Role of p66shc in Renal Toxicity of Oleic Acid

Istvan Arany^a Jeb S. Clark^a Dustin K. Reed^a Luis A. Juncos^{b, c} Mehul Dixit^a

^aDivision of Pediatric Nephrology, Department of Pediatrics, ^bDepartment of Physiology and Biophysics, and

^cDepartment of Medicine, University of Mississippi Medical Center, Jackson, Miss., USA

Key Words

Lipotoxicity · Renal toxicity · p66shc · Reactive oxygen species · Mitochondria · Depolarization · Injury

Abstract

Background/Aims: Adult and childhood obesity is an independent risk factor in development of chronic kidney disease (CKD) and its progression to end-stage kidney disease. Pathologic consequences of obesity include non-esterified fatty acid-induced oxidative stress and consequent injury. Since the serine36-phosphorylated p66shc is a newly recognized mediator of oxidative stress and kidney injury, we studied its role in oleic acid (OA)-induced production of reactive oxygen species (ROS), mitochondrial depolarization and injury in cultured renal proximal tubule cells. **Methods:** Renal proximal tubule cells were used and treated with OA: ROS production, mitochondrial depolarization as well as injury were determined. Transcriptional effects of OA on the p66shc gene were determined in a reporter luciferase assay. The role of p66shc in adverse effects of OA was determined using knockdown, p66shc serine36 phosphorylation and cytochrome c binding-deficient cells. **Results:** We found that OA increased ROS production via the mitochondria – and to a less extent via the NADPH oxidase – resulting in ROS-dependent mitochondrial depolarization and consequent injury. Interestingly, OA also stimulated the promoter of p66shc. Hence, knockdown of p66shc, impairment its Ser36 phosphorylation (mutation of Ser36 residue to alanine) or cytochrome c binding (W134F mutation) significantly attenuated

OA-dependent lipotoxicity. **Conclusion:** These results offer a novel mechanism by which obesity may lead to renal tubular injury and consequently development of CKD. Manipulation of this pathway may offer therapeutic means to ameliorate obesity-dependent renal lipotoxicity.

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Introduction

Prevalence of childhood [1] and adult [2] obesity is a growing health problem: it increases the risk of kidney dysfunction leading to chronic kidney injury (CKD) and ultimately end-stage renal disease [3–6]. Plasma levels of non-esterified fatty acids (NEFA) are chronically elevated in obese individuals [7]: increasing body of evidence demonstrates the role of NEFAs, such as oleic acid (OA) in cellular dysfunction and injury in the kidney [7, 8]. Accumulation of NEFA has a toxic effect on proximal tubular epithelial cells that contributes to pathological changes of the tubulointerstitium [7]. Excess accumulation of lipids causes increased oxidative stress and cell injury via increased reactive oxygen species (ROS) production [9] and consequent mitochondrial dysfunction [10]. Studies also showed that the major source of NEFA-mediated ROS is the mitochondria [9, 11].

p66shc is an enzyme that contributes to generation of ROS in the mitochondria through diverting electrons from cytochrome c [12]. Our previous studies demonstrated that p66shc is an important stimulator of mito-

chondrial ROS production during a variety of cellular stress in renal proximal tubule cells (RPTCs) [13–17].

Accordingly, the aim of this study was to demonstrate whether p66shc is an important regulator of OA-induced ROS release, mitochondrial dysfunction and injury in cultured RPTCs.

Materials and Methods

Cell Lines and Treatment

The immortalized mouse RPTCs (TKPTS, named further as RPTC) were generous gift from Dr. Bello-Reuss [18]. The p66shc knockdown version of TKPTS cells was created [16] and maintained as described elsewhere [16]. Cells were treated with OA (Sigma-Aldrich, St. Louis, Mo., USA) dissolved in ethanol as indicated.

Transfection of Mutant p66shc Plasmids

Semiconfluent RPTCs were transfected with one of the following p66shc plasmids: serine36 phosphorylation mutant (S36A), phosphomimetic mutant (S36D) [15, 16] or cytochrome *c* binding-deficient (W134F) mutant [15]. For transfection, Lipofectamine 2000 (Life Technologies, Grand Island, N.Y., USA) was used as recommended by the manufacturer.

