PGC-1α deficiency causes spontaneous kidney inflammation and increases the severity of nephrotoxic AKI

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ABSTRACT

PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α, PPARGC1A) regulates the expression of genes involved in energy homeostasis and mitochondrial biogenesis. Here we identify the inactivation of transcriptional regulator PGC-1α as a landmark for experimental nephrotoxic acute kidney injury (AKI) and describe the in vivo consequences of PGC-1α deficiency over inflammation and cell death in kidney injury.

Kidney transcriptomic analyses of wild type (WT) mice with folic acid-induced AKI revealed 1398 up- and 1627 down-regulated genes in the second condition. Upstream transcriptional regulator analyses pointed at PGC-1α as the transcription factor potentially driving the observed expression changes that had suffered the highest reduction in activity. Reduced *Pgc-1α* expression was shared by human kidney injury. *Pgc-1α*-/- mice had spontaneous subclinical kidney injury characterized by tubulointerstitial inflammation and increased *Ngal* expression. Upon AKI induction, *Pgc-1α*-/- mice had lower survival and more severe loss of renal function, tubular injury, and reduction in the kidney expression of mitochondrial PGC-1α-dependent genes and an earlier decrease in mitochondrial mass than WT mice. Additionally, surviving *Pgc-1α*-/- mice showed higher rates of tubular cell death, compensatory proliferation, expression of proinflammatory cytokines, NF-κB activation and interstitial inflammatory cell infiltration. Specifically, *Pgc-1α*$^{-/-}$ mice displayed increased M1 and decreased M2 responses and expression of the anti-inflammatory cytokine IL-10. In cultured renal tubular cells, PGC-1α targeting promoted spontaneous cell death and pro-inflammatory responses.

In conclusion, PGC-1α inactivation is a key driver of the gene expression response in nephrotoxic AKI and PGC-1α deficiency promotes a spontaneous inflammatory kidney response that is magnified during AKI.

KEYWORDS

PGC-1α, acute kidney injury, inflammation, mitochondria, proximal tubule, cell death.

INTRODUCTION

Acute kidney injury (AKI) results in an acute and usually transient decrease in renal function [1]. AKI is associated with a high mortality and an increased risk of chronic kidney disease (CKD) progression and there is no satisfactory treatment [1,2]. The main causes of parenchymal AKI are nephrotoxicity, ischemia-reperfusion, and sepsis. Although, they share some pathophysiological mechanisms, etiology-specific features also exist. Tubular cell death and inflammation are early events in AKI that are followed by tubular dedifferentiation, proliferation and regeneration or tubulointerstitial fibrosis [3,4]. A key role of mitochondria in AKI has been suggested, nephroprotective agents targeting mitochondria have been successfully tested in experimental AKI and clinical trials are ongoing [5]. A more in-depth understanding of the factors regulating mitochondrial biogenesis, kinetics and function during AKI and their relationship with key features of AKI such as inflammation and tubular cell death may help to design novel therapeutic approaches.

Tubular cells consume huge amounts of energy to reabsorb the bulk of the glomerular ultrafiltrate. This energy is provided by numerous mitochondria. During AKI, there is evidence of mitochondrial injury and dysfunction [5]. PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator-1α) is a key transcriptional regulator of the expression of mitochondrial proteins, which is involved in mitochondrial biogenesis, energy homeostasis, and oxidative stress [6,7]. *Pgc-1α* expression is downregulated in experimental AKI induced by endotoxemia, nephrotoxic agents and ischemia-reperfusion renal injury (IRI) [8-12] and in models of fibrosis and of AKI-to-CKD transition [13]. Inflammation drove PGC-1α downregulation in tubular cells through NFκB-mediated transcriptional suppression [10]. Moreover, PGC-1α deficiency increased the severity and/or precluded recovery from endotoxemia- and IRI-induced AKI, but the impact on local kidney inflammation was not studied and a recent review by the authors of these manuscripts stated that “the exact mechanisms of PGC1α´s protective effects in AKI are not yet fully elucidated” [7,9,11]. PGC-1α downregulation has been associated to lipid accumulation and fibrosis progression and *Pgc-1α* overexpression reduces renal fibrosis [8,13]. However, whether PGC-1α deficiency directly

modulates cell death and inflammation, key processes in initial stages of AKI, has not been explored in either non-stressed or AKI kidneys.

We have now performed a functional analysis of upstream transcriptional regulators in transcriptomic datasets from mice with experimental nephrotoxic AKI, which revealed PGC-1α as the transcriptional regulator that had suffered the highest reduction in activity. To further gain insight into the role of PGC-1α in kidney physiology and nephrotoxic AKI, we explored for the first time the consequences of genetic *Pgc-1α* deficiency for adult kidneys from healthy mice and for folate-induced AKI. PGC-1α deficiency resulted in spontaneous kidney inflammation and in higher mortality, more severe kidney dysfunction, an earlier decrease in mitochondrial mass and increased cell death and inflammation in mice with AKI. An enhanced inflammatory response may drive the more severe kidney injury in PGC-1α-deficient mice as suggested by the inflammatory response elicited by PGC-1α targeting in cultured tubular cells.

