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## LABORATORY STUDY

# A pan caspase inhibitor decreases caspase-1, IL-1 $\alpha$ and IL-1 $\beta$ , and protects against necrosis of cisplatin-treated freshly isolated proximal tubules

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Caspase-1, IL-1 $\alpha$ , and IL-1 $\beta$  are known to be activated in the NLRP3 inflammasome. The inflammasome is activated mostly in inflammatory cells. The presence of inflammasome proteins in proximal tubules (PTs) and the effect of cisplatin-treatment or caspase inhibition on inflammasome proteins in PTs are not known. The aim of this study was to investigate the effect of cisplatin on inflammasome proteins in freshly isolated PTs and also to determine the effect of caspase inhibition on inflammasome proteins and PT injury. PTs were isolated using collagenase digestion and Percoll centrifugation. After recovery period, freshly isolated PTs were incubated with vehicle, 50  $\mu$ M cisplatin or 50  $\mu$ M cisplatin plus 50  $\mu$ M pan caspase inhibitor, QVD-OPH. PTs treated with 50  $\mu$ M cisplatin showed Propidium Iodide staining indicative of necrosis. Necrotic cells (%) were 2.2 in Vehicle-treated, 37.7 in Cisplatin-treated ( $p < 0.05$  vs. Vehicle), and 3.3 in QVD-treated ( $p < 0.05$  vs. Cisplatin). LDH release (%), a marker of cell membrane damage seen in necrosis was 7.1 in Vehicle-treated, 39.7 in Cisplatin-treated ( $p < 0.05$  vs. Vehicle), and 13.5 in QVD-treated ( $p < 0.05$  vs. Cisplatin). Caspase-1 activity and active caspase-1 protein (10 kDa) were significantly increased in Cisplatin-treated PTs. NLRP3 was strongly expressed in PTs, but there were no significant changes between groups. Pro-apoptotic BID (22 kDa) was unchanged between groups. IL-1 $\alpha$  and IL-1 $\beta$  activity was increased in Cisplatin-treated PTs. QVD-OPH co-treatment decreased caspase-1, IL-1 $\alpha$ , and IL-1 $\beta$ . In summary, caspase inhibition decreases caspase-1, IL-1 $\alpha$ , and IL-1 $\beta$  but not NLRP3 or BID protein and protects against necrosis in cisplatin-treated freshly isolated PTs.

**Keywords**

Acute kidney injury, caspase-1, cisplatin, inflammasomes, kidney tubules proximal

**History**

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**Introduction**

Mammalian cell death is usually classified into two types: apoptosis and necrosis. Apoptosis is a fundamental type of programmed cell death that can occur by genetically controlled processes.<sup>1,2</sup> There is now evidence that necrosis, traditionally considered an accidental form of cell death, can in certain instances be initiated or modulated by programmed control mechanisms.<sup>3–6</sup> The effect of caspase inhibition on apoptosis in proximal tubules (PTs) is well known.<sup>7,8</sup> However, the effect of caspase inhibition on necrosis in PTs is less well known. Within the cytoplasm, NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing protein 3) forms a complex,

NLRP3 inflammasome with ASC (apoptosis-associated speck-like protein containing caspase recruitment domain) and inactive caspase-1 (procaspase-1). Pro-inflammatory caspase-1, activated by inflammasome complexes in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), converts the cytokines interleukin (IL)-1 $\beta$  and IL-18 to their active forms and also participates in apoptosis through the activation of caspase-3.<sup>9–11</sup> The NLRP3 inflammasome is present mainly in inflammatory cells. The presence of inflammasome proteins in PTs and the effect of caspase inhibition on inflammasome proteins in PTs are not known.

It is well appreciated that cells in culture undergo a variety of phenotypic changes to survive *in vitro*. These changes are particularly problematic for studies of the pathogenesis of injury to the PT. PTs change from their normal dependence on oxidative, mitochondrial metabolism to glycolysis under culture conditions.<sup>12</sup> There are some studies on the effect of caspase inhibition in cultured renal PTs treated with cisplatin *in vitro*.<sup>13–17</sup> However, the effects of caspase inhibition in freshly isolated PTs, a model that more closely

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resembles the *in vivo* situation, are less well known. Thus the effect of caspase inhibition was investigated in freshly isolated PTs.

## Materials and methods

### Animals and reagents

For all the mouse studies, 8 to 10-week-old C57BL/6 mice (Harlan, Indianapolis, IN and Jackson Labs, Bar Harbor, ME) weighing 20 to 25 g were used. Mice were maintained on a standard diet and water was freely available. Mice were housed five per cage under a 12-hour light and dark schedule for at least 1 week prior to experiment.