Determination of Intracellular ROS Production

Confluent RPTCs grown in 96-well plates loaded with 100 μM of the oxidant-sensitive 2',7'-dichlorofluorescein-diacetate (Life Technologies): ROS production was calculated as changes in fluorescence/30 min/ 0.2×10^6 cells and expressed as percentage of the corresponding untreated values as described elsewhere [15].

Mitochondrial Depolarization

Mitochondrial depolarization was determined by the polarity sensor fluorescent dye JC-1 (Life Technologies) as described elsewhere [15]. Changes in JC-1 fluorescence was calculated as decrease of red fluorescence/ 0.5×10^6 cells between 10 and 20 min after adding OA and expressed as percentage of the corresponding untreated values.

Determination of Cell Injury

The fluorescent CytoTox-One Homogenous Membrane Integrity assay kit (Promega, Madison, Wisc., USA) was used. LDH release was calculated as the percentage of LDH in the medium compared to the total LDH content (medium + lysate).

Measurement of the p66shc Promoter Reporter Activity

The p66shc-Luc plasmid containing the –1096 to +44 basepair region (relative to the ATG codon) of the human p66shc gene promoter was a gift from Dr. Irani (Cardiovascular Institute, University of Pittsburgh, Pittsburgh, Pa., USA) [19]. The renilla luciferase plasmid was purchased from Promega. Transient plasmid transfection was carried out by using the Lipofectamine 2000 reagent (Invitrogen, Grand Island, N.Y., USA) as recommended by the manufacturer. Firefly and renilla luciferase activities were determined 24 h after treatment with 100 μM OA by the Dual Luciferase Assay System (Promega) in a Modulus luminometer (Turner Bio-Systems, Sunnyvale, Calif., USA). p66shc-Luc activity was normalized to the internal renilla-Luc activity.

Western Blotting

RPTCs were lysed in a RIPA buffer and subjected to SDS/PAGE and Western blotting as described elsewhere [16].

Statistical Analysis

Continuous variables are expressed as mean and SD. Statistical differences between the treated and control groups were determined by Student's *t* test. Differences between means were considered significant if $p < 0.05$. All analyses were performed using the SigmaStat 3.5 (Systat, San Jose, Calif., USA) software package.

Results

OA Dose-Dependently Increases Injury and ROS Production through the Mitochondria and NADPH Oxidase in RPTCs

RPTCs were treated with increasing amounts of OA: cell injury (LDH release) and ROS production was determined. We found that ROS production and LDH release are OA concentration-dependent (fig. 1a, b). For further studies we selected 100 μM OA, which represents physiological level [20]. To determine the source of intracellular ROS, TKPTS cells were pretreated with inhibitors of the xanthine oxidase (allopurinol, Allo, 100 μM), the NADPH oxidase (diphenylpicrylhydrazolium, DPI, 5 μM) or the mitochondrial electron transport chain (antimycin, AntA, 10 μM , and rotenone, Rot, 10 μM) prior to treatment with 100 μM OA. Figure 1c shows that not allopurinol but DPI and to a greater extent AntA/Rot inhibited OA-dependent production of ROS.

These data suggest that the majority of OA-induced ROS originates from the mitochondria and to a lesser extent from the NADPH oxidase system.

OA-Dependent Cell Injury Is ROS- and Mitochondrial Depolarization-Dependent

RPTCs were treated with 100 μM OA and mitochondrial depolarization was determined as described in Materials and Methods. Figure 2a shows that OA significantly increased depolarization of the mitochondria, which was abrogated by pretreatment with the ROS scavenger N-acetylcysteine (NAC-100 μM). Importantly, pretreatment with both NAC and the mitochondrial depolarization inhibitor cyclosporin A (CsA: 5 μM) attenuated OA-dependent LDH release (fig. 2b).

These data imply that OA-mediated mitochondrial depolarization and consequent injury is ROS-dependent and the injury is the consequence of mitochondrial depolarization.

Fig. 1. OA dose-dependently increases cell injury and the intracellular ROS production source of which is the NADPH oxidase/mitochondria in RPTCs. RPTCs were treated with varying concentrations of OA as indicated. LDH release (**a**) and ROS production (**b**) were determined as described in Materials and Methods. $n = 3$, * $p < 0.05$ compared to untreated cells; (**c**) RPTCs were pretreated with 100 μM Allo, 5 μM DPI, 10 μM AntA or 10 μM Rot 1 h prior to treatment with 100 μM OA. ROS production was determined as described in Materials and Methods. $n = 3$, * $p < 0.05$ compared to OA treatment.