METHODS

Transcriptomics arrays

Transcriptomics arrays of kidney tissue (n=3 kidneys obtained 24 h after folic acid and n=3 obtained 24 h after vehicle administration to mice) were performed at Unidad Genómica Moncloa, Fundación Parque Científico de Madrid, Madrid, Spain. Affymetrix microarray analysis followed the manufacturer's protocol [14,15]. Image files were initially obtained through Affymetrix GeneChip® Command Console® Software (AGCC). Subsequently Robust Multichip Analysis (RMA) was performed using Affymetrix Expression Console® Software. Starting from the normalized RMA, the Significance Analysis of Microarrays was performed using the limma package (Babelomics, www.babelomics.org), using a false discovery rate (FDR) of 5% to identify genes that were significantly differentially regulated between the analyzed groups.

Pathway enrichment and upstream regulator analysis

Canonical pathway enrichment and upstream regulator analyses were performed using Ingenuity Pathway Analysis (IPA, Quiagen, Hilden, Germany). p-values were corrected for multiple-testing with the Benjamini-Hochberg method.

Human samples

They were obtained from the IIS-Fundacion Jimenez Diaz Biobank. AKI patients (n=6) corresponded to tissue from non-tumoral nephrectomy (4 males, 2 females, age: 59,7±10,2, serum creatinine: 7±1.2 mg/dl). Controls (n=5) corresponded to healthy kidney tissue from tumoral nephrectomy (4 males, 1 female, age: 63,8±11, serum creatinine: 1±0.3 mg/dl). mRNA was isolated from paraffin samples using XXXX kit (Quiagen) and human PGC-1α expression was amplified by RT-PCR.

Mitochondrial DNA copy number

Half a kidney was lysed in 500 µl buffer containing 1 M Tris-HCl (pH 8), 5 M NaCl, 0.5 M EDTA, 10% SDS and 0.5 mg/ml proteinase K. After 2 h at 55 ºC, 500 µl Phenol/Chloroform/Isoamyl Alcohol was added and mixed by vortexing. Samples were centrifuged at 13000 r.p.m during 10 min and the aqueous phase was transferred to a solution containing 0.7 (v/v) isopropanol and 0.3 sodium acetate 2 M [16]. Samples were centrifuged 15 min at 13000 r.p.m and pellets were washed with 70 % ethanol. After 5 min 13000 r.p.m. centrifugation, pellets were dried and resuspended in water. DNA was amplified by qPCR using SYBER Green PCR Kit (TAKARA, Kusatsu, Japan) and the specific primers (Supplemental materials)[16] and the 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA).

Colorimetric NFκB assay and Southwestern

Nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Rockford, IL). Nuclear RelA was measured using TransAM NFκB Family Kit (Active Motif, Carlsbad, CA). Activated NF-κB was detected by Southwestern histochemistry. Tissue sections fixed in 0.5% paraformaldehyde were incubated with 50 pmol digoxigenin-labeled NF-κB probes (Roche, Basel, Switzerland.) in buffer containing 0.25% bovine serum albumin and 1 µg/ml poly(dI-dC), followed by alkaline phosphatase-conjugated anti-digoxigenin IgG (Roche) and colorimetric detection. A 200-fold excess of unlabeled probe was used to test the specificity of the technique [17].

Adenoviral infection

MCT cells are a cultured line of proximal tubular epithelial cells that have been extensively characterized [18]. Cells ($1.6x10^5$) were seeded in 60 mm dishes in RPMI media (SIGMA, Sant Louis, MO), 10 % FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in 5% CO2 at 37 ºC overnight. Thereafter, they were infected with adenoviruses (shControl or shRNA against *Pgc-1α*) [19]. After infection, cells were serum-depleted for 48 hours and cell death and inflammation were measured. In other experiments, cells were stimulated with 100 ng/ml recombinant TWEAK (Millipore, Burlington, MA) 48 h after infection, the dose being based on detailed dose- and time-response studies from our lab [20].

Statistics

Statistical analysis was performed using the SPSS 11.0 statistical software. Results are expressed as mean ± SD. Significance at the $p<0.05$ level was assessed by nonparametric Mann–Whitney U-test for two groups and analysis of ANOVA for three or more groups. Spearman correlation was used to assess correlation between two continuous variables.

