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Anne Wulf, Angelika Harneit, Joachim M. Weitzel. T3-mediated gene expression is independent of PGC-1 α . *Molecular and Cellular Endocrinology*, 2007, 270 (1-2), pp.57. 10.1016/j.mce.2007.02.008 . hal-00531917

HAL Id: hal-00531917

<https://hal.science/hal-00531917>

Submitted on 4 Nov 2010

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Accepted Manuscript

Title: T3-mediated gene expression is independent of PGC-1 α

Authors: Anne Wulf, Angelika Harneit, Joachim M. Weitzel

PII: S0303-7207(07)00096-2
DOI: doi:10.1016/j.mce.2007.02.008
Reference: MCE 6625

To appear in: *Molecular and Cellular Endocrinology*

Received date: 7-11-2006
Revised date: 20-2-2007
Accepted date: 20-2-2007

Please cite this article as: Wulf, A., Harneit, A., Weitzel, J.M., T3-mediated gene expression is independent of PGC-1 α , *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2007.02.008

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T3-mediated gene expression is independent of PGC-1 α

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Running Title: Knock-down of PGC-1 α

30

Abstract

Thyroid hormone (T3) has a profound influence on normal development, differentiation and metabolism, processes which are known to be regulated by the transcriptional coactivator
35 PGC-1 α (peroxisome proliferator-activated receptor γ coactivator-1 α). Since T3 rapidly induces PGC-1 α expression, we investigated whether reduced PGC-1 α levels lead to alterations in T3-mediated gene expression patterns. Using RNA interference, we reduced PGC-1 α mRNA to \sim 10% of its initial concentration in rat pituitary GC cells. Knock-down of PGC-1 α is accompanied by diminished protein concentration and decreased expression
40 level of PGC-1 α target genes, among them key enzymes involved in gluconeogenesis, mitochondrial biogenesis and fatty acid oxidation. PGC-1 α , PGC-1 β and NRF-1 mRNA molecules were rapidly degraded with a half-life time of \sim 90 minutes, but this was independent of T3 stimulation. Expression of T3-target genes was not changed upon knock-down of PGC-1 α . Our data indicate that complex T3-mediated gene expression patterns are
45 maintained independently of PGC-1 α activation.

50 **Keywords:**

Gene expression, thyroid hormone, coactivator, nuclear receptor, RNA interference, half-life time

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Abbreviations:

T3, thyroid hormone; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; NRF-1, nuclear respiratory factor-1; TRE, thyroid hormone response element; mGPDH,
60 mitochondrial glycerol-3-phosphate dehydrogenase; ANT2, adenine nucleotide translocator 2; G α , stimulatory G protein alpha-subunit

Introduction

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Thyroid hormone (T3) triggers mitochondrial biogenesis, adaptive thermogenesis and hepatic gluconeogenesis (Yen, 2001) which resembles those effects regulated by the transcriptional coactivator peroxisome proliferators-activated receptor γ (PPAR γ) coactivator-1 α (PGC-1 α). PGC-1 α has originally been identified as a coactivator of PPAR γ and of the glucocorticoid signal pathway in a genetic approach (Knutti et al., 2000;Puigserver et al., 1998). Subsequently, PGC-1 α has been shown to bind and coactivate a great variety of transcription factors implicated in the control of cellular energy metabolic pathways; reviewed in (Lin et al., 2005;Finck and Kelly, 2006). Gain-of-function analysis demonstrated that a forced expression of PGC-1 α is sufficient to induce the full program of mitochondrial biogenesis both *in vivo* and *in vitro* (Lehman et al., 2000;Wu et al., 1999). Furthermore, gene expression studies in skeletal muscle of non-diabetic but insulin resistant patients demonstrated reduced PGC-1 α expression rates which are associated with reduced levels of mitochondria-associated genes further underscoring the importance of PGC-1 α for regulation of these target genes (Mootha et al., 2003;Patti et al., 2003). Surprisingly, recent loss of function analysis demonstrated a lean phenotype of knockout mice carrying a target gene disruption of PGC-1 α (Lin et al., 2004;Leone et al., 2005). It is suggested that this phenotype is caused by spongiform lesions in striatum and brain stem leading to a hyperactive phenotype of transgenic animals. In contrast, isolated liver and muscle cells from PGC-1 α knockout mice showed reduced oxygen consumption rates and impaired fasting gluconeogenesis (Leone et al., 2005;Lin et al., 2004;Arany et al., 2005). Similar results have been observed after infusion of an adenovirus carrying a siRNA construct against PGC-1 α (Koo et al., 2004).

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PGC-1 α has been shown to be strongly regulated by T3 *in vivo*, both on transcript and protein levels (Irrcher et al., 2003;Weitzel et al., 2003a;Weitzel et al., 2001b). Holistic gene expression studies of T3-target genes identified at least two different expression patterns. While some target genes were rapidly induced within 6 hours after the administration of T3 ('early' induction), a second subset of T3-target genes were delayed regulated after a lag period of 12 – 24 hours ('late' induction). Whereas numerous thyroid hormone response elements (TREs) have been identified within early induced gene promoters (Weitzel et al., 2001a;Zilz et al., 1990), we failed to detect TREs in late induced gene promoters (Weitzel et

al., 2003a). These late-induced gene promoters might be regulated by an ‘intermediate factor’ in a TRE-dependent or TRE-independent manner. As a candidate factor for such an intermediate factor we suggested PGC-1 α (Weitzel et al., 2003b). PGC-1 α is endogenously regulated by T3, this activation occurs rapidly and PGC-1 α coactivates a variety of transcription factors including TR and NRF-1 probably serving as bridging factors for inducing late T3-regulated genes.