Fig. 2. OA-induced ROS depolarizes the mitochondria, which is responsible for cell injury. **a** RPTCs were treated with 100 μM OA in the presence or absence of 100 μM NAC and mitochondrial depolarization was determined as described in Materials and Methods. $n = 3$, * $p < 0.05$ compared to untreated cells. **b** RPTCs were pretreated with 100 μM NAC or 5 μM CsA 1 h prior to treatment with 100 μM OA and LDH release was determined 24 h later. $n = 3$, * $p < 0.05$ compared to untreated cells, ** $p < 0.05$ compared to OA-treated cells.

OA Increases Expression of p66shc through Activation of Its Promoter

Next, RPTCs were treated with 100 μM OA for 24 h and expression of p66shc was determined by Western blotting. Figure 3a and b shows that OA treatment significantly increased expression of p66shc. To demonstrate whether this effect is transcriptional, RPTCs were transfected with a p66shc promoter-luciferase plasmid [19] together with a renilla plasmid followed by treatment with 100 μM OA for 24 h. Figure 3c demonstrates that OA significantly stimulated the promoter of p66shc. These results imply that p66shc is pivotal in lipotoxicity of OA.

Fig. 3. OA increases expression of p66shc via stimulation of its promoter. **a** RPTCs were treated with 100 μ M OA for 24 h and expression of p66shc was determined by Western blotting. As internal control, blots were rehybridized with an anti-actin antibody. Image shown is representative of three independent experiments. **b** Densitometry of results shown in figure 3a. $n = 3$, * $p < 0.05$ compared to untreated. **c** RPTCs were transfected with a p66shc promoter-luciferase plasmid together with a renilla plasmid as described in Materials and Methods. After 24 h with treatment with 100 μ M OA, luciferase activities were determined. Values are calculated as p66shc-Luc/renilla ratios and expressed as % of the untreated value. $n = 3$, * $p < 0.05$ compared to untreated.

Adverse Effects of OA Depend on Ser36

Phosphorylation and Cytochrome *c* Binding of p66shc

First, we employed p66shc wild-type (w.t.) and p66shc knockdown (p66shc k.d.) RPTCs [16] and treated with 100 μ M OA. Our data show that p66shc k.d. attenuated OA-mediated ROS production, mitochondrial depolarization and LDH release (fig. 4a–c). Similarly, preventing

of Ser36 phosphorylation of p66shc (utilizing the S36A mutant) or mitochondrial cytochrome *c* binding (W134F mutant) also attenuated adverse effects of OA (fig. 4a–c). Importantly, the phosphomimetic S36D mutant does not influence adverse effects of OA, further suggesting a role for serine36 phosphorylation in this process. Additional experiments confirmed that overexpression of the S36A – but not the phosphomimetic S36D mutant – indeed attenuated OA-mediated Serine36 phosphorylation of p66shc (online suppl. fig. 1a, b; for all online suppl. material, see www.karger.com/doi/10.1159/000354357). Similarly, impairment in OA-mediated binding of p66shc to cytochrome *c* through overexpressing the W134F mutant was confirmed (online suppl. fig. 1c, d).

These results demonstrate that Ser36 phosphorylation of p66shc and its binding to mitochondrial cytochrome *c* is essential in renal toxicity of OA.

Discussion

Although fatty acids are an important source of energy in the kidney cortex [21, 22], their excess accumulation leads to organ injury termed lipotoxicity [7, 8]. It is widely accepted that lipotoxicity is – at least partly – due to NEFA-mediated increase in intracellular ROS production [7, 8, 22]. RPTCs are highly sensitive to oxidative stress-induced damage [23–27], thus they are also targets of lipotoxicity [7, 8]. The sites of production of intracellular ROS include NADPH oxidases, xanthine oxidase, P450 mono-oxygenases, cyclo-oxygenases and lipoxygenases, but most importantly, the mitochondria [28–30]. In the mitochondria, complex I and III of the electron transport chain are the major sites for ROS generation [31–33], but lately the redox enzyme p66shc is also recognized as a mediator of non-physiological ROS production [34]. It is that the oxidative stress-activated (Ser36-phosphorylated) p66shc is translocated into the mitochondrial intermembrane space where – after dephosphorylation – it binds mitochondrial cytochrome *c* [35]. This binding diverts electrons from cytochrome *c* and increases production of H_2O_2 within the mitochondria [12]. Our previous studies established a role for p66shc in oxidant-induced mitochondrial ROS production in cultured RPTCs [15].

