Histone lysine-crotonylation in acute kidney injury

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SUMMARY STATEMENT

We have assessed the effect of the epigenetic post-translational modification histone crotonylation during kidney injury in vivo and in cell culture, and the involvement of PGC-1α and SIRT3 in the process.

ABSTRACT

Acute kidney injury (AKI) is a potentially lethal condition for which no therapy is available beyond replacement of renal function. Post-translational histone modifications modulate gene expression and kidney injury. Histone crotonylation is a recently described post-translational modification. We hypothesized that histone crotonylation may modulate kidney injury. Histone crotonylation was studied in cultured murine proximal tubular cells and in kidneys from mice with AKI induced by folic acid or cisplatin. Histone lysine-crotonylation was observed in tubular cells from healthy murine and human kidney tissue. Kidney tissue histone crotonylation increased during AKI. This was reproduced by exposure to TWEAK in cultured tubular cells. Specifically, ChIP-seq disclosed enrichment of histone crotonylation at the genes encoding the mitochondrial biogenesis regulator PGC-1α and the sirtuin-3 decrotonylase in both TWEAK-stimulated tubular cells and in AKI kidney tissue. To assess the role of crotonylation in kidney injury, crotonate was used to increase histone crotonylation in cultured tubular cells or in the kidneys in vivo. Crotonate increased the expression of PGC-1α and sirtuin-3, and decreased CCL2 expression in cultured tubular cells and healthy kidneys. Systemic crotonate administration protected from experimental AKI, preventing the decrease in renal function and in kidney PGC-1α and sirtuin-3 levels as well as the increase in CCL2 expression. For the first time we have
identified factors such as cell stress and crotonate availability that increase histone crotonylation in vivo. Overall, increasing histone crotonylation may have a beneficial effect on AKI. This is the first observation of the in vivo potential of the therapeutic manipulation of histone crotonylation in a disease state.
INTRODUCTION

Post-translational modifications of proteins are involved in chronic kidney disease and cardiovascular disease (Gajjala et al., 2015). Indeed, mounting evidence suggests that histone post-translational modifications, such as methylation, acetylation or phosphorylation, play a key role in diverse biological processes, such as development, cell differentiation, cell death and inflammation (Berdasco and Esteller, 2010). Indeed, aberrant histone post-translational modification contributes to disease (Berdasco and Esteller, 2010). Histone post-translational modifications regulate chromatin-templated processes via two major mechanisms (Kouzarides, 2007): modulating chromatin packaging and regulating chromatin structure and function by recruiting post-translational modification-specific binding proteins, which recognize modified histones. Alternatively, histone post-translational modifications can also inhibit the interaction of specific binders with chromatin. Recent studies identified lysine crotonylation (Kcr) as a novel evolutionarily-conserved histone post-translational modification, present in several somatic tissues from adult mice (Tan et al., 2011). As recently described, histone crotonylation is mechanistically and functionally different from histone lysine acetylation (Tan et al., 2011, Sabari et al., 2015). Histone crotonylation was observed in kidney tissue, suggesting that it might play a role in epigenetic regulation of gene expression during kidney injury (Tan et al., 2011).

There is yet very little knowledge about the regulation and function of histone crotonylation during tissue injury. Crotonate is a short-chain unsaturated carboxylic acid (CH₃CH=CHCO₂H) that increased histone crotonylation in cultured non-renal cells (Tan et al., 2011, Sabari et al., 2015), but its effect in vivo was not addressed (Tan et al., 2011). Sirtuin-3 (SIRT3) is a histone deacetylase, recently identified also as a decrotonylase (Tan et al., 2011). SIRT3 is a member of the sirtuin family of NAD(+)
dependent deacetylases (Huang et al., 2010, Scher et al., 2007) that associates with chromatin to repress nearby genes (Shechter et al., 2007, Iwahara et al., 2012). SIRT3 is present both in mitochondria and nuclei (Scher et al., 2007, Nakamura et al., 2008, Sundaresan et al., 2008). SIRT3 is expressed by kidneys and metabolically active tissues. Under physiologic and stress conditions SIRT3 and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) regulate each other expression (Shi et al., 2005, Palacios et al., 2009, Than et al., 2011, Giralt et al., 2011). PGC-1α regulates gluconeogenesis and mitochondrial biogenesis and respiration. SIRT3 is a mediator of PGC-1α effects on mitochondrial biogenesis (Kong et al., 2010). PGC-1α is downregulated during (AKI) (Ruiz-Andres et al., 2015, Tran et al., 2011), a condition characterized by a sudden, potentially prolonged reduction of the renal glomerular filtration rate causing azotemia. AKI is associated with high morbidity and mortality rates and there is no therapy to treat established AKI beyond replacement of kidney function (Berger and Moeller, 2014). Proximal tubules are rich in mitochondria and are key sites of injury during AKI. An improved understanding of pathogenic pathways involved in AKI may provide clues to design novel therapeutic approaches. Inflammation and cell death are key contributors to AKI (Linkermann et al., 2014, Garcia-Cenador et al., 2013). In this regard, tumor necrosis factor-like weak inducer of apoptosis (TWEAK) was recently shown to be a key contributor to AKI and kidney injury in general (Ortiz et al., 2011, Sanz et al., 2011). TWEAK is a cytokine of the TNF superfamily that activates the Fn14 receptor, and has multiple actions on kidney cells. Thus, TWEAK promotes kidney inflammation by increasing chemokine secretion by renal cells and decreasing the expression of nephroprotective factors such as klotho, promotes tubular cell proliferation in a permissive environment and induces mesangial and tubular cell apoptosis under proinflammatory conditions (Sanz et al., 2011).
TWEAK decreases PGC-1α and target gene expression in tubular cells through NFκB activation and histone deacetylation (Ruiz-Andres et al., 2015). Since TWEAK actions are mediated through the recruitment of signaling mechanisms that include NFκB activation and histone acetylation, we hypothesized that TWEAK may also modulate histone lysine-crotonylation. Furthermore, since epigenetic changes are also observed in AKI and histone deacetylase (HDAC) inhibitors may protect from kidney injury (Van Beneden et al., 2011, Van Beneden et al., 2013), we further hypothesized that histone lysine-crotonylation may be a contributor to and a therapeutic target in AKI.

