DESOGESTREL DOWN-REGULATES PHOX2B AND ITS TARGET GENES IN PROGESTERONE RESPONSIVE NEUROBLASTOMA CELLS

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Abbreviations: Sp1: Specificity Protein 1; CREB: CAMP Responsive Element Binding Protein; c-Jun: c-Jun proto-oncogene; *Ap2α:* Activator protein 2 alpha; CCHS: Congenital Central Hypoventilation Syndrome; 3-KDG: 3-Ketodesogestrel; PGR: human Progesterone Receptor; DRB: 5,6 dichloro-beta-D-ribofuranosylbenzimidazole

ABSTRACT

The paired-like homeobox 2B gene (*PHOX2B*) encodes a key transcription factor that plays a role in the development of the autonomic nervous system and the neural structures involved in controlling breathing. In humans, PHOX2B over-expression plays a role in the pathogenesis of tumours arising from the sympathetic nervous system such as neuroblastomas, and heterozygous *PHOX2B* mutations cause Congenital Central Hypoventilation Syndrome (CCHS), a life-threatening neurocristopathy characterised by the defective autonomic control of breathing and involving altered CO_2/H^+ chemosensitivity. The recovery of CO_2/H^+ chemosensitivity and increased ventilation have been observed in two CCHS patients using the potent contraceptive progestin desogestrel. Given the central role of *PHOX2B* in the pathogenesis of CCHS, and the progesterone-mediated effects observed in the disease, we generated progesterone-responsive neuroblastoma cells, and evaluated the effects of 3-Ketodesogestrel (3-KDG), the biologically active metabolite of desogestrel, on the expression of *PHOX2B* and its target genes. Our findings demonstrate that, through progesterone nuclear receptor PR-B, 3-KDG down-regulates *PHOX2B* gene expression, by a post-transcriptional mechanism, and its target genes and open up the possibility that this mechanism may contribute to the positive effects observed in some CCHS patients.

Keywords: PHOX2B, Desogestrel, congenital central hypoventilation syndrome, neuroblastoma, progesterone receptor

INTRODUCTION

Heterozygous mutations (frameshift mutations and poly-alanine triplet expansions) in the *PHOX2B* gene, a key transcription factor that plays a role in the development of the autonomic nervous system and the neural structures involved in controlling breathing [1,2], cause Congenital Central Hypoventilation Syndrome (CCHS; MIM# 209880), a syndrome of autonomic dysfunction that is characterised by life-threatening sleep-related hypoventilation caused by an abnormal ventilatory response to carbon dioxide: children affected by CCHS require ventilatory support during non-REM sleep (tracheostomy or non-invasive ventilation), although a milder degree of hypoventilation may also be present during REM sleep and wakefulness [3-5]. CCHS may also be associated with a spectrum of non-respiratory symptoms and other conditions (cardiac arrhythmias, ocular and endocrinological disorders) that reflect more generalised autonomic nervous system (ANS) dysregulation, including Hirschsprung's disease and tumours of neural crest origin, and we and others have reported that the complexity of clinical phenotypes is influenced by the type of mutation (frameshift mutations *vs* poly-alanine triplet expansions), the type of translational frame, and gene dosage [6-13]. No pharmacological treatment has yet proved to be effective in improving the disease-related respiratory defects. Recent findings [14] have shown that two CCHS female patients (respectively with +5 and +6 alanine expansions) using the progestin contraceptive desogestrel for contraceptive purposes experienced a partial recovery of chemosensitivity and increased ventilation, thus opening up new prospects for a pharmacological intervention that could at least improve respiratory symptoms. Desogestrel has much greater affinity for progesterone receptors than progesterone itself [14,15].

Progesterone is a steroid that is primarily synthesised in the gonads, adrenal glands and placenta but, as it is also synthesised *de novo* in the central and peripheral nervous system, can be classified as a neurosteroid. It is well known that progesterone is a powerful respiratory stimulant that may be useful for treating adult apnea, and it has been suggested that it could also be used for the treatment of apnea in pre-term neonates [16-18]. Progesterone exerts its biological effects by binding nuclear or membrane progesterone receptors, and recent data provide evidence that nuclear receptors are important modulators of respiratory control during sleep and chemoreflex sensitivity: adult female mice from which the nuclear receptor has been deleted show more frequent sighs and post-sigh apnea during non-REM sleep, and reduced responses to hypercapnia after chronic progesterone treatment [19]. Although the exact molecular mechanism of the pharmacological effect of desogestrel is unknown, recent evidence indicates that multiple pathways, involving both medullary and supramedullary neurons, may play a role in the ventilatory effects of desogestrel [15,20]. It is also worth noting that a number of rodent brain structures (including some expressing

Phox2b in adulthood) are activated by desogestrel and the same structures may be involved in the recovery observed in the two CCHS patients; however, the contribution of PHOX2B and its mutations to respiratory improvement has not yet been investigated.

Given the central role of the *PHOX2B* gene in the pathogenesis of CCHS and the co-expression of progesterone receptors and PHOX2B in neurons that play a role in CO_2 chemosensitivity [21,22], the aim of this study was to determine whether treating PHOX2B-expressing cell lines with the biologically active metabolite of desogestrel, 3-Ketodesogestrel (3-KDG) or etonogestrel [23], affects the expression of *PHOX2B* and its target genes. In order to investigate the relationship between progestin and PHOX2B activity further, we generated a stable progestin-responsive SK-N-BE(2)C neuroblastoma cell line. Neuroblastoma is a universally accepted model of neural crest derivative, with most of the molecular features of a sympathetic ganglionic cell, a kind of structure heavily affected in Phox2b knock-out mice, that likely share a fraction of its PHOX2B-dependent transcriptome with other types of PHOX2B^{+} neurons. In this model, our results provide the evidence of a direct molecular link between PHOX2B and Desogestrel.

MATERIALS AND METHODS

Cell cultures

The SK-N-BE(2)C, IMR32 and SH-SYSY human neuroblastoma cell lines, and the MCF7 and T47D breast carcinoma cell lines were grown in RPMI 1640, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (LONZA), supplemented with 10% foetal bovine serum (FBS, Euroclone) at 37°C with 5% CO_2. The T47D cell line was maintained in a medium with added human Insulin (Sigma) to a final concentration of 5μg/mL.