Expanded methods are presented as supplemental data

RESULTS

AKI expression profile and upstream regulator analyses identify PGC-1α as the transcriptional regulator with the highest activity reduction

A kidney transcriptomics study disclosing reduced expression of *Pgc-1α* at 24 hours in folic acid-induced AKI (FA-AKI) had been previously described [10]. Among 3025 differentially expressed genes (FDR <0.05) between FA-AKI and vehicle-injected mice, 1398 were up-regulated and 1627 downregulated (**SF. 1, Table S1**), and *Pgc-1α* was among the most downregulated genes in terms of mRNA expression (% decrease) [10]. We have now performed functional analyses of the genes detected as differentially expressed to identify biological patterns involved in AKI. Upstream transcription regulator analysis identified PGC-1α as the transcriptional regulator whose activity had decreased more dramatically in AKI, as compared to vehicle-injected kidneys, based on expression changes of downstream target genes (**Fig. 1.A**). That is, in addition to been downregulated at the gene expression levels, PGC-1α was the transcription factor showing evidence of the largest decrease in transcription regulation activity when compared to control kidneys based on the changes in the expression of downstream target genes. **SF. 2** shows a circular plot with the 5 upstream regulators with the most negative z score and their target genes (All data are listed in **Table S2**). Moreover, mitochondrial dysfunction, oxidative phosphorylation and the NFκB signaling pathway, a negative regulator of *Pgc-1α* expression [10], were among the top-ten canonical pathways detected as enriched (**Fig. 1.B**) and 17 differentially regulated genes were both PGC-1α-regulated and contributed to these three pathways (**Fig 1.C**). The relation between PGC-1α activity reduction and the observed expression changes for three of those genes (two downregulated genes representative of the mitochondrial dysfunction and oxidative phosphorylation pathways, *Ndufs1* and *Sdha*; and one upregulated gene representative of the NFκB pathway, *Tnfα*) was validated in cultured tubular cells, or in vivo, during AKI, upon *Pgc-1α* shRNA targeting in experiments described below. In addition, *PGC-1α* mRNA was downregulated in kidney tissue from patients with AKI, supporting the clinical relevance of the findings (**Fig. 1.D**). Based on the predicted reduction of PGC-1α transcriptional factor activity during AKI, and the observed decrease in *Pgc-1α* expression during human and experimental kidney injury, we have explored the consequences of PGC-1α deficiency for the kidney under spontaneous conditions and during nephrotoxic FA-AKI.

Spontaneous kidney inflammation in *Pgc-1α*$^{-/-}$ mice

Tubulointerstitial inflammation is a key contributor to kidney injury [20,21]. In this regard, kidneys from healthy, vehicle-injected *Pgc-1α*$^{-/-}$ mice displayed mild inflammation when compared to WT mice. Specifically, expression of genes encoding cytokines, chemokines and cytokine receptors contributing to kidney inflammation and renal injury, such as *Il-6*, *Fn14*, *Tnfα* and *Ccl2*, was higher in *Pgc-1α*$^{-/-}$ than in age-matched WT mice (**SF. 3.A**). Moreover, this was associated with inflammatory infiltrates consisting of F4/80+ macrophages and $CD3^+$

lymphocytes in *Pgc-1α*$^{-/-}$ mice as compared to WT mice (**SF. 3.B**). Gene expression and urine levels of the tubular injury and inflammation marker *Ngal* was also increased (**SF. 3.C**) and there was a trend towards a higher baseline rate of tubular cell death **(SF. 3.D**), both suggesting subclinical kidney damage. However, serum creatinine and albuminuria did not differ between WT and *Pgc-1α*$^{-/-}$ mice, which is expected for mild inflammation not resulting in loss of >50% of renal mass (**Fig 2.B, SF. 3.E**). There was no evidence of tubulitis, glomerulitis, vasculitis or perivascular infiltrates or tertiary lymphoid tissues formation nor was evidence of interstitial fibrosis in sections stained with PAS. These results suggest that PGC-1α is a physiological negative modulator of the inflammatory response in the kidney.

Impact of PGC-1α deficiency on survival, renal function and mitochondrial biogenesis during AKI

Upon induction of AKI, survival of *Pgc-1α*$^{-/-}$ mice was lower than for WT mice (4/7, 57% vs 6/6, 100% survival at 48 hours after AKI induction) (**Fig. 2.A**). The lower survival appeared related to the severity of AKI, since surviving *Pgc-1α*$^{-/-}$ mice, which may be expected to have less severe injury that non-surviving mice, had significantly worse renal function at 72 hours than WT mice, assessed as plasma creatinine levels (**Fig. 2.B**), as well as higher kidney expression of the renal tubular injury marker *Ngal* (**Fig. 2.C**). To assess the impact of PGC-1α deficiency on mitochondrial biogenesis, we quantified the expression of PGC-1α targets *Tfam*, *Ndufs1* and *Sdha*. In WT mice with AKI, these genes were downregulated in a time-dependent manner, and gene expression started to recover by 72 h, while in *Pgc-1α*$^{-/-}$ mice downregulation persisted at 72 hours (**Fig. 2.D**). Moreover, the expression of some of these genes was lower at baseline in *Pgc-1α*$^{-/-}$ mice (e.g. *Ndufs1* and *Sdha*). Immunohistochemistry of TOMM22, a translocase of the outer mitochondrial membrane which is used as a marker of mitochondrial mass, also indicated earlier mitochondrial mass loss during AKI in *Pgc-1α*$^{-/-}$ mice than in WT mice (**Fig 2.E**). Indeed, there was a significant negative correlation between TOMM22 staining and plasma creatinine levels (**Fig. 2.F**). In addition, levels of mitochondrial DNA (mtDNA) were decreased in AKI at 72 hours and they decreased earlier in *Pgc-1α*$^{-/-}$ mice (**Fig. 2.G**) and accumulation of DNA deletions was more evident in *Pgc-1α*$^{-/-}$ mice at 72 hours of AKI (**SF.4**) These results suggest that PGC-1α maintains mitochondrial levels and possibly mitochondrial activity during nephrotoxic AKI, and this may have a nephroprotective effect.