We have now explored histone crotonylation regulation and function in cultured kidney tubular epithelial cells and during kidney injury in vivo. Specifically, we have observed that histone lysine-crotonylation is increased during AKI and by inflammatory cytokines such as TWEAK in tubular cells. Crotonate increased histone lysine-crotonylation and PGC-1α expression in cultured tubular cells and in the kidney in vivo and protected from AKI.

RESULTS

Histone crotonylation is increased in kidney tubular cells during acute kidney injury

The histone crotonylation pattern during renal injury was explored in detail in an established mouse model of AKI induced by a folic acid overdose and the results confirmed in experimental cisplatin-induced AKI. In folic acid-induced AKI, as in other experimental models and human AKI, loss of renal function, tubular cell injury and interstitial inflammation were observed (Sanz et al., 2008a).

Consistent with prior reports (Tan et al., 2011), histone crotonylation was observed in healthy murine kidney tissue when assessed by Western blot (Fig. 1.A) or
immunohistochemistry (Fig. 1.B). Western blot identified histones as crotonylated proteins, while immunohistochemistry localized lysine-crotonylation mainly to tubular cell nuclei (Fig. 1.B). Western blot disclosed an increase in overall histone crotonylation in folic acid AKI kidney tissue (Fig. 1.A). Similar results were observed in cisplatin-induced AKI at 72h (Fig. S1). However, the rest of the detailed studies discussed in the manuscript were obtained in the folic acid model. We also explored the histone H3 crotonylation (H3k9cr) pattern during renal injury. Consistent with the finding of overall histone crotonylation, western blot disclosed an increase in kidney histone H3 crotonylation in folic acid AKI tissue (Fig. S2.A). Immunohistochemistry identified tubular cells as sites of lysine-crotonylation during AKI (Fig. 1.B). Nuclear localization of lysine crotonylation consistent with histone crotonylation was also observed in cultured murine proximal tubular cells by immunofluorescence (Fig. 1.C). As it was the case in vivo, there were different degrees of lysine crotonylation in individual cultured tubular cells, suggesting that this is a regulated and dynamic process. Cell separation into nuclei and cytosol disclosed a faint crotonylated protein band in nuclei that corresponded in size to histones (Fig. 1.D). Thus, histones appear to be the most abundant crotonylated proteins in tubular cells. Immunohistochemistry also identified nuclear lysine-crotonylation in diseased human kidney tubular cells (Fig. 1.E).

**TWEAK increases histone crotonylation in cultured kidney tubular cells**

Next, we explored the hypothesis that inflammatory mediators of AKI could modulate histone crotonylation. TWEAK is a key mediator of AKI that promotes inflammatory responses in cultured tubular cells but has no direct cytotoxicity if used in the absence of other inflammatory mediators (Sanz et al., 2010b, Izquierdo et al., 2012)
and we studied the effect of TWEAK on histone crotonylation in kidney cells. TWEAK increased histone crotonylation at 6 and 24 hours in cultured tubular cells (Fig. 2). These results suggest that inflammatory cytokines may regulate the histone crotonylation status in kidney cells.

At this point, we also explored the hypothesis that direct cytotoxicity may promote histone crotonylation. As is the case for folic acid-induced AKI, ischemia-reperfusion-induced AKI and other forms of AKI; in vivo cisplatin recruits an inflammatory response that amplifies kidney injury (Zhang et al., 2008, Baek et al., 2015) However, contrary to TWEAK, cisplatin has a direct toxic effect on cultured tubular cells. In this regard, cisplatin at concentrations that induced direct toxicity in cultured tubular cells, did not modify histone crotonylation (Fig. S3). These results indicate that cytotoxicity and histone crotonylation can be dissociated in cultured tubular cells and argue for the involvement of additional factors in vivo.