To generate SK-N-BE(2)C neuroblastoma cell line stably expressing human progesterone receptors (PGRs), 7.5 x 10^5 cells were plated in 100 mm dishes and transfected with 10 μg *PGR*-pCDNA 3.1 myc-His plasmid using a FuGene HD (Promega) transfection reagent in a ratio of 1:4 (DNA:FuGene). Forty-eight hours after transfection, the SK-N-BE(2)C cells were diluted 1:10 in 100 mm dishes and grown under 700 μg/mL geneticin selection (G-418 Sulfate, Gibco), and the individual colonies were transferred to multi-well plates for further propagation. The selected clones were screened for the expression of intracellular progesterone receptor protein by means of a Western blot assay. The stable SK-N-BE(2)C 5.8 and 6.30 clones were maintained under selection by adding geneticin (G-418 Sulfate, Gibco) at a final concentration of 0.4 mg/mL, with selective medium changes every second day. The desogestrel treatments were carried out by adding 1 nM of the active 3- Keto-desogestrel metabolite (3-KDG, USBiological) for the times indicated in the figure legends. Transcript and protein stability were respectively measured by means of treatment with 75 μM 5,6 dichloro-beta-D-ribofuranosylbenzimidazole (DRB, Sigma) and 1 μg/mL Cycloheximide (CHX, Sigma) for the times indicated in the figure legend.

Total RNA extraction, reverse transcription and qPCR

To measure *PHOX2B* and *PGR-B* mRNA expression level, total RNA was extracted using the RNeasy™ Mini kit or RNA plus Micro kit and accompanying QIAshredder ™ (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Five hundred nanograms of RNA were reverse transcribed using the GoScript™ Reverse Transcriptase kit (Promega), and *PHOX2B*, *PGR-B* and *GAPDH* transcripts were quantitatively analysed using an ABI Prism Thermocycler QuantStudio 5 (Applied Biosystems, CA). The TaqMan® primer and probe assays (Life Technologies, Inc.) were human *PHOX2B* (ID #Hs00243679_m1), human *PGR* (ID #Hs01556702_m1), and the endogenous control *GADPH* (ID #Hs99999905_m1). Each sample was run in triplicate, and the results were calculated using the $2^{-\Delta CT}$ and the $2^{-\Delta\Delta CT}$ methods in order to allow the normalisation of each sample to *GAPDH* and comparisons with the calibrator of each experiment (set to a value of 1) as indicated in the figure legends.

Transient transfections and Luciferase assays

To evaluate the *TLX2*-luc or *DBH*-luc reporter vectors activity, the cells were transiently transfected or co-transfected by means of lipofection using FuGENE HD transfection reagent (Promega) as previously described [24] using 1.7 x 10^5 SK-N-BE(2)C cells. Twenty-four hours after transfection, the cells were treated with 1 nM of 3-KDG (USBiological) or 3 nM progesterone (Sigma) for the times indicated in the figure legends.

Luciferase was assayed using the Dual Luciferase Reporter Assay System (Promega) in a GloMax Discovery Luminometer (Promega), and the results were analysed as previously described [24]. All of the transfections were performed in triplicate, and each construct was tested in at least three independent experiments using different batches of plasmid preparation. The results are given as mean values ± standard deviation (SD), and the data were analysed using an unpaired two-tailed Student's *t* test or one-way ANOVA and GraphPadPrism 5 Software (GraphPad Software, Inc.) as described in the figure legends; p values of <0.05 were considered significant.

Plasmid construction

The h*PGR*-pCDNA 3.1 myc-His plasmid, containing the human intracellular progesterone receptor and used to generate the stable SK-N-BE(2)C clones, was obtained by re-cloning the region corresponding to the human *PR-A* encoding sequence (kindly gifted by Angelo Poletti, University of Milan) into the Bam HI sites of the pCDNA 3.1 myc-His vector (InVitrogen). The region encoding the N-terminus of the *PR-B* isoform was amplified by means of PCR using cDNA extracted from T47D cells and the GC-rich PCR system (Roche Applied Science), and sub-cloned into the Kpn I and BstE II sites of the pCRII vector (InVitrogen). The oligonucleotide sequences were: forward primer, 5'-CGT CAG ACT GAG CTG AAG GCA AAG- 3'; and reverse primer, 5' - GGT GGA TCT TCG GGA AGT TCG GG- 3'. In order to obtain the full-length h*PGR*-pCDNA 3.1 myc-His construct, the *5'PR-B* fragment was cloned upstream of the *hPR-A* fragment in the *hPR-A*-pCDNA 3.1 myc-His plasmid, after double enzymatic digestion with Kpn I/Pf1M I restriction enzymes.

The *TLX2* (-1185bp) and *DBH* promoter constructs, used to evaluate the effect of desogestrel on the transcription activity of PHOX2B target genes, have been previously described [25,26]. The progesterone responsive *MMTV* promoter reporter construct contained the murine mammary tumour virus long terminal repeat (*MMTV* LTR) inside the pGL3-basic vector (Promega).

All of the constructs were checked by means of restriction analysis and partial sequencing of both strands. All of the enzymes used for the cloning were purchased from New England Biolabs.

Protein preparation and Western Blot analyses

For evaluating the effect of desogestrel on PHOX2B and its target genes protein level, total protein extract was prepared from SK-N-BE(2)C cells and the 5.8 and 6.30 clones stably over-expressing PGR using the freeze and thaw method, and was analysed by means of Western blotting as previously described [27]. Table S1 shows the primary and secondary antibodies used.

After appropriate washes, the bands were revealed using Super Signal West Dura (Thermo Fisher Scientific), for the secondary antibodies conjugated with horseradish peroxidase, or the Odyssey CLx Infrared Imaging System for infrared-conjugated IgG IRDye (LI-COR Bioscience). A standard molecular weight (PageRuler^{TM} Broad Range Unstained Protein Ladder, Thermo-Fisher Scientific and Prestained Protein SHARPMASST^M VII, EuroClone) was loaded in parallel. Densitometric analysis of the obtained signals was carried out using NIH ImageJ 1.61/fat, or Image Studio (LI-COR Biosciences) software, and the results are shown as the mean values ± standard deviation (SD) of at least three independent experiments.

The statistical analyses were made using ANOVA or an unpaired two-tailed Student's *t* test and GraphPad Prism 5 Software (GraphPad Software, Inc.) as indicated in the figure legends. P values of < 0.05 were considered significant.