PGC-1α deficiency increased tubular cell death and compensatory tubular cell proliferation during AKI

Tubular cell death is a key feature of AKI. Tubular cell death, assessed by TUNEL staining, was higher in the *Pgc-1α*$^{-/-}$ mice that survived up to 72 h and could be analyzed than in WT mice (**SF. 5**). Recovery from AKI is characterized by tubular cell dedifferentiation and compensatory proliferation to restore kidney function. Compensatory tubular cell proliferation was assessed by PCNA staining. *Pgc-1α*$^{-/-}$ mice presented a higher number of PCNA-stained cells than WT mice at 24 hours of AKI (**SF. 6**). These results are consistent with the higher severity of AKI in *Pgc-1α*$^{-/-}$ mice and suggest that PGC-1α deficiency does not interfere with compensatory tubular cell proliferation, a requirement for recovery of renal function.

Increased kidney inflammation in *Pgc-1α*$^{-/-}$ mice during AKI

During AKI, infiltration by F4/80^{+} macrophages and CD3^{+} lymphocytes was more severe in *Pgc-1α*$^{-/-}$ mice than in WT mice, at 24 and 72 hours, respectively (**Fig. 3**).

Next, we analyzed the expression of cytokines, chemokines and their receptors. Fn14 expression is upregulated in AKI where it mediates cell death and inflammation in response to TWEAK [22]. During tissue injury, a dramatic increase in the Fn14 receptor expression is the main driver of TWEAK/Fn14 signaling, while changes in TWEAK expression are milder [22]. In *Pgc-1α*$^{-/-}$ mice with AKI, Fn14 mRNA and protein were higher at 24 and 72 h than in WT mice with AKI (**Fig. 4.A, B**). Similar results were observed for additional inflammatory mediators of kidney injury, such as TNFα, IL-6 and CCL2. Their expression was higher in *Pgc-1α*$^{-/-}$ mice than in WT mice with AKI at the early time point of 24h or 72 hours (**Fig. 4.C),** suggesting that they may contribute to the amplification of injury and the increase in inflammatory cells (**Fig. 3).** Interestingly, the kinetics of inflammatory mediator expression differed between WT and *Pgc-1α*$^{-/-}$ mice. In WT mice, their expression peaked at 24h or was already decreasing at 72h (**Fig. 4**). By contrast, in *Pgc-1α*$^{-/-}$ mice, expression of inflammatory

factors decreased more slowly (e.g. *Fn14*) (**Fig. 4.A, B**) or even further increased at 72 h (e.g. *Tnfα* and the chemokine *Ccl2*) (**Fig. 4.C**), suggesting persistence or amplification of inflammation.

The NFκB transcription factor is a key regulator of the expression of inflammatory factors in AKI and is activated in tubular cells by various cytokines, including TWEAK and TNFα [14,20]. NFκB activation was assessed by nuclear translocation of p65, a subunit involved in the canonical pathway of NFκB activation, and by Southwestern histochemistry. Nuclear translocation of p65 clearly differed between *Pgc-1α*$^{-/-}$ and WT mice at 72 hours (**Fig. 4.D**) consistent with the wider differences in the expression of *Tnfα*, a key driver of canonical NFκB activation [23], at this time point (**Fig. 4.C**). Furthermore, Southwestern histochemistry localized NFκB activation mainly to tubular cell nuclei and these results were consistent with higher NFκB activation in *Pgc-1α*$^{-/-}$ than in WT mice (**Fig. 4.E**). These results suggest that in addition to being a negative physiological regulator of kidney inflammation, PGC-1α is also a negative modulator of the inflammatory response in AKI.

PGC-1α and the macrophage M1-to-M2 transition

Macrophage phenotype influences their function as promoters of further inflammation and injury or of repair. M1 macrophages favor inflammation while M2 macrophages have anti-inflammatory effects and could favor tissue recovery after AKI [24]. Therefore, we tested the effect of PGC-1α deficiency on M1-M2 macrophage phenotypes, assessed by the expression of iNOS (M1) and M (M2). *iNos* mRNA expression peaked at 24h and was higher at this time point in *Pgc-1α*$^{-/-}$ mice than in WT with AKI (**Fig. 5.A**). By contrast, *Mannose receptor (Mannose R)* expression was increased at 72 h in WT mice but not in *Pgc-1α*$^{-/-}$ mice (**Fig. 5.B).** Consequently, the M1/M2 (*iNos*/*MannoseR*) ratio was higher in *Pgc-1α*$^{-/-}$ than in WT mice and the difference was more evident at 24h (**Fig. 5.C**). Moreover, mRNA levels of the anti-inflammatory cytokine *Il-10* were lower in *Pgc-1α*$^{-/-}$ than in WT mice also at 72h (**Fig. 5.D**), also consistent with lower levels of M2 macrophages. In line with this, MannoseR staining disclosed more infiltrating M2 macrophages in WT mice at 72 hours (**Fig. 5.E**). In addition, we analyzed protein levels of Arginase 2 and Arginase 1, markers of M1 and M2 respectively. Arginase 2 levels (M1) are higher in *Pgc-1α*$^{-/-}$ mice while arginase 1 (M2) levels are higher in WT mice (**SF. 7.A, B**).