Crotonate increases histone crotonylation and elicits biological responses in cultured tubular cells

Then, we searched for potential target genes of histone crotonylation whose expression is differentially regulated in AKI. PGC-1α is a regulator of mitochondrial biogenesis that is decreased in AKI, while SIRT3 is a deacetylase, and both regulate each other expression. Therefore, as representative downregulated gene we chose PGC-1α, since it regulates SIRT3 expression (Giralt et al., 2011, Kong et al., 2010, Bell and Guarente, 2011). Moreover, TWEAK decreases PGC-1α expression by epigenetic mechanisms involving histone acetylation (Ruiz-Andres et al., 2015). ChIP-seq analysis using the pan anti-crotonyl-lysine antibody showed that PGC-1α and SIRT3 were more enriched in crotonylated histones in tubular cells treated with TWEAK and in kidneys
with AKI (Fig. 3.F-G). To study the effect of crotonylation on PGC-1α and SIRT3 expression, cells were pretreated with crotonate since exogenous crotonate increased histone crotonylation in cultured tubular cells (Fig. 3.A). This is consistent with findings in non-renal cells (Tan et al., 2011, Sabari et al., 2015). Crotonate increased tubular cell PGC-1α mRNA and protein levels (Fig. 3.B-C). As representative upregulated gene we chose CCL2 since it encodes the MCP-1 chemokine, a promoter of kidney injury (Sanz et al., 2010a). Crotonate decreased tubular cell CCL2 mRNA in cultured cells (Fig. 3.D). Taken together, these results suggest that histone crotonylation could play an overall protective role in kidney injury by promoting upregulation of some protective genes and downregulation of genes involved in tissue injury. Crotonate also increased SIRT3 mRNA levels in cultured tubular cells in a time-dependent manner (Fig. 3.E), suggesting the activation of a negative feed-back loop.

Crotonate did not promote tubular cell death nor proliferation as assessed by the presence of hypodiploid cells or cells in the S/M phases by flow cytometry studies (Fig. S4), nor did it increase cell detachment as observed by contrast phase microscopy (not shown). Mannitol, an osmolarity control did not modify histone crotonylation at concentrations equimolar to the crotonate concentrations used, arguing against a role of osmolarity in modulating histone crotonylation (Fig. S5).

**Crotonate increases histone crotonylation and modulates regulated gene expression in mouse kidney**

We explored whether crotonate modulates kidney histone crotonylation in vivo. Systemic administration of crotonate increased histone crotonylation in mouse kidney in a dose- and time-dependent manner (Fig. 4.A-B). The 6 mmol/kg crotonate dose did not significantly change kidney histone crotonylation (Fig. S6.A) or PGC-1α mRNA
expression (Fig. S6.B) at 24h. Thus, 12 mmol/kg crotonate was used for further experiment and found to increase whole kidney histone crotonylation (Fig. 4), PGC-1α mRNA levels (Fig. 5.A) and PGC-1α protein (Fig. 5.B), and to decrease kidney CCL2 mRNA levels (Fig. 5.C).

Thus, the potential nephroprotective actions of crotonate observed in cultured tubular cells (increased expression of the nephroprotective gene PGC-1α and decreased inflammatory gene expression) were reproduced in vivo.

Since the SIRT3 decrotonylase (Bao et al., 2014) and PGC-1α each regulate each other under physiologic and stress conditions (Shi et al., 2005, Palacios et al., 2009, Than et al., 2011, Giralt et al., 2011), we studied the effect of exogenous crotonate over kidney SIRT3 expression in vivo and found that crotonate increased whole kidney SIRT3 mRNA (Figure 5.D) and protein levels (Figure 5.E). This is consistent with the effects observed in cultured tubular cells and is again suggestive of activation of a negative feed-back loop.

**Crotonate protects from experimental AKI**

We next explored whether crotonate was nephroprotective in vivo. Mice were pretreated with 12 mmol/kg crotonate and 24 hours later was induced by a folic acid overdose and mice were euthanized at 72h, when renal failure peaks (Sanz et al., 2010b). Firstly, we observed that crotonate resulted in lower serum levels of BUN and creatinine, markers of renal dysfunction severity, and in lower KIM-1 mRNA levels, a marker of kidney injury (Fig. 6.A). PAS-stained kidney sections revealed a trend towards decreased tubular injury in crotonate-treated mice (Fig. S7). AKI was associated with increased CCL2 expression (Sanz et al., 2010b) and reduced whole kidney SIRT3 expression (Fig. S8) within the time points studied. In this line, systemic
administration of crotonate prevented the decrease in kidney PGC-1α and SIRT3 levels in AKI (Fig. 6.B,C,E,F) as well as the increase in CCL2 mRNA expression (Fig. 6.D). This suggests a protective effect of crotonate, and thereby of histone crotonylation, against inflammation and mitochondrial stress during AKI.

**TWEAK downregulates SIRT3 and this is prevented by crotonate**

As recently described, increased histone crotonylation in response to crotonate loading may depend on increased substrate (crotonyl-CoA) availability (Sabari et al., 2015). However, the mechanism of increased histone crotonylation following TWEAK stimulation remained unclear. Thus, we explored whether TWEAK regulated the expression of the SIRT3 decrotonylase and observed that TWEAK downregulated SIRT3 at mRNA and protein levels in cultured tubular cells (Fig. 7.A-B) and in whole kidney in vivo (Fig. 7.C.D). TWEAK-induced SIRT3 downregulation was prevented by crotonate in cultured tubular cells (Fig. 7.E-F). These data suggest that decreased SIRT3 expression may be one of the factors contributing to increased kidney cell histone crotonylation in response to TWEAK.

**DISCUSSION**

The main findings of this study are that the degree of histone crotonylation in kidney tubular cells is modified by certain cell stressors or crotonate. Furthermore, increasing histone crotonylation was overall beneficial in AKI. This is the first observation of the in vivo potential of the therapeutic manipulation of histone crotonylation in a disease state.