Since T3 activates PGC-1 α and forced over-expression of PGC-1 α mimics T3-mediated effects, we analysed the impact of PGC-1 α to regulate T3-mediated gene expression patterns by a knock-down approach of PGC-1 α in cell culture. PGC-1 α mRNA was rapidly degraded with a half-life time of \sim 90 min and mRNA levels were reduced to \sim 10% of its initial concentration. Surprisingly, we did not observe any changes in T3-mediated gene expression patterns. Our data indicate that PGC-1 α expression is dispensable for maintaining T3-mediated gene expression patterns.

Materials and Methods

115 *Animals and treatments*

Adult male Wistar rats (Charles River Deutschland GmbH, Sulzfeld, Germany) were housed in the animal care facility of the University Hospital Hamburg-Eppendorf according to the UKE animal care guidelines. Hypothyroidism was provoked by an intraperitoneal injection of Na¹³¹I (Amersham Biosciences, Freiburg, Germany) (250 μ Ci/100 g body weight) and hyperthyroidism was provoked by i.p. injection of T3 (50 μ g/ 100 g body weight) (Weitzel et al., 2003a; Weitzel et al., 2001b). Zero, 6, 24 and 48 hours after thyroid hormone injection, rats were killed by decapitation and livers were isolated for RNA preparation.

125 *RNA interference experiments*

Rat pituitary GC cells (a gift of Danielle Gourdj, Paris, France) were cultured in a 1: 1 mixture of Dulbecco’s modified Eagle’s and Ham’s F12 medium plus 10% fetal calf serum under standard conditions. Twenty-four hours before the experiment, medium was changed

to serum-depleted medium. Double stranded RNA interference oligonucleotides (forward: 5'-uugaagagcggccgugugau-3' and reverse: 5'-aucacacggcgccucuucaa-3'; Qiagen, Hilden, Germany) were designed according to the rat PGC-1 α mRNA sequence (accession number NM_031347) and were tested against the database for the absence of any cross-
135 hybridisation signals to other sequences. For control experiments, GC cells were also mock-incubated with a non-specific siRNA oligonucleotide under identical experimental conditions. For RNA interference experiments, 220 nM of double-stranded siRNA oligonucleotides were mixed with 6.6 μ l TKO reagent (Mirus, Madison, WI, USA) in 220 μ l serum-free medium and incubated for 20 min at RT. Two hundred μ l of this solution was
140 applied to approximately 6×10^5 GC cells per 9.6 cm² dish and incubated for 4 hours. Cells were treated with 100 nM T3 or mock incubated for various time points before cells were harvested and RNA was prepared.

mRNA expression analysis

145 Total RNA was prepared using RNA-Clean (Hybaid-AGS, Heidelberg, Germany) according to the recommendations of the manufacturer. For Northern blot analysis, 10 μ g total RNA was analysed and normalized to ethidium bromide staining and to hybridization signals of the housekeeping genes ubiquitin, G α or 18S rRNA as described (Weitzel et al.,
150 2001b; Weitzel et al., 2003a). Quantitative 'real-time' PCR was conducted using the LC-DNA Master SYBR Green kit in a LightCycler (Roche Applied Science, Mannheim, Germany). Crossing points of target gene amplification products were normalized to the crossing points of the housekeeping genes described above using the standard adjustments recommended by the supplier.

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Determination of mRNA half-life times

Rat pituitary GC cells were cultured under standard conditions and incubated in serum-depleted medium for 48 hours before 5 μ g/ml actinomycin D (Sigma, Taufkirchen,
160 Germany) were added to block RNA synthesis. RNA was prepared and analysed as described above at time of actinomycin D addition (0 hours) and at various time points after the administration. The signals obtained with the corresponding target genes were normalized for those obtained with 18S rRNA, taking into account a previous determination of 65 h for the half-life of 18S rRNA (Nwagwu and Nana, 1980).

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Detection of PGC-1 α protein by immunoprecipitation

For detection of PGC-1 α protein GC cells were harvested 48 hours after the incubation with the siRNA construct. A nuclear extract was performed as described (Weitzel et al., 2001a).
170 Fifty μ g 50% slurry of protein A sepharose CL-4B (Amersham Biosciences) were coupled to a rabbit anti-PGC-1 α antibody (Calbiochem #516557, directed against a C-terminal peptide from amino acids 777 to 797) and washed according to the instructions of the manufacturer. One mg of nuclear extract from GC cells was loaded, washed 3 times and applied to a SDS polyacrylamid gel. PGC-1 α was detected by western blot analysis using a
175 rabbit anti-PGC-1 α antibody directed against the N-terminal \sim 300 amino acids (a kind gift of Natasha Kralli, La Jolla, USA). In vitro translated PGC-1 α was synthesised by a transcription/ translation-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions.