***Pgc-1α* knockdown promotes a lethal and pro-inflammatory response in cultured tubular cells**

Finally, we studied whether tubular cells are directly stressed by *Pgc-1α* knockdown. MCT cells were infected with adenoviruses expressing shRNA against *Pgc-1α* or control shRNA and PGC-1α downregulation was confirmed by western blot (**Fig. 6.A**). PGC-1α downregulation increased spontaneous tubular cell death as assessed by flow cytometry, and increased cell detachment as assessed by phase contrast imaging (**Fig. 6.B**). Moreover, *Pgc-1α* knockdown also induced an inflammatory response, characterized by increased baseline mRNA and protein expression of Fn14 (**Fig. 6.C, D**), which may sensitize to TWEAK-induced inflammation [20], as well as by increased baseline and TWEAK-stimulated gene expression of *Il-6*, *Tnfα* and *Ccl2* (**Fig. 6.E**). The dependence of TNFα upregulation on PGC-1α downregulation was thus validated in both experimental AKI and in cultured tubular cells. These results suggest that tubular cells may be direct targets of PGC-1α-induced nephroprotection and that PGC-1α deficiency in tubular cells may contribute to the observed inflammation in *Pgc-1α*$^{-/-}$ mice.

DISCUSSION

The main findings are that PGC-1α is the transcriptional regulator whose activity is most decreased in FA-AKI when compared to normal kidneys, and that PGC-1α deficiency leads to spontaneous kidney inflammation, likely driven by tubular cell pro-inflammatory responses, and to a more severe nephrotoxic AKI that may be lethal. The progressive increase in AKI severity over time does not appear to be due to insufficient tubular cell proliferation. Rather, there is a failure to recruit repair-associated macrophages. Overall, these data suggest a key nephroprotective role of PGC-1α, dependent on negative modulation of kidney inflammatory responses as well as on promoting mitochondrial biogenesis.

We have verified the functional relevance of PGC-1α in the kidney under physiological and pathological conditions as a constitutive suppressor of inflammation. During renal injury,

transcriptional regulator analysis identified PGC-1α factor whose activity is most extremely reduced in comparison to healthy mice, potentially explaining changes in the expression of multiple genes. Such a decrease in activity was associated to changes in biological processes related to mitochondrial dysfunction and oxidative phosphorylation as well as with inflammation-related NFκB responses, which were among the top-ten canonical pathways detected as enriched. In this regard, the loss of mitochondrial mass during AKI correlated positively with renal injury and inflammation. PGC-1α deficiency resulted in higher mortality during nephrotoxic AKI, likely a consequence of more severe AKI, since kidney injury was already more severe at 24h post-induction of AKI. In this regard, *Pgc-1α*$^{-/-}$ mice studied at 72h may a have a milder kidney injury than *Pgc-1α*$^{-/-}$ mice that died before 72h. These results are consistent with reports of nephroprotection in mice overexpressing *Pgc-1α* [11,13]. However, given the whole body *Pgc-1α* deficiency and the essential role of PGC-1a in metabolism, we cannot exclude that systemic consequence of *Pgc-1α* deficiency may have contributed to the observed mortality or other features of kidney injury in *Pgc-1α*$^{-/-}$ mice.

Systemic or local kidney inflammation lead to kidney PGC-1α downregulation [9,10], but the effects of PGC-1α downregulation or deficiency over renal inflammation had not been well characterized before. Anti-inflammatory effects of PGC-1α have been observed in the skeletal muscle atrophy program, where *Pgc-1α* overexpression reduced *Fn14* expression and TWEAK-induced inflammation [25]. TWEAK/Fn14, IL-6, TNFα and CCL2 are known mediators of kidney injury, as demonstrated by preclinical and/or clinical interventional studies [26-29]. Moreover, TWEAK promotes the expression of these cytokines during AKI [20]. We have now observed that *Pgc-1α* knockdown increased baseline renal inflammation, and this effect occurred, at least in part, by exacerbating pro-inflammatory tubular cell responses to cell stress and to cytokines such as TWEAK. The increased baseline expression of the proinflammatory receptor *Fn14* in PGC-1α deficient cells was likely responsible for the increased sensitivity to TWEAK-induced proinflammatory responses, since receptor levels are key regulators of cell sensitivity to TWEAK [22]. Since inflammatory mediators, such as TWEAK and TNFα, decrease kidney PGC-1α [9,10,12] and, as shown here, PGC-1α deficiency exacerbates the inflammatory response to cell stress, including TWEAK stimulation, the resulting vicious circle may amplify tissue injury. Understanding the intracellular drivers of PGC-1α downregulation may facilitate the development of therapeutic strategies. In this regard, canonical NFκB activation, histone acetylation and downregulation of the HNF-1β transcriptional network have been reported to contribute to PGC-1α downregulation in response to inflammatory cytokines [10,12].