RESULTS

Identification of a cell model to study the effect of 3-KDG on PHOX2B

In order to investigate the mechanism of action of the desogestrel and whether it can affect the expression or activity of PHOX2B and some relevant PHOX2B target genes, we carried out preliminary experiments using the T47D human breast adenocarcinoma cell line, because it has the advantage of expressing intracellular and membrane-associated progesterone receptors, but lacks *PHOX2B* expression. The preliminary experimental evidence obtained by co-transfecting *PHOX2B* cDNA and the *TLX2*-luc PHOX2B target gene promoter into the T47D cells showed that 3-KDG (the active metabolite of desogestrel) up-regulates the transcription of the *TLX2* promoter reporter vector, an effect that is mainly mediated by the intracellular progesterone receptor PR-B (data not shown).

This interesting finding and the notion that neurons that play a central role in CO_2 chemosensitivity co-express progesterone receptors and PHOX2B [21,22] prompted us to study the effect of desogestrel on the PHOX2B pathway in a cell model whose cellular background is more closely associated with the physiological PHOX2B environment also in terms of co-activators and co-repressors. To this end, we used real-time PCR to characterise *PHOX2B* expression (Fig. S1A, grey bars) and progesterone receptor (*PGR*, the main receptor mediating 3-KDG activities; see Fig. S1A, black bars) in various neuroblastoma cell lines. The IMR32, SK-N-BE(2)C and SH-SY5Y cell lines showed *PHOX2B* expression, but only IMR32 and SK-NBE(2)C also expressed *PGR*, albeit at a level that was 30,000 and 200,000 times lower than its expression in the T47D cell line (Fig. S1A, black bar). Despite their *PGR* expression, these cell lines were not responsive to progesterone or 3-KDG treatment, as confirmed by transient transfection with the PGR-responsive reporter construct *MMTV*-luc (Fig. S1B, left bars), due to the absence of the PGR protein (Fig. 1A, lanes 1 and 2), but they could be made responsive by transiently co-transfecting the PGR expression vector (Fig. S1B, white striped and cross-hatched bars *vs* white empty bar). One possibility of the observed loss of progesterone receptor is that neuroblastoma cells express alternatively spliced PGR mRNA variants that give rise to PGR that miss functional domains and make them undetectable by the antibodies used in this study, as already described [28]. Consequently, although the IMR32 cell line expressed higher basal *PHOX2B* and *PGR* transcript levels than SK-N-BE(2)C (Fig. S1A), we decided to use SK-N-BE(2)C because of its greater capacity for transfection.

3-KDG reduces the expression of PHOX2B and of its target genes

Western blot and densitometric analyses of SK-N-BE(2)C cells transiently transfected with *PGR* cDNA (h*PGR*-pcDNA3.1 myc-His) (Fig. 1A, lanes 3 and 4) or pCDNA3 control vector (Fig.

1A, lanes 1 and 2) and treated with 1 nM of 3-KDG for 48 hours (Fig. 1A, lanes 2 and 4) showed that PHOX2B protein levels were significantly reduced 3-fold in the cells transfected with h*PGR* and treated with 3-KDG in comparison with untreated cells (Fig. 1B, white striped bar *vs* white bar), whereas no significant effect was observed in the cells transfected with the control vector (Fig. 1B, striped *vs* plain black bars), thus confirming that 3-KDG has a direct effect on the expression of PHOX2B protein in this cell line. Similarly, the PHOX2B mutant protein carrying one of the most frequent poly-alanine expansion (+7) observed in CCHS was down-regulated upon treatment with 3-KDG (data not shown).

The down-regulation of PHOX2B protein expression after 3-KDG treatment affects the promoter activity of two known PHOX2B target genes, T-cell leukaemia homeobox 2 (*TLX2*), and dopamine beta-hydroxylase (*DBH*), both expressed in the SK-N-BE(2)C cell line. Figures 1C and 1D respectively show a significant 8- and 5-fold decrease in luciferase activity in the cells co-transfected with h*PGR* and the *TLX2*-luc or *DBH*-luc reporter vectors and treated with 3-KDG (white striped bars *vs* white empty bars). Progesterone had a slightly less efficient but still significant effect (Fig. 1C and 1D, white cross-hatched bars *vs* white empty bars), as it led to a 4-fold and 2-fold reduction in *TLX2* and *DBH* promoter activity. The control cells transfected with luciferase promoter-less reporter constructs pGL3b (*vs TLX2*-luc) and pGL4b (*vs DBH*-luc) did not show any significant reduction induced by progestin treatment (Fig. 1C and 1D, striped and cross-hatched *vs* empty grey bars), thus confirming the consistency of the effect of 3-KDG on the two PHOX2B target genes, and suggesting that this reduction may be due to decreased PHOX2B protein availability.

3-KDG down-regulates endogenous PHOX2B protein and target genes in stable progestin-responsive SK-N-BE(2)C cells

In order to investigate the relationship between progestin and PHOX2B activity further, we generated a stable progestin-responsive SK-N-BE(2)C neuroblastoma cell line that allowed us to avoid the limiting step of the transient *PGR* transfection (Fig. S2A). The *PGR* expressing vector used for the stable transfection encodes the two isoforms of intracellular progesterone receptors: the full-length B isoform (PR-B) and the N-terminal truncated isoform (PR-A). Two clones, that had different relative expression levels of the PR-A and PR-B isoforms and named 5.8 and 6.30, were chosen for further analyses (Fig.S2A).

Figure 1A showed that 3-KDG treatment reduced PHOX2B protein levels in SK-N-BE(2)C cells transiently transfected with *PGR*. In order to investigate the mechanism underlying this reduction further and analyse the possible effect of 3-KDG on the PHOX2B pathway, the stable

SK-N-BE(2)C clones 5.8 and 6.30 were treated with 1 nM 3-KDG for 8, 24 and 48 hours, and the endogenous level of PHOX2B and of some known PHOX2B target genes was evaluated (Fig. 2 and Fig.3). Western blotting (Fig. 2A) and quantification analyses (Fig. 2B) showed that treatment significantly reduced the level of PHOX2B protein in both clones (Fig 2B, grey and white striped bars *vs* grey and white empty bars) in comparison with the native SK-N-BE(2)C cells used as controls (Fig 2B, black striped and empty bars), albeit with different kinetics between clones. PHOX2B protein level decreased more rapidly in clone 5.8 than in clone 6.30 (Fig 2A, lanes 6-8 *vs* lanes 10-12), as it was 35% in clone 5.8 and 90% in clone 6.30 after eight hours treatment (Fig. 2B, grey *vs* white striped bars).