Histones were the most abundant crotonylated proteins. The fact that increased histone crotonylation was found under stress conditions, be it AKI or exposure to a
proinflammatory cytokine, begs the question of what is the overall role of crotonylation in kidney injury. As is the case with other histone post-translational modifications, it is expected that in response to the microenvironment, some genes may increase while other genes may decrease the degree of histone crotonylation. Despite this expected heterogeneity, therapeutic agents targeting other histone post-translational modifications have been beneficial in diverse pathological conditions, including kidney injury, even when potentially impacting the expression of multiple genes with diverse or even opposing functions. For example, HDAC inhibitors such as trichostatin A were protective in experimental models of kidney fibrosis (Van Beneden et al., 2013) and selectively mitigate the stimulatory effect of lipopolysaccharide on inflammatory cytokine expression (Munro et al., 2013). We hypothesized that overall interference with histone crotonylation might have a beneficial or deleterious effect in AKI. We have now shown that increasing overall histone crotonylation by exposure to crotonate has potentially beneficial effects on tubular cells in culture and in vivo, including an increased expression of the mitochondrial biogenesis regulator PGC-1α and decreased chemokine expression. Consistent with these findings, crotonate protected from injury and loss of renal function in AKI. The genes studied in the present manuscript were chosen as representative of up and downregulated genes shared by both AKI and stressor-stimulated (i.e. TWEAK-stimulated) cultured tubular cells. Since histone post-translational modifications may impact the expression of multiple genes, it remains to be explored whether changes in the expression of these specific genes or other genes are the key drivers of the observed beneficial effect of crotonate.

We identified and characterized two maneuvers that increased overall histone crotonylation in kidney cells: cell stress by inflammatory cytokines or during AKI, and increasing the crotonate substrate availability. By contrast, direct cytotoxicity by
cisplatin in culture did not modulate histone crotonylation. However both maneuvers had a differential effect on the expression of the studied genes. Thus, we identified two specific genes, PGC-1α and SIRT3, which undergo increased histone crotonylation during AKI and in tubular cells stressed by TWEAK. Under stress conditions, mRNA and protein levels of both genes are decreased, suggesting decreased transcription. One possible explanation is that increased histone crotonylation results in decreased gene transcription. However, increasing overall histone crotonylation by addition of crotonate increased the expression of PGC-1α and SIRT3. Thus, alternative explanations should be sought. One possibility is that the effect of histone crotonylation may be context-dependent: within the increased crotonate substrate availability context, histone crotonylation promotes gene expression while under a proinflammatory or cell stress context, histone crotonylation may decrease expression of certain genes. In this regard, the same histone post-translational modification may be associated with increased or decreased gene expression depending on the gene and context (Bao et al., 2014, Sabari et al., 2015). Further studies should clarify this issue in the specific case of crotonylation. Up to now, higher levels of histone crotonylation at the promoters of genes activated by LPS, such as Il6, Gbp2, Ifit1, and Rsad2, were associated with increased gene expression (Sabari et al., 2015). In this regard, an alternative potential explanation is that increased histone crotonylation at the genes encoding SIRT3 and PGC-1α following cell stress may be a compensatory mechanism that limits the fall in gene expression, rather than the driver of gene suppression. Further studies are needed to unravel the role of histone crotonylation in the regulation of gene expression in different cellular contexts and for specific genes.

It was recently reported that crotonate increased intracellular crotonyl-CoA availability, thus stimulating gene transcription through p300-catalyzed histone
crotonylation (Sabari et al., 2015). In this regard, histone crotonylation can be catalyzed by either p300 or the p300/CBP (acyetyltransferase p300/CREB-binding protein) complex (Sabari et al., 2015). Interestingly, SIRT3 and PGC-1α regulate each other, through CREB-mediated gene expression mechanisms (Shi et al., 2005, Palacios et al., 2009, Than et al., 2011, Giralt et al., 2011). Our results suggests that crotonate, by increasing crotonyl-CoA availability, leads to increased histone crotonylation (Sabari et al., 2015), and increased gene transcription of SIRT3, that acts as a decrotonylase. The increased expression of a decrotonylase in response to crotonate may be teleologically interpreted as the activation of a negative feed-back mechanism. Increased SIRT3 levels and histone crotonylation at the PGC-1α gene may increase PGC-1α levels, limiting cell injury in response to stress.

By contrast, under stress conditions such as AKI or in tubular cells stressed by inflammatory cytokines (e.g. TWEAK), the decrease in SIRT3 expression, and, potentially, in SIRT3 decrotonylase activity, may lead to increased histone crotonylation, thus limiting the downregulation of protective genes such as PGC-1α (Fig. 8). Thus, decreased SIRT3 expression may be an additional pathway leading to increased histone crotonylation in an inflammatory milieu. Further research should explore the changes of histone crotonylation in relation to the functional status of the cell, as well as identify changes in histone crotonylation for individual genes.