Tubular cell death is an early feature of AKI which was more severe in the absence of PGC-1α in vivo and in cultured tubular cells. Several pathways link cell death to inflammation [30,31]. However, the trend towards higher baseline tubular cell death rate in vivo, did not reach statistical significance, presumably because of adaptive responses, while inflammatory gene expression and cell infiltration were clearly increased in *Pgc-1α*$^{-/-}$ mice under baseline conditions. This suggests that additional drivers of inflammation may be more important than cell death. PGC-1α deficiency also promotes oxidative stress, a key driver of NFκB activation and inflammatory responses [23]. Finally, during renal IRI, PGC-1α deficiency also causes deficiency of nicotinamide, a precursor of nicotinamide adenine dinucleotide (NAD) biosynthesis [11]. Nicotinamide administration allowed NAD synthesis by NAMPT [11]. The improvement of IRI-induced AKI via NAD synthesis by NAMPT is in line with recent observations of an adaptive role NAMPT activity that protected from tubular cell death and dampened chemokine expression, thus decreasing pro-inflammatory responses [32].

There was previous evidence for a protective role of PGC-1α in some forms of kidney disease, but the cellular and molecular mechanisms were only partially explored and the role of inflammation and cell death had not been characterized in depth, as recently reviewed [7]. Thus, Tran et al observed worse renal function, greater fat accumulation, more severe tubular injury and metabolite disturbances in *Pgc-1α*$^{-/-}$ than in WT mice at 24h following AKI, but inflammatory mediators or inflammatory cells were not studied [11]. Interestingly, they show in the figures a significantly higher baseline serum creatinine in *Pgc-1α*$^{-/-}$ than in WT mice, although this is not discussed in the text. In a model of systemic inflammation and AKI

characterized by absence of kidney cell death (thus differing from the model reported in the present manuscript), and induced by LPS administration, Tran et al observed persistence of kidney injury in *Pgc-1α*$^{-/-}$ mice, but whether early kidney injury differed between *Pgc-1α*$^{-/-}$ and WT mice was not explored [9]. Based on our results, we suggest that the spontaneous inflammation observed in *Pgc-1α*$^{-/-}$ mice may have predisposed them to more severe renal injury after endotoxemic (i.e. inflammation-related) AKI induction, although, the impact of PGC-1α deficiency on inflammatory mediators or inflammatory cells was not studied by Tran et al. In this LPS study, Tran et al reported the same values for baseline serum creatinine in *Pgc-1α*$^{-/-}$ and in WT mice [9]. Overall, their finding of increased baseline serum creatinine in *Pgc-1α*$^{-/-}$ mice in only some experiments is consistent with our observation of subclinical kidney injury in *Pgc-1α*$^{-/-}$ mice [9,11]. Funk et al observed that activation of the SIRT1/PGC1a axis favors renal recovery following ischemia/reperfusion injury [33]. They suggested that protection was dependent on mitochondrial biogenesis, but renal inflammation was not explored [33]. Pgc-1α overexpression may be beneficial in cultured renal cells and in some experimental models of renal disease [10,11,13]. However, *Pgc-1α* overexpression in podocytes caused collapsing glomerulopathy, suggesting that a tight regulation in at least some cell types is required [34].

In nephrotoxic AKI, we observed higher compensatory proliferation in *Pgc-1α*$^{-/-}$ mice than in WT mice, consistent with a response to a more severe injury. Based on the increased number of PCNA positive cells and the higher severity of kidney injury in *Pgc-1α*$^{-/-}$ we hypothesize that the increased PCNA staining represents an expected compensatory proliferation to more severe kidney injury and further interpret that this suggests that compensatory cell proliferation is not compromised by Pgc-1α deficiency. However, we cannot exclude that indeed Pgc-1α deficiency promotes cell proliferation, or that cell division is not actually completed due to cell cycle arrest or endocycle [35]. In this regard, the severity of inflammation progressively increased overtime in PGC1α$^{-/-}$ mice, as assessed by increasing lymphocyte infiltration, inflammatory cytokine expression and persistence of a pro-inflammatory pattern of macrophages with failure to develop a repair phenotype, and this may have contributed to persistence of injury despite preserved tubular proliferative responses, given the known role of inflammation, and specifically, of TWEAK and TNFα, in AKI [14,20,36]. In endotoxemia-associated AKI, and in renal IRI, kidney injury was more severe or persistent in *Pgc-1α*$^{-/-}$ mice and it was associated to low oxygen consumption, but whether there was an impact on regeneration or inflammation was not clarified [9,11]. Indeed, as indicated above, no significant cell death was observed in endotoxemia-induced AKI, so no tubular cell significant regeneration was expected [9]. Additionally, since these prior reports assessed only one time-point, it was unclear whether injury persists because it was more severe or because recovery was impaired. In this regard, a study of folic acid-induced fibrosis at a unique time-point of 6 weeks, did not explore whether there were differences in inflammation or early kidney injury [13]. Our findings of more severe AKI in *Pgc-1α*$^{-/-}$ mice within a few days of folate administration suggest that fibrosis findings at later time points may be conditioned by early events immediately post folate administration. Our findings of decreased *Pgc-1α* expression in human kidney disease, in line with previous reports [11,13,37], emphasize the clinical relevance of the present observations.