In our experiments we used free instead of albumin-bound OA, even though fatty acids were present in albumin-bound complexes in vivo [36]. However, dissociation from albumin precedes fatty acid uptake [36], thus the intracellular effects of fatty acids are attributed to their free form. Also, this way we were able to exclude the un-

Fig. 4. Adverse effects of OA require Ser36 phosphorylation and cytochrome *c* binding of p66shc. **a** w.t. or p66shc k.d. RPTCs or cells that were transfected with a phosphorylation-deficient (S36A), a corresponding phosphomimetic mutant (S36D) or a cytochrome *c* binding-deficient (W134F) p66shc plasmid were treated with 100 μ M OA and ROS production was determined. Values are expressed as % of control (untreated) cells. $n = 3$, * $p < 0.05$ compared to treated w.t. values. **b** Mitochondrial depolarization was determined in cells treated similar to figure 4a. Values are expressed as % of control (untreated) cells. $n = 3$, * $p < 0.05$ compared to treated w.t. values. **c** LDH release was determined in cells treated similar to figure 4a. Values are expressed as % of total LDH content. $n = 3$, * $p < 0.05$ compared to w.t. values.

Fig. 5. Proposed role of p66shc in OA-dependent renal toxicity. OA stimulates transcription of p66shc leading to increased expression of the p66shc protein, which in turn is Ser36-phosphorylated by ROS generated through NADPH oxidase- or other non-p66shc-related pathways. The Ser36-phosphorylated p66shc is translocated into the mitochondria, where – after dephosphorylation – it binds cytochrome *c*. The result is increased mitochondrial ROS production and consequently mitochondrial depolarization as well as injury.

wanted effects of albumin toxicity [9]. Anyway, our pilot studies also showed that free OA acts similar to OA-BSA (data not shown).

Our present studies established that (a) OA dose-dependently increases intracellular production of ROS and injury in RPTCs (fig. 1a, b) and (b) the major source of this ROS is the mitochondria (fig. 1c). Since the mitochondria is the major source of NEFA-induced ROS [9, 37] (fig. 1c), p66shc may play a pivotal role in mitochondrial ROS release and in renal lipotoxicity. Indeed, knock-down of p66shc, mutation of its Ser36 residue (S36A) or its cytochrome *c*-binding site (W134F) significantly attenuated OA-dependent ROS production, mitochondrial depolarization and injury (fig. 4). In contrast, phosphomimetic mutation of Ser36 (S36D) did not affect ROS production.

Excess generation of ROS could increase mitochondrial membrane permeability by a process called *mitochondrial permeability transition* or MPT [38, 39]. Development of MPT depolarizes the mitochondria leading to cell death [39]. Our studies confirmed this phenomenon in cultured RPTCs under oxidative stress [15]. The present study confirms that (a) OA – through ROS – increases mitochondrial depolarization (fig. 2a) and (b) OA-mediated cell injury is ROS/mitochondrial depolarization-dependent (fig. 2b).

However, the impact of NEFA on p66shc is virtually unknown. Some studies suggested that deletion of the p66shc gene reduces systemic oxidative stress in mice that were fed with high-fat diet [40] and p66shc-induced ROS promotes fat accumulation [41]. We found that OA increases expression of p66shc through stimulating its promoter (fig. 3a–c). The nature of this stimulus is to be determined. Others and us showed that the p66shc promoter is inducible through p53 [42, 43] or epigenetic modification [13, 44]. Considering that obesity/fatty acids increase levels of p53 in various cell types [45, 46], p53 may be a good candidate for OA-dependent stimulation of the p66shc promoter.

Based on our results, we hypothesize the following (fig. 5): OA stimulates transcription of p66shc leading to

increased expression of the p66shc protein, which in turn, is Ser36-phosphorylated by ROS generated through NADPH oxidase- or other non-p66shc-related pathways. The Ser36-phosphorylated p66shc is translocated into the mitochondria, where – after dephosphorylation – it binds cytochrome *c*. The result is increased mitochondrial ROS production and consequently mitochondrial depolarization and associated cell injury. In the long term this injury could lead to development of CKD and ultimately end-stage kidney disease in obese individuals.

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Disclosure Statement

The author have no conflicts of interest to disclose.

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