SIRT3 is the physiological deacetylase that antagonizes p300-mediated histone acetylation (Wang et al., 2012). Although sirtuins were initially described as NAD-dependent deacetylases (Imai et al., 2000, Landry et al., 2000, Sauve et al., 2006), some sirtuins with weak deacetylase activity may have substrate specificity towards other acyl groups attached to lysine residues. For example, SIRT5 can preferentially hydrolyze malonyl and succinyl lysine (Jiang et al., 2013, Du et al., 2011, Peng et al.,
2011), and SIRT6 can remove long chain fatty acyl groups from lysine residues (Jiang et al., 2013). More recently, SIRT1 to SIRT3 were reported to behave as decrotonylases, thus regulating histone crotonylation dynamics and gene transcription (Bao et al., 2014).

A staining pattern compatible with histone crotonylation was also observed in human kidney tissue, including diseased kidneys. Although we were not able to study early stages of human AKI, this observation supports the potential clinical relevance of the findings.

In conclusion, for the first time we have shown that the pattern of histone crotonylation changes during AKI and in cultured tubular cells stressed by an inflammatory cytokine, suggesting a role of histone crotonylation in kidney injury. Furthermore, we have shown that the degree of kidney cell histone crotonylation may be manipulated therapeutically by administering crotonate and that increasing overall histone crotonylation was nephroprotective. While the precise mechanisms for the protective effect of crotonate remains to be further clarified, results reported here are consistent with the hypothesis that histone crotonylation-mediated regulation of gene transcription plays a role. This is the first observation of the in vivo potential of the therapeutic manipulation of histone crotonylation in a disease state. Further studies are needed to define the role of histone crotonylation in additional models of kidney disease and in injury to other tissues.
METHODS

Cells and reagents

For in vitro experiments we use MCT murine proximal tubular epithelial cells. This cell line was originated from the kidneys of SJL mice in the University of Pennsylvania and obtained from Eric G Neilson. Cells were authenticated and tested for mycoplasma contamination before use. MCT cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), in 5% CO2 at 37°C. RPMI-1640, penicillin, streptomycin, and trypsin-EDTA were from BioWhittaker (Waltham, MA) and FBS from Gibco (Carlsbad, CA) (Haverty et al., 1988). For experiments, cells were serum-depleted for 24 hours, and then stimulated. Recombinant human TWEAK (Millipore, Billerica, MA) was used at 100 ng/mL unless otherwise specified, based on previously reported dose-response experiments in the same cells (Sanz et al., 2008b). Crotonate was used at 50 mM and 100 mM, based on previous studies on dynamics of histone lysine-crotonylation in response to crotonate (Tan et al., 2011). Crotonic acid (Sigma) was dissolved in water at a concentration of 100 mg/ml to yield a clear, colorless solution. A 1M stock solution in H2Odd was adjusted to neutral pH (pH= 7.5) with sodium chloride. From this stock solution the necessary dilutions were made in cell culture media (RPMI-1640, Sigma). Following the same protocol, we use mannitol (Sigma), at concentrations equimolar to the crotonate concentrations used, as osmolarity control in experiments in vitro. Mannitol is an inert molecule generally used as osmolarity control for experimental conditions that increase osmolarity, as during the study of the effects of high glucose concentration on cell biology. Cisplatin (Sigma) was used at concentrations of 50 and 100 µM.
RNA extraction and Real-Time Polymerase Chain Reaction

Total RNA was extracted by the TRI Reagent method (Sigma) and 1 µg of RNA was reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Assays were from Applied Biosystems. Quantitative PCR was performed in a 7500 Real Time PCR System with the Prism 7000 System SDS Software (Applied Biosystems) and RNA expression was corrected by GAPDH expression.

Western blot

Cell samples were homogenized in lysis buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM PMSF and 1 µg/ml pepstatin A) then separated by 10% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to Nitrocellulose membranes (BioRad), blocked with 5% skimmed milk in PBS/0.5% v/v Tween 20 for 1 hour, washed with PBS/Tween, and incubated with mouse polyclonal anti-PGC-1α (1:1000, CalBiochem) or rabbit polyclonal anti-SIRT3 (1:1000, Cell Signaling). Anti-PGC-1α was diluted in 5% milk PBS/Tween and anti-SIRT3 in 5% BSA PBS/Tween. Blots were washed with PBS/Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham, Aylesbury, UK). After washing with PBS/Tween blots were developed with the chemiluminescence method (ECL) (Amersham) using ImageQuant LAS 400 system (GE Healthcare). Then, the images were analyzed with Quantity One software (BioRad). Blots were then probed with mouse monoclonal anti-α-tubulin antibody (1:10000, Sigma) and levels of expression were corrected for differences in loading.

Histones were isolated using the epiQuik Total histone Extraction kit (Epigentek), and then separated by 10% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to PVDF membranes (Millipore), blocked
with 5% BSA in PBS/0.5% v/v Tween 20 for 1 hour, washed with PBS/Tween, and incubated with rabbit polyclonal pan anti-crotonyl-lysine antibody (1:1000, PTM Biolabs) and anti-crotonyl-Histone H3 (Lys9) antibody (anti-H3K9, 1:1000, PTM Biolabs). Both antibodies were diluted in 5% BSA PBS/Tween. Blots were then probed with rabbit polyclonal anti-histone H3 antibody (1:2000, Cell Signaling) after stripping with a buffer that removes primary and secondary antibodies from membranes (Yeung and Stanley, 2009). Anti-Kcr staining was normalized for anti-Histone H3 or Ponceau staining for each sample to correct for differences in loading. Then, the mean of the normalized control values was considered to be 100% and the normalized expression levels in other samples were expressed as % change over control.