The present results are in line with prior descriptions of organ injury in PGC-1a KO that suggest that the spontaneous inflammatory phenotype is not limited to the kidneys. PGC-1a KO mice also show spontaneous inflammatory infiltrated in pancreas and this is enhanced during acute pancreatitis [38]. Similar observations have been described in liver, where PGC-1a KO mice present higher number of macrophages than WT mice [39]. In this regard, PGC-1a KO mice present a reduced oxidative metabolism in both pancreas and liver. However, the present manuscript was not focused on oxidative metabolism.

In conclusion, the present study provides evidence that PGC-1α plays an anti-inflammatory role in normal kidneys and during nephrotoxic AKI. In this regard, PGC-1α deficiency sensitized tubular cells to death and resulted in exacerbated pro-inflammatory responses both in culture and in vivo, increasing the severity and mortality of AKI (**Figure 7**).These results are consistent with prior descriptions of the role of PGC-1α in kidney injury but add a hitherto unexplored role of this molecule in the regulation of kidney inflammation and

may be relevant to design therapeutic approaches that modulate PGC-1α activity or the consequences of PGC-1α deficiency during kidney injury.

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AUTHOR CONTRIBUTIONS

All the authors reviewed the manuscript and approved the final version. MF-B contributed to experiment planning; acquisition, analysis and interpretation of all data; JMM-M, OR-A, DM-S, and SC contributed to the data acquisition and analysis. MM and CS-R provided *Pgc-1α-/-* mice, shRNA against *Pgc-1α* or control shRNA, and contributed to design the experiments to assessed mitochondrial injury. MJG carried out bioinformatics analysis and generation of figures. MDS-N and MRO contributed to the critical review. RC and CGE provided human samples. AO contributed to the designing research studies, the writing the manuscript and financial support. ABS contributed to designing research studies, analyzing data, writing the manuscript and financial support.

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LEGENDS TO FIGURES

Figure 1. Upstream regulator and canonical pathway enrichment analyses, using IPA, suggest that PGC-1α and mitochondrial function may play a key role during AKI. Upstream regulator and canonical pathway enrichment analyses, using IPA, suggest that PGC-1α and mitochondrial function may play a key role during AKI. **A)** Top 25 upstream regulators with the highest absolute z-score and p value < 0.05, predicted from the analysis of 3025 genes detected as differentially expressed after comparison of AKI vs control transcriptomic profiles. Significance of upstream-regulator target-gene enrichments is expressed as -log10(p value) and represented in a color scale. Z-score values, as calculated by IPA, represent a prediction on the activity of regulators; positive and negative values imply activation and inhibition, respectively, and are represented on the X axis. PGC-1α is the transcriptional regulator whose activity is most dramatically reduced in AKI, as indicated by a z-score of -5.66. **B)** Mitochondrial dysfunction, oxidative phosphorylation and NFκB signaling are among the top-ten canonical pathways detected as enriched in the same set of 3025 differentially expressed genes. Bars lengths are proportional to enrichment significance, expressed as the negative logarithm of Benjamini-Hochberg adjusted p-values. **C)** Circular plot representing the connections between 17 differentially expressed genes regulated by PGC-1α and three key pathways affected by AKI: mitochondrial dysfunction, oxidative phosphorylation pathways and NFκB signaling. PGC-1α-dependence was validated for the genes labelled in bold type, as described in Figures 5, 6 and SF3. **D)** *PGC-1α* mRNA expression assessed by RT-qPCR in human kidney with AKI. Upstream regulator and pathway enrichment analyses were performed on previously published transcriptomics data [10] (**Table S1**).