We then investigated whether a reduced PHOX2B protein level could affect the expression of some of its already known target genes, *DBH* and *TFAP2A (Ap2α)*. As shown in Figure 3A, as in the case of PHOX2B, a significant reduction in proteins level at each time point was observed in clone 5.8 (Fig. 3A, lanes 6-8 *vs* lane 5), in comparison with the treated native cells (Fig. 3A, lanes 2-4 *vs* lane 1). In particular, after 48 hours 3-KDG treatment DBH was almost undetectable (Fig. 3B, grey striped bar), whereas TFAP2A was reduced by 95 % only after eight hours (Fig. 3C, grey bars). DBH and AP2α levels were also reduced in clone 6.30 (Fig. 3B and C, white bars), but to a lesser extent, which led us to hypothesise that the amount of PR-B may affect the expression of these genes differently.

For control purposes, we analysed the expression of proteins that are not direct PHOX2B target genes, such as SP1, CREB and c-JUN (Fig. S3A). There was no significant change in their expression in either of the clones (Fig. S3B to D) or in the control cells, thus confirming the direct effect of desogestrel on PHOX2B expression and/or activity.

In conclusion, our findings demonstrated that 3-KDG directly affects PHOX2B and its target genes by reducing their expression, thus indicating that it has a specific effect on the PHOX2B pathway.

3-KDG partially affects *PHOX2B* mRNA degradation

In order to investigate the mechanism underlying these effects further, we tested whether the decrease in protein level in clone 5.8 (which showed the most rapid kinetic reduction in PHOX2B protein) was due to reduced transcription or a post-transcriptional level mechanism.

Quantitative PCR analysis of *PHOX2B* mRNA expression level (Fig. 4A) in SK-N-BE(2)C clone 5.8 and native SK-N-BE(2)C cells treated with 1 nM 3-KDG for eight, 24 and 48 hours showed that 3-KDG significantly reduced *PHOX2B* mRNA level by 50%, (Fig. 4A grey bars) and, unlike the protein, which showed a significant reduction of up to 10% (Fig. 2A), this was maintained during

the following 48 hours of treatment. No change in the level of mRNA was observed in the native SK-N-BE(2)C cells (Fig 4A, black bars). As shown in Figure 4B (grey bars), *PHOX2B* mRNA levels were reduced after as little as two hours of treatment, thus supporting the hypothesis that the reduction in PHOX2B protein was partially due to a reduction in mRNA, which may occur because of reduced transcription or reduced transcript stability.

In order to clarify this, we measured the stability of *PHOX2B* mRNA in clone 5.8, by determining its decay after using the RNA pol II inhibitor DRB to inhibit transcription in the presence and absence of 3-KDG treatment (Fig. 4C). Before adding DRB, the cells were pre-treated for 24 hours with 3-KDG or vehicle alone (Fig. 4C). The level of *PHOX2B* mRNA was reduced by 50% in approximately one hour regardless of the presence (Fig. 4C, solid line) or absence of 3-KDG (Fig. 4C dashed line) but, after two hours, it became significantly different because the mRNA was no longer degraded in the absence of 3-KDG (Fig. 4C). This was not due to the induction of transcription of the factor required to degrade *PHOX2B* mRNA by 3-KDG because the same result was obtained when 3-KDG was added to DRB (Fig. 4D).

These findings suggest that 3-KDG partially affects *PHOX2B* mRNA stability, and indicate that the 50% reduction in mRNA was due to greater degradation in the treated sample.

3-KDG does not affect PHOX2B protein stability

The reduction in *PHOX2B* mRNA levels in experiments in Figures 4A and 4B does not totally explain the drastic reduction in protein levels in clone 5.8 after 3-KDG treatment (Fig. 2A). In order to investigate the underlying mechanism further, we tested whether 3-KDG also affects PHOX2B protein stability by pre-treating SK-N-BE(2)C clone 5.8 cells with 1μg/mL cycloheximide (CHX) for one hour, followed by the addition of 3-KDG or DMSO for different times (Fig. 5A), and found no difference in the PHOX2B degradation rate (Fig. 5B, dashed line *vs* solid line). Pre-treatment with 3-KDG before adding CHX confirmed that 3-KDG does not induce a factor acting on the degradation of PHOX2B protein.

In conclusion, our findings demonstrated that the effects of 3-KDG on PHOX2B are mediated by increased *PHOX2B* mRNA degradation (Figs. 4C and D). As the drastic reduction in PHOX2B protein cannot be exclusively attributed to increased protein degradation, we hypothesise that 3-KDG reduces PHOX2B protein by also blocking its translation.

DISCUSSION

The findings of this study demonstrate that the biologically active metabolite of desogestrel, 3-KDG, modulates *PHOX2B* gene expression via progesterone nuclear receptor PR-B. Treatment with 3-KDG decreased both *PHOX2B* mRNA and protein levels as a result of an increased mRNA degradation rate and possibly concomitant translation inhibition. Apparently, the increase in mRNA degradation rate and inhibition of translation do not require the synthesis of new molecules, and so the molecular factor(s) involved in the two processes remain to be identified and further investigated. Moreover, PHOX2B protein down-regulation is paralleled by a decrease in two of its well known target genes (DBH and TFAP2A), whereas other genes not regulated by PHOX2B are not affected.

Transcription block experiments showed that the kinetics of *PHOX2B* mRNA degradation is biphasic: almost one half is rapidly degraded in one hour but, after that, the degradation slows and there is no further decrease in mRNA level, thus suggesting the existence of two *PHOX2B* mRNA pools. Our findings indicate that 3-KDG increases the rate of *PHOX2B* mRNA degradation, and in particular that of the more stable pool of mRNAs. Given that the *PHOX2B* 3' untranslated region (3'UTR), and the microRNAs (miRNAs) targeting it are important for the post-transcriptional regulation of the *PHOX2B* gene, a possibility is that various isoforms of different 3′ UTR lengths are synthesised and have distinct properties and different degradation rates [29]. However, mRNA does not completely disappear and is maintained at a certain level by means of compensatory transcriptional up-regulation. As we observed very little induction of the *PHOX2B* promoter reporter construct (data not shown), we cannot exclude the possibility that the degradation induces a more general synthesis [30]. The different steady-state levels of PHOX2B protein at different time points of treatment and in two stable clones differently expressing progesterone receptors influence the degree of down-regulation of PHOX2B target genes in a gene specific manner. A slight decrease in PHOX2B is not sufficient to down-regulate all PHOX2B target genes, and it has been previously reported that the partial silencing of *PHOX2B* only slightly affects PHOX2B target gene expression [31]. Interestingly, progesterone induces the up-regulation of tyrosine hydroxylase, a well-known PHOX2B target gene, in the SK-N-SH neuroblastoma cell line (which is negative for *PHOX2B* and expresses very low levels of *PHOX2A*) and in the noradrenergic CAD cell line (which expresses higher levels of *PHOX2A* than *PHOX2B*), thus suggesting that PHOX2B and the cellular context play a critical role in determining positive or negative progesterone-mediated effects [32].