Animal models

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of IIS-FJD. Folic acid nephropathy is a classical model of AKI (Sanz et al., 2008a, Ortega et al., 2006, Fang et al., 2005, Doi et al., 2006) which has been described in humans (Metz-Kurschel et al., 1990). Mice received a single intraperitoneal injection of 250 mg/kg folic acid (Sigma) in 0.3 mol/l sodium bicarbonate or vehicle control and were sacrificed at days 1, 3 or 7 (n=5 per day and group).

As a second model of AKI another set of mice (n=5 per group) was injected intraperitoneally with 20 mg/kg Cisplatin (Sigma) or vehicle (saline control)(Zhang et al., 2008). Mice were sacrificed at 72 hours. Nephrotoxicity is the dose-limiting side effect of the chemotherapeutic agent cisplatin in humans.

A further set of mice was injected intraperitoneally with crotonate. For dose-response experiments mice received 3, 6 or 12 mmol/kg crotonate (Sigma) or vehicle (saline control) and were sacrificed at 48 hours. For time-course experiments mice were
injected intraperitoneally with 12 mmol/kg crotonate or vehicle (saline control) (Hawkins et al., 1973, Lemieux et al., 1979, Lemieux et al., 1977) and killed at 24, 48 or 72 hours (n=4 per group and time-point). Crotonate dose was calculated based on in vitro experiments for an extracellular volume of 6.5 ml/ mouse and further refined by the dose-response studies. In other set of experiments, mice were pretreated with 12 mmol/kg crotonate and 24 hours later AKI was induced by folic acid injection and mice were sacrificed at 72 hours.

Kidneys were perfused in situ with cold saline before removal. One kidney was snap-frozen in liquid nitrogen for RNA and protein studies and the other fixed and paraffin-embedded. Blood was collected from femoral vein before kidneys perfusion. For all the experiments C57/BL6 mice, 12 to 14 weeks old were used.

For histological assessment, PAS-stained kidney sections were evaluated by an experienced pathologist blinded as to the nature of the samples, using a semiquantitative histological score on a 0 (normal) to 3 scale (severely affected) evaluating the following items: tubular cell injury, tubular cell regeneration, tubular atrophy, calcification, tubule dilatation, leukocyte casts, hyaline casts. For each mice, the sum of the individual score for each item yielded the total score.

**ChIP-seq**

ChIP-seq for histone lysine-crotonylation was carried out as previously described with 100 µg fractionated cell or kidney tissue chromatin and 5 µg anticrotonyl-lysine antibody (Tan et al., 2011). ChIP-seq libraries for sequencing were prepared following Illumina protocols (Illumina, San Diego, CA) with minor modifications. Libraries for input samples were generated using 20 ng of corresponding input chromatin. Briefly, ChIPed DNA was first blunted with END-IT DNA repair kit (Epicenter Biotechnology, Madison, WI) and then incubated with Klenow fragment
(3’→5’ exo-) (New England Biolabs, MA) and dATP to generate single base 3’-dA overhang. Illumina sequencing adapter was then ligated to the resulting DNA, followed by size selection (180–400 bp) in an 8% acrylamide gel. This size-selection step was repeated after PCR amplification with DNA primers (Illumina). Libraries were sequenced using Illumina GAI or HiSeq machine as per manufacturer's protocols. Following sequencing cluster imaging, base calling were conducted using the Illumina pipeline. Reads were mapped to mouse mm10 genome build with a bowtie software package. Total mapped tags were paired down to unique, monoclonal tags. These are tags that mapped to one location in the genome and each sequence is represented once.

**Flow cytometry**

10,000 cells were seeded in 12-well plates (Costar, Cambridge, MA) in 10% FBS RPMI overnight and rested in serum-free medium for 24h before crotonate addition. Thereafter, stimuli were added to subconfluent cells. For assessment of apoptosis by flow cytometry adherent cells were pooled with spontaneously detached cells, and incubated in 100 µg/mL propidium iodide (PI), 0.05% NP-40, 10 µg/mL RNAse A in PBS at 4°C for >1 h. This assay permeabilizes the cells, thus PI stains both live and dead cells. The percentage of apoptotic cells with decreased DNA staining (hypodiploid cells) was counted by flow cytometry using BD CellQuest Software (BD Biosciences) (Sanz et al., 2009, Justo et al., 2006, Lorz et al., 2000).

**Immunohistochemistry and immunofluorescence**

Kidney tissue immunohistochemistry was performed as previously described (Hazzouri et al., 2000) in 3 µm thick sections of paraffin-embedded tissue using a PT-link device (with a low pH solution, 95°C, 20 min). Sections were washed with wash buffer for 5 minutes and blocked by incubation with PBS containing 5% milk for 30 minutes. For immunostaining sections were incubated with anti-crotonyl-lysine
antibody (PTM Biolabs) diluted at 1/250 in TBS 0.5% milk for 2 hours, washed in TBS thrice, incubated with biotinylated secondary antibody (1/2000 in PBS containing 0.5% milk) for 30 minutes, washed and incubated with AB streptavidin-complex. Then the slides were washed with TBS and final detection was performed using DAB (Dako Diagnostics) according to the manufacturer’s instructions. Sections were counterstained with Carazzi’s hematoxylin. Negative controls included incubation with isotype IgG. Immunohistochemistry was performed in 5 human kidney samples from the IIS-FJD Biobank, corresponding to males, aged 56 to 80 years, serum creatinine 0.7 to 1.7 mg/dl.