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Results

We previously described differential gene expression patterns after the administration of thyroid hormone into hypothyroid rats *in vivo*. We detected a first expression pattern in
185 which T3 target genes were differentially regulated within 6 hours ('early' induction) and a second expression pattern in which a differential gene expression has been observed after a lag period of 12 to 24 hours ('late' induction). Detailed promoter analysis identified TREs in early induced genes, but failed to detect a TRE in late induced gene promoters. An alternative regulation mechanism suggested the action of an intermediate factor regulating
190 the late induced genes, which may activate and coordinate these target genes TRE-independently (Weitzel et al., 2003b; Kelly and Scarpulla, 2004; Scarpulla, 2006). Based on T3-induction data and gain-of-function analysis, we suggested PGC-1 α to be a candidate for an intermediate factor of T3 action, which is rapidly regulated after hormonal treatment. In order to investigate the underlying mechanism(s) and to reduce the complexity of the
195 system, we screened for a cell line mimicking the *in vivo* investigated expression patterns. Rat pituitary GC cells have been shown to maintain the kinetic features which have been observed in liver. Although the gene expression in GC cells is not identical to those of hepatocytes they show identical T3-mediated kinetics of gene expression (Weitzel et al.,

2001b;Weitzel et al., 2003a;Miller et al., 2001). To verify this observation, we tested
200 various T3-regulated genes (ANT2, mGPDH, Tfam, NRF-1, PGC-1 α , PGC-1 β ; among
others) which all show similar expression kinetics in rat liver and GC cells. In particular, we
investigated the gene expression patterns of mitochondrial glycerol-3-phosphate
dehydrogenase (mGPDH, an early induced gene) and adenine nucleotide translocator 2
(ANT2, a late induced gene). Both genes respond with similar kinetics both in rat liver *in*
205 *vivo* and in the cell line (Figure 1).

To investigate the role of PGC-1 α we designed a RNA interference approach to knock-
down the mRNA of PGC-1 α by siRNA. By using siRNA oligonucleotides directed against
the PGC-1 α mRNA which were transfected into GC cells with a liposome-mediated
210 transfection reagent (see Materials and Methods) we received transfection efficiency rates
of > 99% and survival rates of ~ 75% of the cells, as estimated by transfection of a
fluorescence labelled oligonucleotide and detection the viability by 7-aminoactinomycin D
staining (data not shown). PGC-1 α -siRNA-treated and mock-incubated cells were harvested
after an incubation time of 6, 15, 24, 48 and 72 hours. RNA was prepared and adjusted to
215 real-time PCR and normalized to the housekeeping genes Gsa (Figure 2A) and 18S rRNA
with similar results. As shown in Figure 2A, RNA of PGC-1 α was down-regulated to \leq 10%
within 6 to 24 hours and remained down-regulated for at least 3 days. PGC-1 α protein is
undetectable after siRNA treatment (Figure 2B). Similar results have been observed using
two alternative siRNA constructs against PGC-1 α whereas mock-incubation with a non-
220 specific siRNA oligonucleotide did not alter PGC-1 α concentrations (Figure 2B). To test the
functional consequences of PGC-1 α knock-down, we investigated the expression levels of
various PGC-1 α target genes. As shown in Figure 2C, glucose-6-phosphatase (G6-Pase),
cytochrome c (Cyt c), acyl coenzyme A oxidase (AOX) and adenine nucleotide translocator
2 (ANT2) were down-regulated within 48 hours after siRNA treatment, whereas
225 mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) is not a PGC-1 α target gene.
Thus our siRNA approach against PGC-1 α was successful.

We next asked whether a knock-down of PGC-1 α is associated with any alteration in T3-
mediated gene expression. After knock-down of PGC-1 α , GC cells were treated with T3 or
230 mock-incubated as described above. After various time points (from 6 up to 72 hours) cells
were harvested and RNA was investigated by real-time PCR. Surprisingly, although we
reproducibly knock-down PGC-1 α RNA (see Figure 2) we did not detect any changes in

T3-mediated gene expression patterns, both in early and in late induced genes (Figure 3). This has been observed for T3-target genes which are also PGC-1 α -target genes (like
235 ANT2, Cyt c, G6-Pase) but also for non-PGC-1 α -target genes (like mGPDH) (Figure 3 and
data not shown). PGC-1 α RNA was not induced by T3 in knock-down cells, but remains
regulated by T3 in mock-siRNA-treated cells (Figure 2D). Furthermore, we failed to detect
any alterations in expression levels of PGC-1 β or NRF-1 indicating the absence of any
240 compensatory activation by these functionally related proteins, consistent with data from
knockout animals (Lin et al., 2004; Leone et al., 2005).

To exclude an influence of T3 other than de novo transcription rates, we investigated the
half-life time of various genes. GC cells were treated with actinomycin D to block
transcription and cells were harvested after various time points. RNA was prepared,
245 quantified by real-time PCR and normalized to RNA concentrations of 18S rRNA with a
known half-life time (Nwagwu and Nana, 1980). As indicated in Figure 4, PGC-1 α , PGC-
1 β and NRF-1 have short half-life times of 70 – 90 min which are typically observed for
transcription factors whereas ANT2 showed a longer half-life time of ~ 24 hours (Figure
5B). We finally investigated whether T3 modifies the half-life times of PGC-1 α and ANT2.
250 As shown in Figure 5B, we did not detect any T3-dependent changes in $t_{1/2}$ of ANT2. Very
similar observations have been observed for PGC-1 α , PGC-1 β and NRF-1 (Figure 5A, and
data not shown).