Unlike what has been observed in neuroblastoma cell lines in which *PHOX2B* over-expression plays a pathogenic role, the respiratory and autonomic symptoms of CCHS patients, and

their predisposition to Hirschsprung's disease and intestinal developmental and functional defects, are due to *PHOX2B* haploinsufficiency [12,33]. In this perspective, the positive effects of desogestrel on ventilation and chemosensitivity observed in two CCHS patients [14,15] are somewhat unexpected because, at molecular level, 3-KDG decreases *PHOX2B* expression. However, *in vivo* findings argue against the idea that CCHS is due to a pure loss-of-function mechanism, and it is likely that toxic functions gained by mutant proteins also contribute to the respiratory defects [34-39]. In this light, as we observed that PHOX2B mutant protein levels decrease similarly to those of its wild-type counterpart after 3-KDG treatment (data not shown), the beneficial effect of decreasing the level of the mutant PHOX2B may clinically compensate for the potential pathogenic effect of insufficient PHOX2B expression, especially after birth when the differentiation of a number of PHOX2B$^+$ cell types and autonomic nervous system components such as the enteric nervous system (ENS) has already occurred, at least in patients carrying mild PHOX2B polyAla expansions. It is worth noting that the role of *Phox2b* and its mutations has been widely investigated during embryonic development, but their exact role in adulthood is still unclear. *Phox2b* gene expression persists in various structures in adult rats and human [21,40], and various findings indicate that it plays a role in maintaining the function of noradrenergic neurons in the adult [41]. Recent studies have also shown residual cardiorespiratory responses in prevalently younger CCHS patients, thus suggesting age-dependent mechanisms such as the developmental reconfiguration of respiratory circuits or the continuing loss of vulnerable cell populations within them due to the expression of an altered PHOX2B transcription factor [42]. In mouse models of CCHS, the development of the retrotrapezoid nucleus (RTN), which expresses *Phox2b*, is particularly vulnerable to different *Phox2b* mutations (frameshift mutations and poly-alanine triplet expansions) that cause CCHS in humans [34,38,39], and dysfunctioning CO_2/H^+ chemosensitive/Phox2b-positive neurons in the RTN/pFRG is generally thought to play a critical role in perinatal respiratory control in rodents [43]. However, the fact that mice can survive the selective genetic deletion of the RTN PHOX2B-positive neurons and the loss of breathing stimulation by CO_2 suggests that the neurological basis of CCHS is not confined to the absence of the RTN and that compensatory mechanisms may partially restore CO_2 sensitivity [36]. Other structures, including some expressing *Phox2b* in adulthood, are grossly unaltered in *Phox2b* mutant mice (and reasonably in humans), and may be involved in the recovery of the two CCHS patients. One putative structure is the nucleus of the solitary tract (NTS), which expresses *Phox2b* in adult rats, is not affected by *Phox2b* poly-alanine mutations, and contains neurons that are inhibited by hypoxia but whose activity is restored by progesterone administration [34,44-46]. Consistently, neurons in the commissural part of the NTS are activated by 3-KDG [15,20]. C1 neurons may also

be involved in the recovery of the two CCHS patients as PHOX2B has been identified in selected populations of TH-expressing neurons in adult rats, including the C1 neurons [44] that regulate the breathing network at multiple levels [47,48]. It has also been shown that 3-KDG activates catecholaminergic neurons in the ventrolateral medullary reticular nucleus [15].

In conclusion, although our results are based on an *in vitro* system such as neuroblastoma cell lines, the findings of this study provide the evidence of a direct molecular link between PHOX2B and Desogestrel by showing that 3-KDG down-regulates the expression of *PHOX2B* and its target genes and open up the possibility that the down-regulation of PHOX2B and mutant proteins may contribute to the recovered chemosensitivity and increased ventilation observed in the two CCHS patients taking desogestrel. However, further research is needed to evaluate the usefulness of pharmacological approaches targeting *PHOX2B* and possibly attenuating the deleterious effects of *PHOX2B* mutations in CCHS patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Figure 1. Effects of 3-KDG on the expression of PHOX2B protein and its transcriptional activity on the TLX2 and DBH target promoters

A and B) Western blot analysis and quantification of the effect of 3-KDG on PHOX2B endogenous protein in SK-N-BE(2)C cells. *A*) Total protein extracts of SK-N-BE(2)C cells transfected with the PGR expression vector (lanes 3 and 4) or the empty vector pCDNA3 (lanes 1 and 2), and treated with 3-KDG for 48 hours (lanes 2 and 4). Extracts of the IMR32 (lane 5) and T47D cell lines (lane 6) were respectively used as positive controls of PHOX2B and PGR expression. *B*) Relative quantification of PHOX2B protein in untreated (black and white bars) and 3-KDG treated cells (hatched bars). The results are the mean PHOX2B values ($\pm$ SD, error bars) normalised to that of β-tubulin (TUB) in two independent experiments, and are expressed as fold-induction over untreated cells (=1). *$p<0.05$, significant differences in PHOX2B levels between the treated (white hatched bar) and untreated (white bar) cells transfected with the hPGR vector (Student's *t* test). *C and D*) Luciferase assay. The bars indicate the transcriptional activity of reporter constructs driven by the *TLX2* (C, right) and *DBH* promoter (D, right) upon the co-transfection of the PGR expression vector in SK-N-BE(2)C cells treated with 1 nM 3-KDG (hatched bars) or 3 nM progesterone (cross-hatched bars) for 48 hours. The empty vectors pGL3b (C, left) and pGL4b (D, left) were used as negative controls (grey bars). The results are the mean values $\pm$ SD (error bars) of the transcriptional activity of the constructs in at least five experiments performed in duplicate, and are expressed as RLU normalised to that of *Renilla*. ***$p<0.001$, significant differences in activity between the treated and untreated cells transfected with *TLX2* or *DBH* promoters (one-way ANOVA, Tukey's test).

Figure 2. Effects of 3-KDG on endogenous PHOX2B protein in stable SK-N-BE(2)C hPGR clones.