For immunofluorescence one million cells were seeded on coverslips in RPMI medium supplemented with 10% FBS and were serum starved for 24 hours prior to experiments. Cells were washed with ice-cold PBS three times and fixed in neutral buffered 10% formalin (Sigma) in PBS for 20 min at room temperature. After three brief PBS rinses, cells were permeabilized with 0.2 % Triton X-100 in PBS for 10 min on ice followed by PBS rinses. Permeabilized cells were then blocked with 5% BSA in PBS for 30 min at room temperature and then incubated with pan anti-crotonyl-lysine antibody (PTM Biolabs) 1/500 in 5% BSA/PBS at 4°C overnight, followed by incubation with AlexaFluor488-goat anti-rabbit IgG (1/300 in 5% BSA/PBS, Invitrogen) for 1 hour at 37 °C. Then cells were incubated with AlexaFluor 555 phalloidin (Life technologies) (1/1500 in 5% BSA/PBS) for 30 minutes at room temperature, counterstained with DAPI and mounted.
Statistics

Statistical analysis was performed using the SPSS 11.0 statistical software (Chicago, IL). Results are expressed as mean ± SEM. Significant differences between mean values were determined with the Mann-Whitney U-test for comparison of two groups or paired Student’s t-test if appropriate (normal distribution and n≥5). 2-tailed test values p<0.05 were considered significant. For small samples sizes (n<5) nonparametric Mann-Whitney U-test was assessed.
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Competing interests:
Non-financial competing interests

Authors’ contributions
ORA contributed to the design of the experiments, acquisition, analysis and interpretation of all data, writing and drafting of the manuscript. PCO provided paraffin human tissue. MDSN and MRO contributed to critical review of the manuscript. JE contributed to critical review of the manuscript and financial support of the study. AO and ABS contributed to the design of experiments, analysis and interpretation of the data, writing the manuscript and financial support of the experiments. All the authors reviewed the manuscript and approved the final version to be published.

Translational impact
Acute kidney injury (AKI) has a mortality of 50% and there is no satisfactory therapy. The incidence of AKI is increasing as an aging population is exposed to more complex medical procedures. For the first time, we describe the pattern of histone lysine crotonylation in AKI and in tubular kidney cells, establish a model to assess the consequences of histones crotonylation in the kidney by the parental administration of crotonate, and uncover the therapeutic potential of crotonate for AKI. These results identify a new model to assess the consequences of histone crotonylation and identify a new therapeutic approach to AKI.
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Fig. 1. Histone crotonylation in kidney tubular cells A) Western blot of murine healthy control kidneys and acute kidney injury (AKI) induced by a folic acid overdose, and quantification: Mean ± SEM of 5 animals per group. Histone-3 was used as loading control. *p<0.05 vs control. Results were expressed as percentage change of
crotonylated histones over control. P values were calculated by nonparametric Mann-Whitney U-test. B) Immunohistochemistry localized histone crotonylation mainly to tubular cell nuclei in murine kidneys. Scale bar, 100 µm (Original magnification x1000), detail scale bar, 50 µm (original magnification x400). C) Nuclear localization of histone crotonylation (green) was also observed in cultured murine proximal tubular cells. Nuclei were stained with DAPI (blue) and the actin cytoskeleton with fluorescent phalloidin (red). Note several degrees of histone crotonylation: some cells have a lower staining intensity (white arrows) than others (yellow arrows). Scale bar, 5 µm. D) Cultured tubular cells. Strong Kcr signal in histones corresponding to the faint band in nuclei and low cytosol signal. Western blot and Coomassie blue stain. E) Immunohistochemistry localized histone crotonylation to tubular cells also in human kidneys. Scale bar, 50 µm (Original magnification x200), detail: scale bar 50 µm (original magnification x400) and scale bar 20 µm (original magnification x1000).
Fig. 2. **TWEAK increases histone crotonylation in kidney tubular cells.** Histone crotonylation in cultured murine proximal tubular cells stimulated with 100 ng/ml TWEAK. Western blot. Data from 5 independent experiments, expressed as mean ± SEM; *p<0.05 vs control. P values were calculated by Student’s t-test. Results were expressed as percentage change of crotonylated histones over control.
Fig. 3. Crotonate increases histone crotonylation and modifies gene expression in cultured proximal tubular epithelial cells. Cells were stimulated with 0, 50 or 100 mM crotonate for 24h. A) Quantification of histone crotonylation and representative western blot. Mean ± SEM of three independent experiments; *p<0.05 vs 0 mM.
crotonate. P values were calculated by nonparametric Mann-Whitney U-test. B, D) PGC-1α and CCL2 mRNA levels. Data from 8 independent experiments expressed as mean ± SEM; * p<0.05 vs control. P values were calculated by Student’s t-test. C) PGC-1α western blot of whole cell extracts. Data from 4 independent experiments expressed as mean ± SEM. *p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test. E) SIRT3 mRNA levels in tubular cells expose to 50 mM crotonate. Data from 4 independent experiments expressed as mean ± SEM; * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test. F,G) ChIP-seq analysis was performed using a pan anti-Kcr antibody in (F) tubular cells incubated with 100 ng/ml TWEAK for 6h (data of 3 independent experiments) and (G) kidney tissue from mouse AKI (6 animals per group).
**Fig. 4.** Crotonate increases histone crotonylation in mouse kidney. Mice were treated with crotonate at different doses and for different times. Histone crotonylation was measured by western blot. 