255 Discussion

PGC-1 α integrates a great variety of physiological signals by coactivating different
transcription factors in a tissue-dependent manner. E.g. fasting induced PGC-1 α expression
in liver triggers key enzymes of the gluconeogenic pathway, via coactivating transcription
260 factors such as HNF-4 α , FOXO1 and GR (Herzig et al., 2001; Knutti et al., 2000; Yoon et al.,
2001). In turn, down-regulation of PGC-1 α in liver or isolated hepatocytes leads to reduced
expression of gluconeogenic key enzymes associated with a fasting hypoglycemia
phenotype in these transgenic animals (Koo et al., 2004; Leone et al., 2005; Lin et al.,
2004; Handschin et al., 2005). Blunted expression rates of gluconeogenic key enzymes
265 have been reproduced in our cell model (Figure 2C). Furthermore, PGC-1 α knockout mice
have reduced mitochondrial function and defects in oxidative phosphorylation, highlighted

by a reduced thermogenic capacity. In turn over-expression of PGC-1 α induces the full program of mitochondrial biogenesis (Arany et al., 2005; Lehman et al., 2000). Consistent with this observation, we detected reduced expression levels of mitochondrial biogenesis marker genes cytochrome c, cytochrome c oxidase and mitochondrial transcription factor A (Figure 2C, and not shown). Moreover, investigations in non-diabetic but insulin resistant patients detected reduced expression rates of PGC-1 α in muscle, correlating with reduced expression rates of mitochondria-associated genes (Mootha et al., 2003; Patti et al., 2003). Based of these and similar data one would suggest that mice carrying a target gene disruption of PGC-1 α are rather hypothyroid and have an obese phenotype. However, unexpectedly these mice turned out to show a lean phenotype (Leone et al., 2005; Lin et al., 2004). It is suggested that this phenotype is based on spongiform lesions in brain, leading to a hyperactive phenotype and/or by blunted fasting gluconeogenesis in liver, a major contributor to the hyperglycaemic phenotype in diabetic patients.

To get inside the regulation mechanisms of PGC-1 α and to reduce the complexity of the system, we designed a RNA interference approach in order to knock-down PGC-1 α in cell culture. To test whether PGC-1 α mRNA degrades fast or slowly, we blocked transcription by adding actinomycin D to rat pituitary GC cells. As shown in Figure 4A, PGC-1 α degrades with a short half-life time of \sim 90 min. This short half-life time is typical for transcription factors (Reichwald et al., 2000) and has also been detected for NRF-1 and PGC-1 β (Figures 4B and 4C). Using an RNA interference approach, we were able to knock-down the RNA of PGC-1 α to \sim 10% of its initial expression rates accompanied by undetectable protein concentrations. Furthermore, steady-state levels of PGC-1 α target genes involved in gluconeogenesis, mitochondrial biogenesis and fatty acid oxidation were down-regulated indicating the functionality of the cell system (Figure 2).

Since PGC-1 α is rapidly activated by T3 (both in rat liver and GC cells) and forced expression of PGC-1 α mimics T3-mediated effects (see above) we focussed on T3-mediated expression patterns which have previously been detected by DNA microarray analysis. Investigation of T3-mediated gene expression analysis detected at least two different gene expression patterns. Whereas \sim 50% of T3-regulated genes were rapidly induced within 6 hours after the administration of T3 *in vivo*, another \sim 50% of T3 target genes were differentially regulated after a gap period of 12-24 hours within 48 hours (Weitzel et al., 2001b; Weitzel et al., 2003a). Interestingly, similar expression patterns have been observed

in the tail of *Xenopus laevis* tadpoles and several cell systems (Helbing et al., 2003; Moeller et al., 2005; Shih et al., 2004). This observation leads to the assumption that the delayed expressed genes may be indirectly regulated via an intermediate factor. Based on a candidate gene approach we suggested PGC-1 α to be an intermediate factor of T3 action (Weitzel et al., 2003b). Using rat pituitary GC cells we identified a cell system mimicking the T3-induced expression kinetics. As representative examples we analysed mGPDH (an early induced gene) and ANT2 (a late induced gene) which show very similar kinetics of T3-stimulated expression patterns in the cell line and *in vivo* (Figure 1). To verify that administration of T3 did not alter the half-life time of target genes, we investigated the half-life time of various transcripts in addition to T3. As shown in Figure 5 the half-life of different transcripts is not influenced by changing T3 concentrations. Surprisingly, T3-mediated gene expression patterns in the siRNA-treated cells versus mock-incubated cells did not show any differences. The investigation included several known T3-target genes (ANT2, mGPDH, Tfam, NRF-1, PGC-1 α , PGC-1 β ; (Weitzel et al., 2003a; Weitzel et al., 2001b)) and known PGC-1 α -target genes (glucose 6-phosphatase, cytochrome c, acyl-CoA oxidase; (Koo et al., 2004)); however, we could not rule out that other genes may differentially regulated. Taken together our data indicate that PGC-1 α is dispensable for coordinating complex T3-mediated gene expression patterns. Residual concentrations of PGC-1 α might be sufficient to maintain the late-induced expression patterns or a back-up system compensates for the loss of PGC-1 α . However, neither several transcription factors (e.g. NRF-1, PPARs, TRs) nor coactivators (e.g. PGC-1 β , PRC) showed any alteration in PGC-1 α knock-down cells. Our data are consistent with recent data from PGC-1 α knockout mice which show only subtle alterations in mitochondrial biogenesis and architecture (Arany et al., 2005; Lin et al., 2004; Leone et al., 2005) and with recent data on exercise-induced mitochondrial biogenesis which begins before PGC-1 α expression (Wright et al., 2007).