A) Representative Western blot of endogenous PHOX2B in SK-N-BE(2)C cells stably over-expressing hPGR (clones 5.8 and 6.30, lanes 5 to 12) and untransfected SK-N-BE(2)C cells (lanes 1 to 4) after treatment with 1 nM 3-KDG for eight, 24 or 48 hours. IMR32 nuclear extract (IMR32 NE, lane 13) and T47D (lane 14) were respectively used as positive and negative controls for PHOX2B. *B*) The bar chart shows the relative quantification of PHOX2B protein in native SK-N-BE(2)C cells (black bars), SK-N-BE(2)C clone 5.8 cells (grey bars) and SK-N-BE(2)C clone 6.30 cells (white bars) after 3-KDG treatment (hatched bars). The data show the relative fold induction in the treated samples in relation to the untreated samples (=1), and are expressed as the mean values ($\pm$ SD, error bars) of the densitometry intensity of each lane normalised to β-tubulin (TUB) in at

least three independent experiments. ***p <0.001, **p<0.01 and *p<0.05, significant differences in PHOX2B protein levels between treated and untreated (-) clones 5.8 and 6.30 (one-way ANOVA, Tukey's test).

Figure 3. Effects of 3-KDG on PHOX2B target genes.

A) Representative Western blots of PHOX2B target genes expression in stable SK-N-BE(2)C clones 5.8 (lanes 5-8) and cl. 6.30 (lanes 9-12), and native SK-N-BE(2)C cells (lanes 1-4) after treatment with 1 nM 3-KDG for eight, 24 or 48 hours. The DBH and TFAP2A signal were normalised to that of β-tubulin (TUB). *B, and C*) The bar charts show the relative quantification of DBH (B) and TFAP2A (AP-2 alpha, C) protein levels in native SK-N-BE(2)C cells (black bars), clone 5.8 (grey bars) and clone 6.30 cells (white bars) after 3-KDG treatment (hatched bars). The data indicate the relative fold induction in the treated samples in relation to the untreated samples (=1), and are expressed as the mean values ± SD (error bars) of at least three independent experiments. **p<0.01 and *p<0.05, significant differences in DBH (B) and TFAP2A (C) protein levels between the treated and untreated (-) cells (one-way ANOVA, Tukey's test).

Figure 4. 3-KDG affects *PHOX2B* mRNA stability

A) Time course qPCR analysis of endogenous *PHOX2B* mRNA expression normalised to that of the *GAPDH* gene in native SK-N-BE(2)C (black bars) and clone 5.8 cells (grey bars) treated with 1 nM 3-KDG (hatched bars) for the indicated times. The bars indicate the relative fold induction in the treated samples in relation to the untreated samples (=1) calculated using the $2^{-\Delta\Delta CT}$ method, and are expressed as the mean values ± SD (error bars) of at least three independent experiments performed in triplicate. *p <0.05, statistically significant differences in *PHOX2B* mRNA expression in SK-N-BE(2)C clone 5.8 cells, between the treated and untreated samples (one-way ANOVA, Tukey's test). *B*) The same experiment as in *A* was performed for a shorter time. The bars represent the mean values ± SD (error bars) of fold induction in comparison with the untreated sample (=1 grey bar) in a single representative experiment performed in triplicate. ***p<0.001, statistically significant differences between the treated and untreated samples (one-way ANOVA, Tukey's test). *C and D*) *PHOX2B* mRNA decay determined by qPCR analysis. *C*) Top: schematic representation of the protocol. SK-N-BE(2)C clone 5.8 cells were pre-treated with 1 nM 3-KDG (graph solid line), or vehicle (DMSO; graph dashed line) for 24 hours before the addition of 75 μM DRB for different times. *D*) Top: schematic representation of the protocol. SK-N-BE(2)C clone 5.8 cells were treated with 1 nM 3-KDG (solid line) or DMSO (dashed line) together with DRB (75 μM) for different times. Data are represented as mean values ± SD (error bars) of three (panel C) and two (panel D) independent experiments performed in triplicate, and normalised to the endogenous *GAPDH* gene.

$PHOX2B$ mRNA levels at each time point were compared to those in untreated cells (initial level, T0=1 in panel C; or =1 in panel D), and are expressed as fold induction calculated using the $2^{-\Delta\Delta CT}$ method. Non-linear regression analysis was used to calculate one-phase exponential $PHOX2B$ mRNA decay (GraphPad Prism 5.0). ***$p<0.001$, **$p<0.01$ and *$p<0.05$, statistically significant differences in PHOX2B mRNA levels between each time point and T0 (one-way ANOVA, Tukey's test). ##$p<0.01$ and #$p<0.05$, statistically significant differences in $PHOX2B$ mRNA levels between treated and untreated cells after two and four hours of DRB treatment (Student's t test).