Figure 2. Effect of PGC-1α deficiency on mice survival, renal function and mitochondrial mass during AKI. A) Mouse survival. PGC-1α deficient mice had lower survival than WT mice at 48 hours after AKI. n=6 animals for WT and n=7 for *Pgc-1α*$^{-/-}$ mice. **B)** Renal function assessed by plasma creatinine levels. Kidney failure was more severe in surviving *Pgc-1α*$^{-/-}$ mice. **C)** *Ngal* mRNA expression was higher in *Pgc-1α*$^{-/-}$ than in WT mice. NGAL is a marker of renal tubular injury. **D)** Renal mRNA expression of PGC-1α targets *Tfam*, *Ndufs1* and *Sdha* was lower in *Pgc-1α*$^{-/-}$ mice than in WT mice. RT-qPCR. **E)** TOMM22 staining in renal tissue. TOMM22 levels decreased earlier in *Pgc-1α*$^{-/-}$ AKI mice than in WT AKI mice. Original magnification, x200. Scale bars, 50 µm. **F)** Scatter plot showing the significant negative correlation between TOMM22 staining and plasma creatinine (n=26). **G)** Mitochondrial DNA/nuclear DNA ratio (mtDNA/nDNA), expressed as % change over WT control. **B-E, G)** Mean ± SD of 4-8 mice per group. # p<0.05 vs control WT, ## p<0.01 vs control WT, *p<0.05 vs control *Pgc-1α*$^{-/-}$, **p<0.01 vs control *Pgc-1α*$^{-/-}$ † p<0.05, †† p<0.01.

Figure 3. PGC-1α deficiency was associated with increased tubulointerstitial inflammation in AKI. A) F4/80 staining in renal tissue. *Pgc-1α*$^{-/-}$ mice presented more severe F4/80^{+} macrophage infiltration at 24 hours after AKI compared with WT mice. Original magnification, x200. Scale bars, 50 µm. **B)** CD3 staining in renal tissue. *Pgc-1α*$^{-/-}$ mice presented more severe CD3^{+} lymphocyte infiltration at 72 hours after AKI compared with WT mice. Original

magnification, x200. Scale bars, 50 μm. **C)** Quantification of $F4/80^+$ cells and $CD3^+$ lymphocytes. Mean ± SD of 4-8 mice per group. # p<0.05 vs control WT, *p<0.05 vs control *Pgc-1α*$^{-/-}$, **p<0.01 vs control *Pgc-1α*$^{-/-}$, † p<0.05, †† p<0.01.

Figure 4. PGC-1α deficiency increased kidney expression of cytokines and chemokines and canonical NFκB activation, and modulated macrophage M1-to-M2 transition markers in AKI. A) *Fn14* mRNA levels assessed by RT-qPCR. **B)** Fn14 protein levels. Representative western blot and quantification of Fn14 protein levels. The sensitivity of Western blot was optimized to display Fn14 during AKI, not in vehicle injected animals. **C)** *Il-6*, *Tnfα* and *Ccl2* mRNA levels. **D)** Nuclear NFκB p65 levels, assessed by ELISA, are higher in *Pgc-1α*$^{-/-}$ mice than in WT mice with AKI at 72 hours. **E)** Nuclear staining for NFκB p65 assessed by Southwestern histochemistry at 72 hours localized the increased NFκB p65 levels to tubular cell nuclei, most notably in *Pgc-1α*$^{-/-}$ mice. Representative images. Original magnification, x200. Scale bars, 50 μm.

Figure 5. PGC-1α deficiency is associated with modulation of the macrophage M1-to-M2 transition. A) Kidney *iNos* mRNA levels during AKI were higher in *Pgc-1α*$^{-/-}$ than in WT mice at 24 hours. **B)** Kidney *Mannose R* mRNA levels during AKI were lower in *Pgc-1α*$^{-/-}$ than in WT mice at 72 hours. **C)** The M1/M2 (*iNos*/*Mannose R* mRNA levels) ratio was higher in *Pgc-1α*$^{-/-}$ than in WT mice. **D)** mRNA levels of the anti-inflammatory cytokine *Il-10* was lower in *Pgc-1α*$^{-/-}$ than in WT mice. **E)** Mannose receptor (Mannose R) staining in renal tissue. *Pgc-1α-/-* mice presented a significantly lower mannose R+ macrophage M2 infiltration at 72 hours after AKI than WT mice**.** Original magnification, x400. Scale bars, 50 μm. **A-E)** Mean ± SD of 4-8 mice per group. # p<0.05 vs control WT, ## p<0.01 vs control WT, *p<0.05 vs control *Pgc-1α*$^{-/-}$, **p<0.01 vs control *Pgc-1α*$^{-/-}$, † p<0.05, † p<0.01.

Figure 6. PGC-1α downregulation promotes cell death and sensitizes to TWEAK-induced pro-inflammatory responses in tubular cells. Murine proximal tubular MCT cells were infected with adenoviruses expressing shRNA against *Pgc-1α* or shControl. **A)** Downregulation of PGC-1α was tested by western blot. Representative western blot. **B)** Cell death assessed by flow cytometry of propidium iodide-stained cells and by contrast phase microscopy of unstained cells. **C, D)** Fn14 expression at the mRNA **(C)** and protein levels **(D). E)** 48 hours after infection, MCT cells were stimulated with 100 ng/mL TWEAK for 6 hours and mRNA levels of *Il-6*, *Tnfα* and *Ccl2* were measured. **B-E)** Mean ± SD of four independent experiments. *p<0.05 vs shControl. **p<0.01 vs shControl.

SUPPLEMENTARY MATERIAL:
Supplementary methods
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6
Supplementary Figure 7
Supplementary Figure 8

Table S1
Table S2