Acknowledgements

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We are indebted to Danielle Gourdji for kind gift of rat pituitary GC cells, to Natasha Kralli for kind gifts of anti-PGC-1 α antibodies and expression plasmids, to Meike Kröger for help with the LightCycler and to Hans J. Seitz for continued support during the project.

335 The work was supported by grants from the Deutsche Forschungsgemeinschaft (WE2458/3-
2, GRK336) to JMW.

Figure Legends

340 **Figure 1**

Comparison of thyroid hormone-mediated gene expression patterns in rat liver and pituitary GC cells. (A) + (C) Hypothyroid male rats were treated with T3 for 0, 6, 24 and 48 hours. Livers were prepared, RNA isolated and quantified by real-time PCR. Expression levels of mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) (A) and adenine nucleotide translocator 2 (ANT2) (C) were normalized to the expression levels of the housekeeping gene ubiquitin. **(B) + (D)** Rat pituitary GC cells were treated with T3 for 0, 6, 15, 24, 48 hours. Cells were lysed, RNA isolated and quantified by real-time PCR. Expression levels of mGPDH (B) and ANT2 (D) were normalized to the housekeeping gene $G\alpha$. Data are mean \pm SD of at least three independent experiments performed in
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duplicates and are expressed as fold activation data relative to un-stimulated expression values.

355 **Figure 2**

Knock-down of PGC-1 α in GC cells. (A) Rat pituitary GC cells were treated with a siRNA construct against PGC-1 α at time $t = 0$ hours. RNA was prepared at 6, 15, 24, 48 and 72 hours after the treatment with the RNA interference construct and quantified by real-time PCR. Data are mean \pm SD of three to five independent RNA interference experiments and are expressed as fold repression data relative to un-inhibited expression values. **(B)** Mock siRNA-treated (mock) or PGC-1 α siRNA-treated (siRNA) GC cells were immunoprecipitated with an anti-PGC-1 α antibody directed against the C-terminus and detected by Western blot analysis using an anti-PGC-1 α antibody directed against the N-terminus of the protein. In vitro translated (IVT) PGC-1 α was used as positive control. **(C)**
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365 GC cells were treated with a siRNA construct against PGC-1 α or mock incubated. The expression levels of PGC-1 α target genes glucose-6-phosphatase (G6-Pase), cytochrome c (Cyt c), acyl coenzyme A oxidase (AOX), and adenine nucleotide translocator 2 (ANT2)

and non-PGC-1 α target gene mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) were quantified by real-time PCR. **(D)** GC cells were treated with a siRNA construct against PGC-1 α or mock incubated and stimulated with or without thyroid hormone (T3). The expression rates of PGC-1 α were determined by quantitative PCR.

Figure 3

Consequences of knock-down of PGC-1 α on T3-mediated gene expression patterns. GC cells were treated with a siRNA construct against PGC-1 α or mock incubated and stimulated by T3 at time t = 0 hours. Expression levels of mGPDH **(A)** and ANT2 **(B)** 6, 15, 24 and 48 hours after the treatments were quantified by real-time PCR and normalized to un-stimulated expression levels.

380

Figure 4

Determination of mRNA half-life times of (A) PGC-1 α , (B) PGC-1 β and (C) NRF-1. GC cells were treated with actinomycin D to block RNA synthesis at time t = 0 hours. After 15, 30, 45, 60, 90 and 120 minutes cells were harvested, RNA was prepared and quantified by real-time PCR. Amplification signals were normalized to the amplification signals of 18S rRNA and expressed as percentage versus unblocked expression levels (0 hours). Data are mean +/- SEM of three independent real-time PCR experiments.

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Figure 5

Influence of different T3 concentrations on mRNA half-life times. GC cells were treated with actinomycin D in the presence (crosses) or absence (circles) of T3. Expression levels of PGC-1 α **(A)** were determined by real-time PCR at various time points after the administration of actinomycin and normalized to the expression levels of 18S rRNA. For determination of ANT2 expression levels **(B)** unblocked (0 hours) and 6, 12 and 24 hours treated cells were analysed by Northern blot hybridization. Ten μ g of RNA per lane were separated by agarose gel electrophoresis, blotted to nylon membranes and incubated with a radioactively labelled cDNA probe against ANT2.

