism observed in cancer cells.¹¹ However, this high glycolytic activity likely yields high quantities of MG as a toxic byproduct, especially under the influence of T/FSH exposure, placing even greater importance on protective mechanisms that mitigate the effects of MG-induced carbonyl stress. As the principal enzyme for the detoxification of MG, the up-regulation of Glo1 in T/FSH-treated SCs appears to represent an important way in which the metabolic demands of spermatogenesis are matched by protective mechanisms in SCs to vouchsafe their viability and, thereby, spermatogenesis itself.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.013>

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