**A)** Dose response curve at 48h. Mice received vehicle, 3, 6 or 12 mmol/kg crotonate ip. Mean ± SEM of 4 mice per group. * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test.

**B)** Time-course curve. Mice were euthanized 24, 48 or 72 h following ip injection of 12 mmol/kg crotonate ip or vehicle (control). Mean ± SEM of 4 mice per group. * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test.
Fig. 5. Crotonate increases PGC-1α and SIRT3 mRNA and decreases CCL2 mRNA expression in mouse kidney. Mice were treated with 12 mmol/kg crotonate for different times. A, B) Crotonate increases kidney PGC1α expression at mRNA levels as assessed by qRT-PCR (A) and at protein levels, as assessed by Western blot (B). Mean ± SEM of 4 per group; * p<0.05 vs control. P values were calculated by nonparametric
Mann-Whitney U-test. C) Crotonate decreases whole kidney CCL2 mRNA levels as assessed by qRT-PCR. Mean ± SEM of 4 per group; *p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test. D,E) Crotonate increases whole kidney SIRT3 mRNA levels as assessed by qRT-PCR (D) and SIRT3 protein, as assessed by Western blot (E). Mean ± SEM of 4 per group; * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test.
Fig. 6. Crotonate prevents downregulation of kidney PGC-1α and SIRT3 as well as CCL2 upregulation in experimental AKI. AKI was induced by a folic acid overdose in mice pretreated with or without 12 mmol/kg crotonate. All mice were sacrificed at 72 hours. A) Crotonate prevented the increase in serum BUN and creatinine levels and in Kim-1 mRNA expression observed in AKI. Mean ± SEM of 5 mice per group. *p<0.05
vs vehicle control, # p<0.05 vs vehicle AKI. P values were calculated by nonparametric Mann-Whitney U-test. B-D) Kidney PGC-1α mRNA decrease (B), SIRT3 mRNA decrease (C), and CCL2 mRNA increase (D) were prevented by pretreatment with crotonate. Mean ± SEM of 5 animals per group. *p<0.05 vs vehicle control, # p<0.05 vs vehicle AKI. P values were calculated by nonparametric Mann-Withney U-test. E, F) Kidney PGC-1α (E) and SIRT3 (F) protein levels decreased in AKI and this was prevented by pretreatment with crotonate. Western blot of whole kidney protein. Mean ± SEM of 5 animals per group. *p<0.05 vs vehicle control, # p<0.05 vs vehicle AKI. P values were calculated by nonparametric Mann-Whitney U-test.
Fig. 7. TWEAK downregulates SIRT3 in cultured tubular cells and this is prevented by crotonate. A) SIRT3 mRNA levels in tubular cells incubated with 100 ng/ml TWEAK. Data from 3 independent experiments and expressed as mean ± SEM; * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test.
B) Total protein levels of SIRT3 in MCT cells incubated with 100 ng/ml TWEAK for different periods of time. Mean ± SEM of three independent experiments * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test. C) Kidney SIRT3 mRNA levels in mice treated with TWEAK. Mean ± SEM of 5 animals per group. *p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test. D) Kidney SIRT3 protein levels in mice treated with TWEAK for different times. Mean ± SEM of 5 animals per group. * p<0.05 vs control. P values were calculated by nonparametric Mann-Whitney U-test.

E) SIRT3 mRNA levels were tested in tubular cells incubated with 100 ng/ml TWEAK with or without 50 mM crotonate for 6 hours. Data from 3 independent experiments and expressed as mean ± SEM; * p<0.05 vs vehicle control; #p<0.05 vs vehicle TWEAK. P values were calculated by nonparametric Mann-Whitney U-test. F) Total SIRT3 protein levels in tubular cells incubated with 100 ng/ml TWEAK with or without 50 mM crotonate for 48 hours. Data from 4 independent experiments and expressed as mean ± SEM. * p<0.05 vs vehicle control, # p<0.05 vs vehicle TWEAK. P values were calculated by nonparametric Mann-Whitney U-test.
Fig. 8. Working hypothesis. Histone lysine-crotonylation in kidney injury. A) Kidney cell stressors. Cell stressors, such as TWEAK, decrease PGC-1α and increase CCL2 expression. These changes may contribute to tissue injury. We hypothesize that the decreased PGC-1α expression may contribute to decreased expression of the crotonylase SIRT3 and, this, in turn, limit the decrease in PGC-1α and SIRT3.
expression by promoting histone crotonylation at the PGC-1α and SIRT3 genes, as observed in cultured cells. B) Therapeutic response to crotonate. Crotonate increased overall histone crotonylation and increased the expression of PGC-1α and SIRT3, and decreased CCL2 expression. We hypothesize that these changes may contribute to the observed nephroprotection afforded by crotonate. The increased SIRT3 expression could, in turn, limit histone crotonylation as a negative feed-back mechanism.