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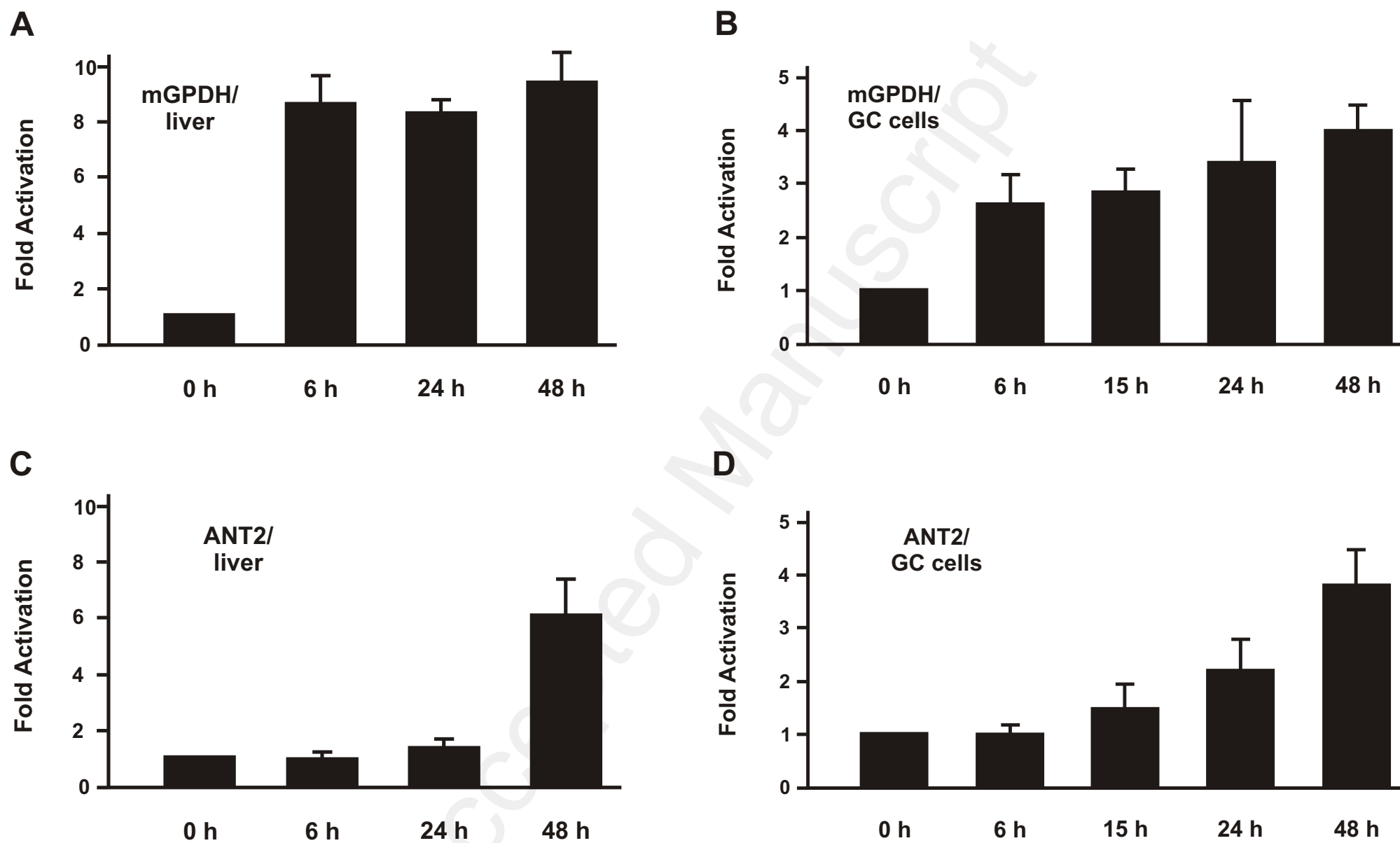