Figure 5. 3-KDG does not affect PHOX2B protein stability

A and C) Top: schematic representation of the protocol. Bottom: representative Western blots. *A*) SK-N-BE(2)C clone 5.8 cells were pre-treated with 1μg/mL CHX for one hour (lane 3) and the treatment was continued with (lanes 4-7) or without 1 nM 3-KDG (lanes 8-11) for the indicated times. Lane 2: untreated cells. IMR32 nuclear extract (IMR32 NE, lane 1) was used as a positive control for PHOX2B. The PHOX2B signal was normalised to that of β-tubulin (TUB). *C*) SK-N-BE(2)C clone 5.8 cells were pre-treated with (lanes 2-7) or without (lanes 8-13) 1 nM 3-KDG for two hours before the addition of 1μg/mL CHX. IMR32 nuclear extract (IMR32 NE, lane 1) was used as a positive control for PHOX2B. *B and D*) Quantification. The graphs show the relative quantification of PHOX2B protein levels at each time point in comparison with T0 (=1): i.e. before the addition of DMSO/3-KDG (protocol A) or CHX (protocol C) in the treated (solid line) and untreated samples (dashed line) expressed as the mean densitometry intensity values ± SD (error bars) of each lane normalised to that of β-tubulin in two independent experiments. Non-linear regression analysis was used to calculate one-phase exponential decay (GraphPad Prism 5.0).
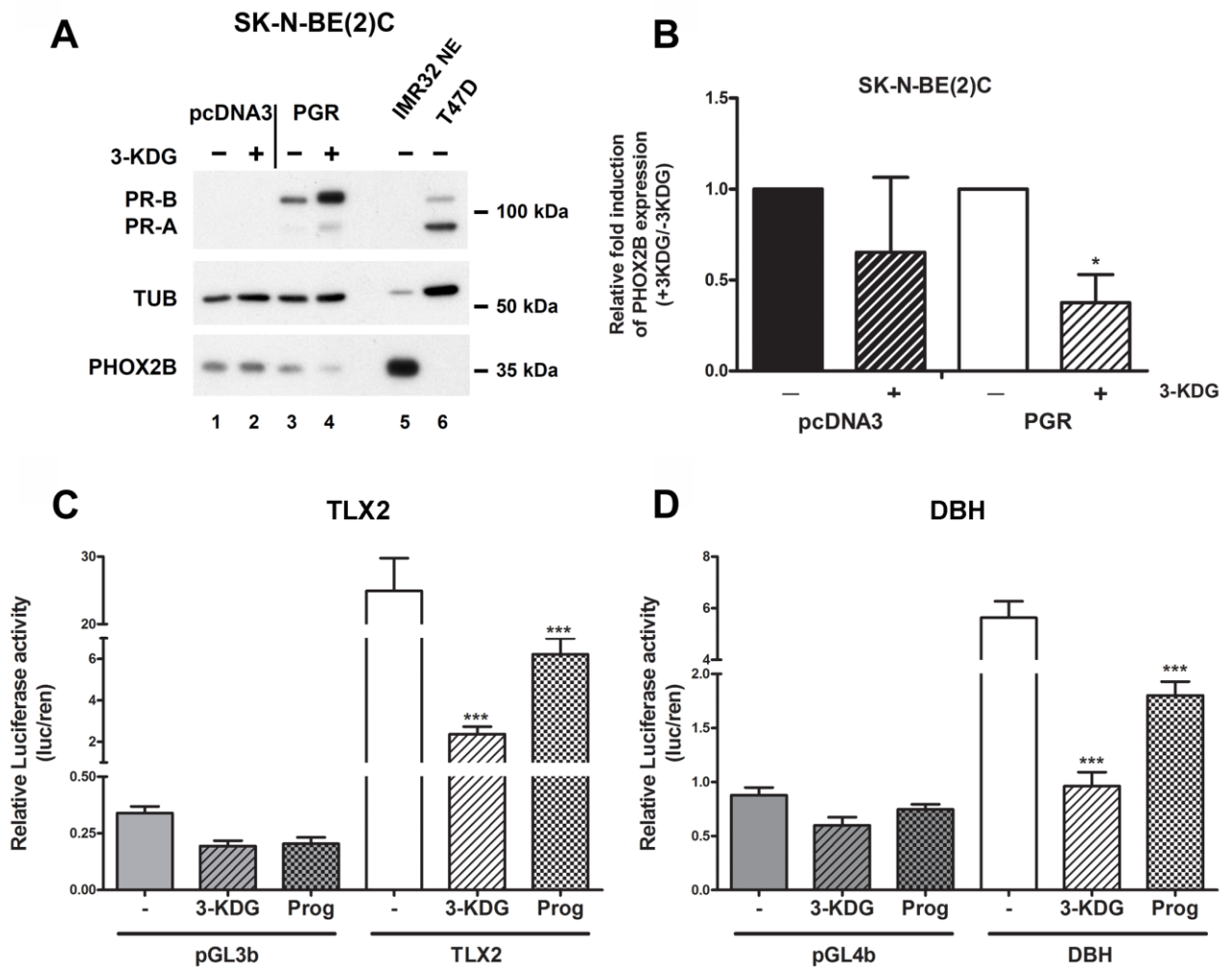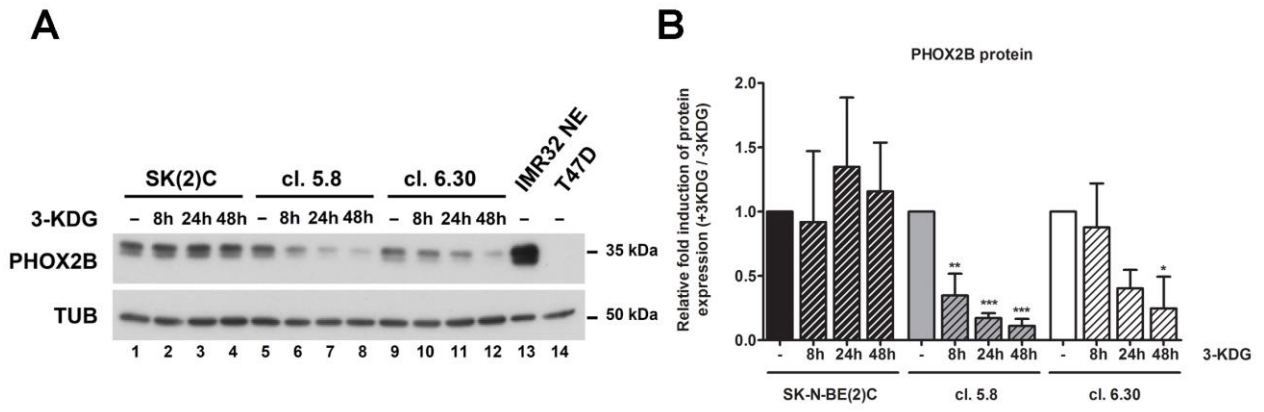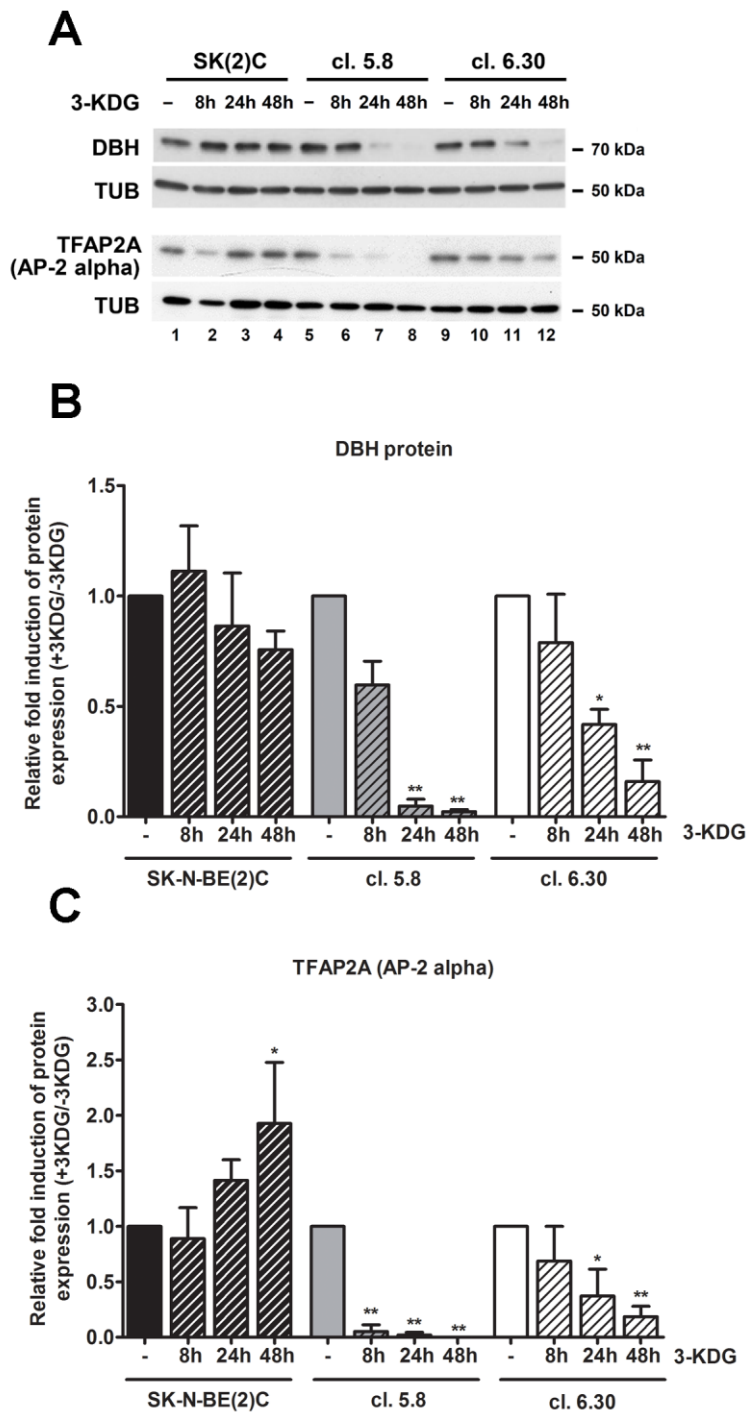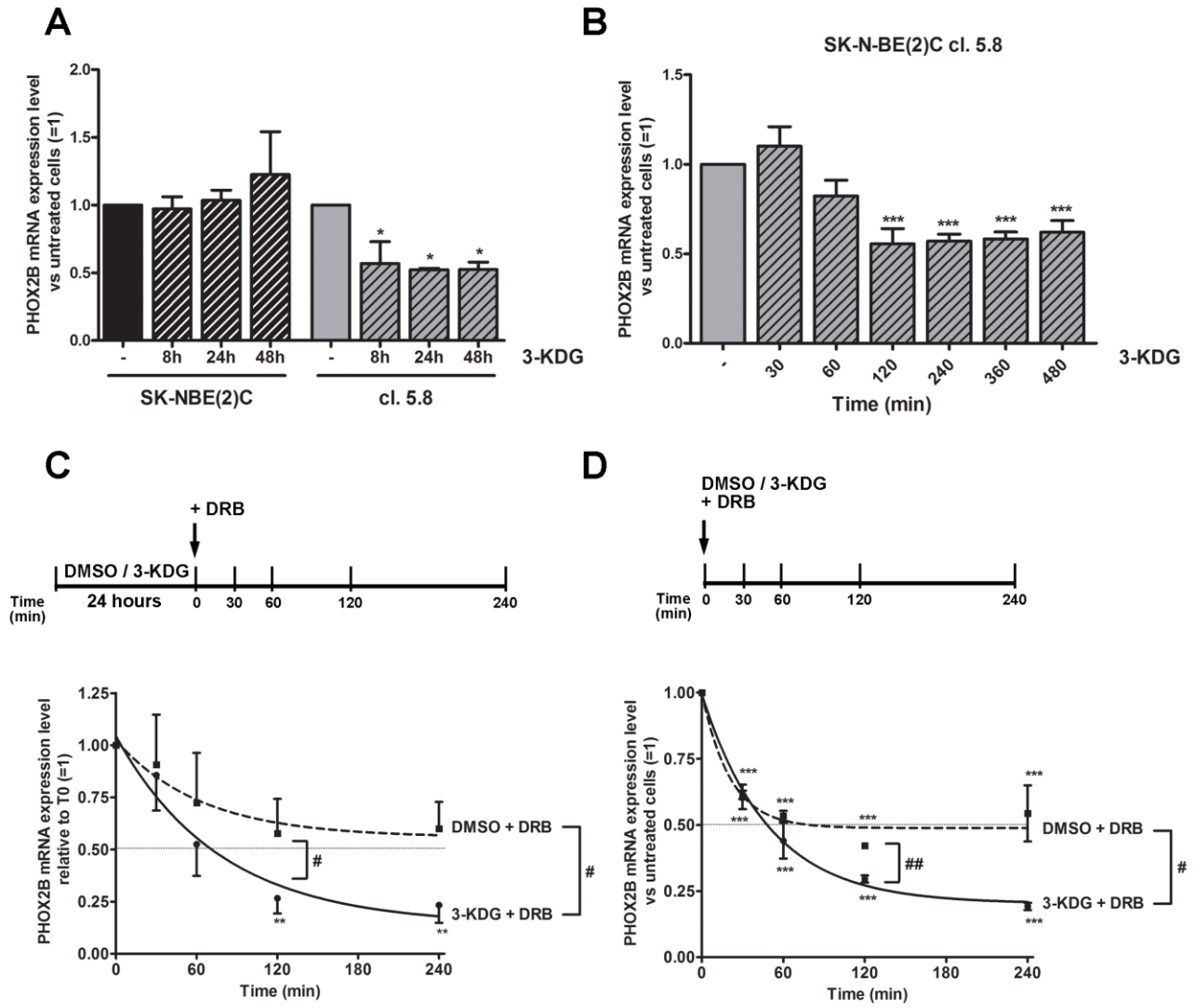
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Fig. 1

A

SK(2)C cl. 5.8 cl. 6.30

3-KDG   – 8h 24h 48h   – 8h 24h 48h   – 8h 24h 48h   –   –

PHOX2B   — 35 kDa

TUB   — 50 kDa

         1 2 3 4   5 6 7 8   9 10 11 12 13 14

B

PHOX2B protein

Fig. 2

Fig. 3

Fig. 4

Fig. 5