Figure 1

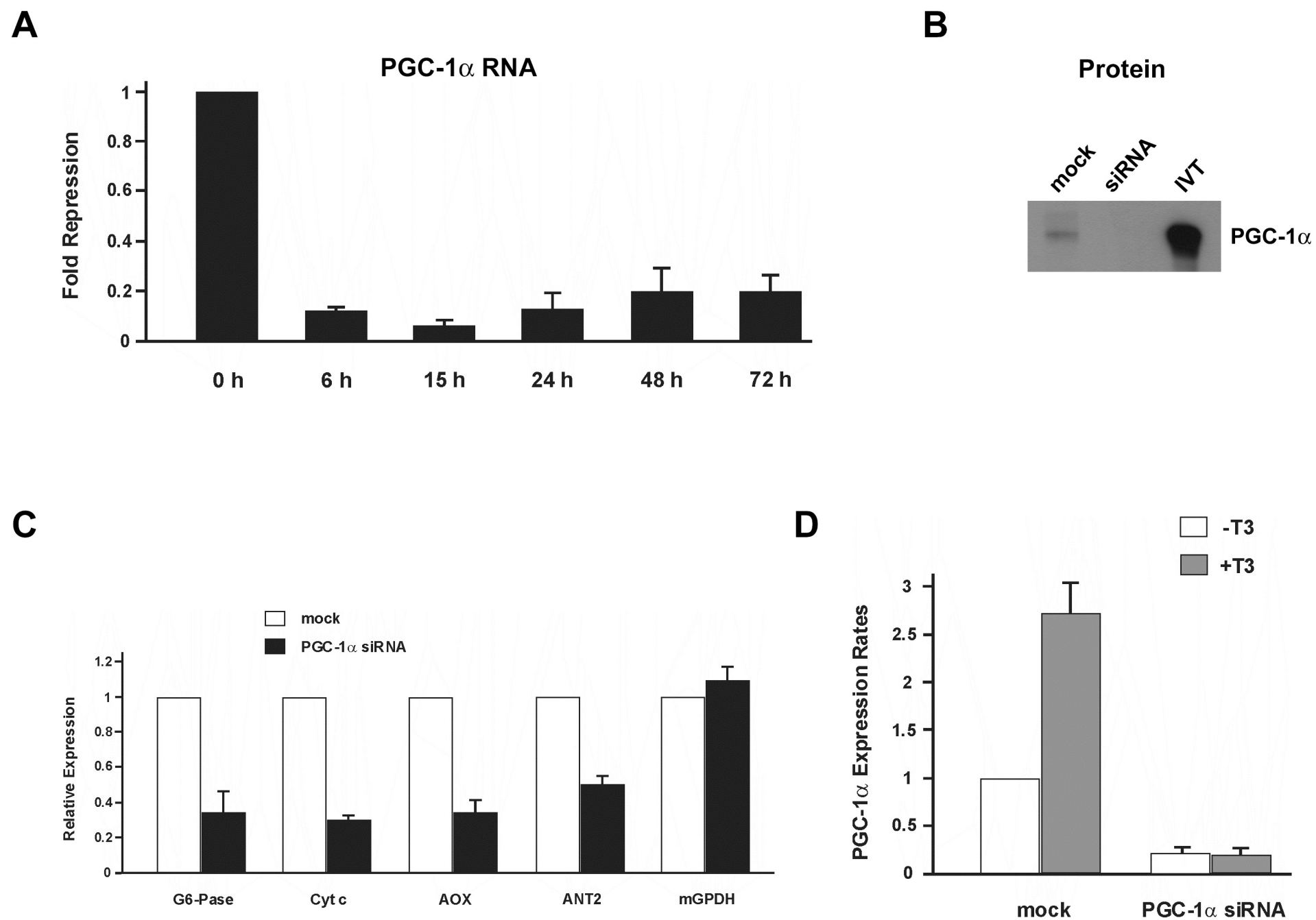


Figure 2

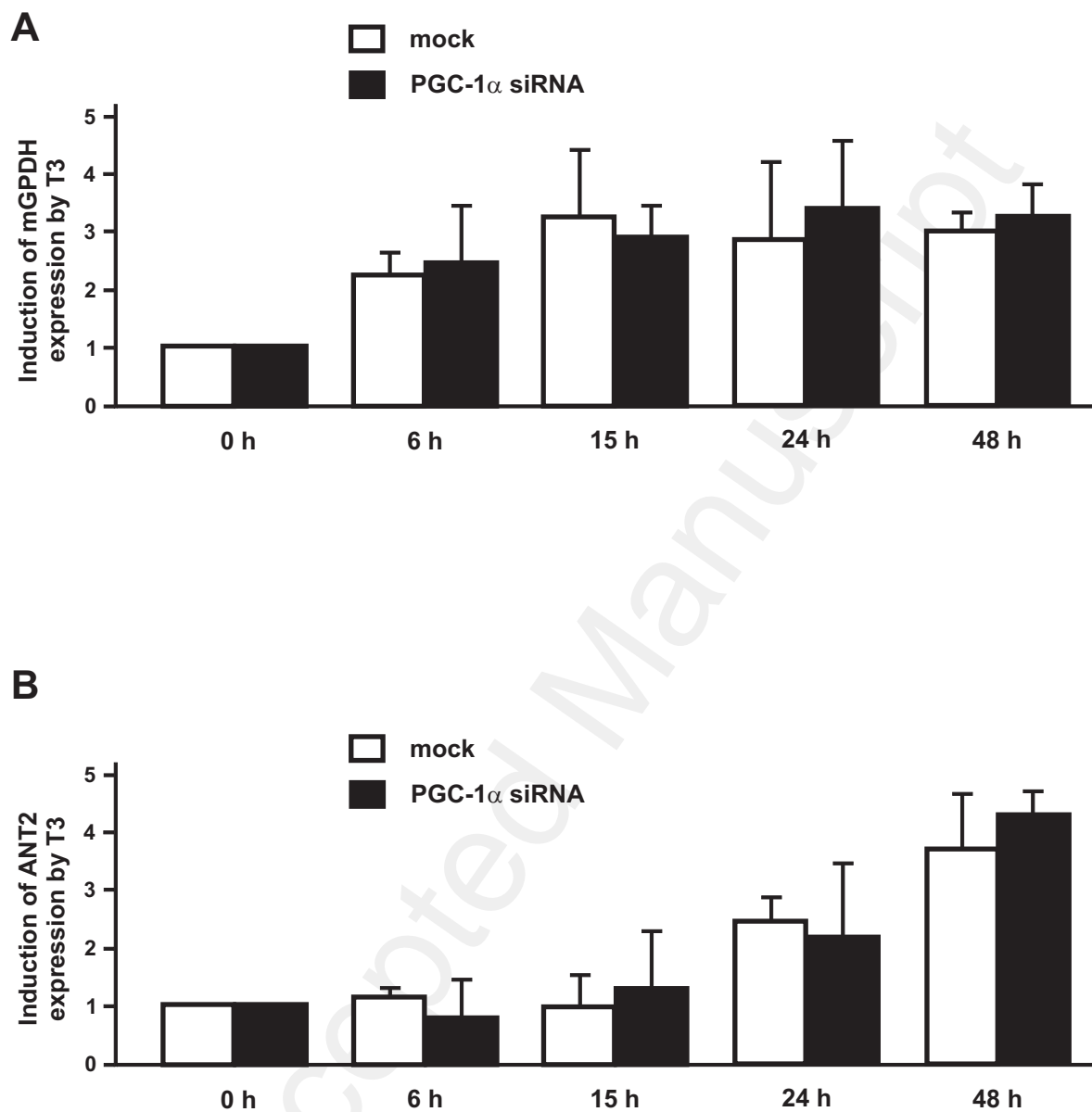


Figure 3

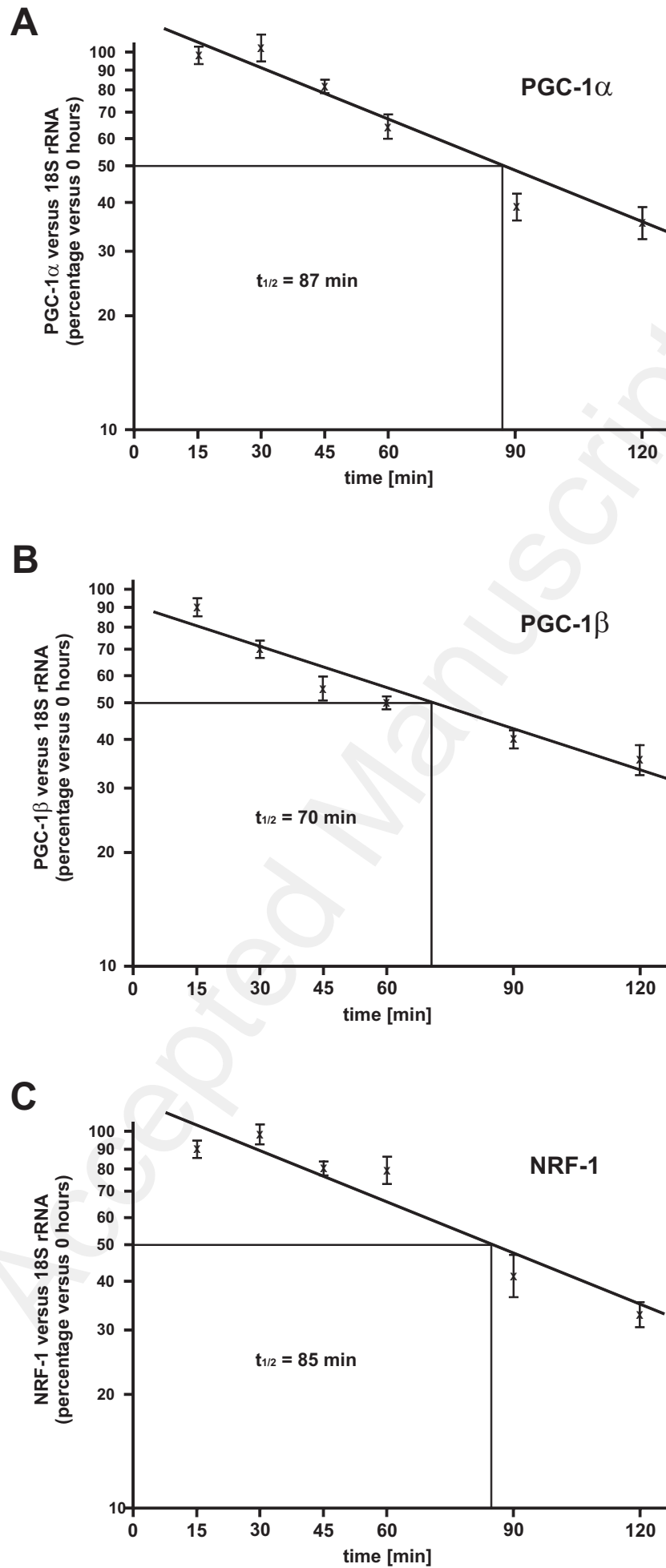
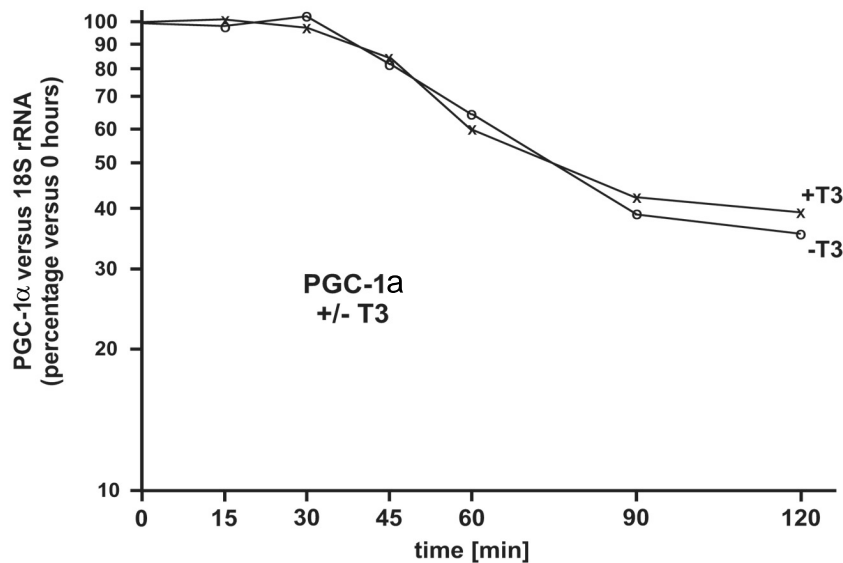
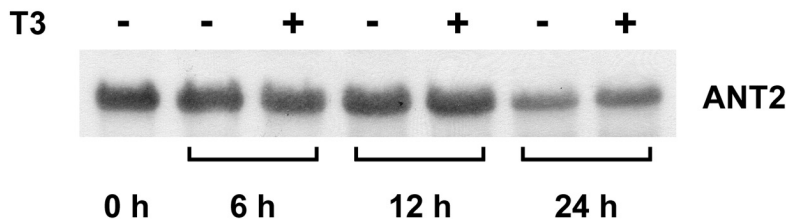


Figure 4

**B****Figure 5**