Cisplatin was purchased from Sigma-Aldrich (Milwaukee, WI). Pan Caspase inhibitor, Q-Val-Asp-Oph (QVD-OPH) was purchased from MP Biochemicals (Aurora, OH). DMSO was obtained from Aldrich. The vital nuclear dye bisbenzamide (Hoechst 33342) and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR).

### Preparation of renal PTs

PTs were isolated from kidney cortex of male C57BL/6 mice using collagenase digestion and Percoll centrifugation as we have previously described in detail.<sup>18,19</sup> Six-milliliter aliquots of tubule suspension (approximately 1 to 2 mg/mL) were placed in siliconized 25 mL Erlenmeyer flasks for a “recovery” period, which included gassing with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 5 min on ice. Flasks were then capped with rubber stoppers and kept at room temperature for 5 min, then placed in a shaking water bath at 37 °C for 10 min. After adding the reagents, the flasks were regassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 5 min and then closed and kept in the shaking water bath for 25 min. At the end of the “pre-incubation and experiment” period, 1 mL of tubule suspension was sampled for measurement of lactate dehydrogenase (LDH), 5 mL for caspase activity, ELISA, or immunoblots.

### Cisplatin treatment

After “recovery” period, PTs were incubated with cisplatin (final concentration 50 µM). Cisplatin was freshly prepared and dissolved in DMSO (final concentration 0.1%) at the time of the experiment. Control PTs were treated with the vehicle (0.1% DMSO).

### Caspase inhibition

To determine the role of inflammasome and caspase-1 in cisplatin-treated PTs, PTs were incubated with 50 µM pan caspase inhibitor, QVD-OPH (catalogue number OPH109, MP Biochemicals, Aurora, OH).

### Measurement of LDH release and protein

Leakage of lactate dehydrogenase (LDH) was measured as an index of lethal membrane injury.<sup>19</sup> The incubation medium was collected and the remaining pellet was dissolved in 1.5% Triton X-100 to the original volume. The percentage of LDH released from tubules was calculated by determining the ratio of LDH in the supernatant compared to that in the lysed tubule pellet plus the supernatant. Tubule protein was

measured by the Lowry method, using bovine serum albumin as standard.

### Immunofluorescence study

Nuclear morphology of the PTs was examined to identify the mechanism of cell death. The cells were stained with the DNA-specific dyes Hoechst 33342 and Propidium Iodide (PI). Fluorescent microscopy was used to determine necrosis. Cells were washed in PBS and then stained with Hoechst 33342 (final concentration 5 µM) and PI (final concentration 0.5 µM) for 10 min at 37 °C. Cells were washed again in PBS and then fixed with 3% paraformaldehyde. Fields of cells were photographed (magnification ×400), by using appropriate filters to examine Hoechst 33342 and PI fluorescence staining. Nuclear morphology was assessed with the cell membrane-permeant supravital DNA dye Hoechst 33342 (excitation wavelength 348 nm; emission 479 nm). Hoechst 33342, unlike PI, enters and stains the nucleus of both viable cells and cells that have died by apoptosis or necrosis. Plasma membrane integrity was assessed using the cell membrane-impermeant DNA dye PI (excitation wavelength 535 nm; emission 617 nm). Necrosis was determined on the basis of positive PI staining in red color, indicative of loss of membrane integrity.

### Caspase-1 activity

The caspase assay in PTs was measured as we have previously described in detail.<sup>7</sup> Proximal tubule extracts for the caspase assay were prepared as follows. After exposure to normoxia or to hypoxia, the tubule pellet was separated from 2 mL of tubule suspension by centrifugation. The tubule pellet was mixed with 1 mL of lysis buffer on ice for 10 min. The lysis buffer used contained 20 mM Hepes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 1% Triton X-100, pH 7.2. The lysate was then centrifuged at 5000 g for 10 min. The supernatant was mixed with an equal quantity of 50% glycerol. The caspase assay was then performed on this supernatant as follows: 200 µL of supernatant (200–400 µg protein) and 10 µL of the substrate (final concentration, 50 µM) were added to the caspase assay buffer. The total volume of the assay solution was adjusted to 2 mL using the assay buffer. The assay buffer contained 100 mM Hepes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, and 10% glycerol, pH 7.4. Ac-YVAD-AMC was used as a susceptible substrate for caspase-1. Fluorescence was measured at 380 nm excitation and 460 nm emission was determined using a Hitachi F2000 spectrophotometer. Caspase activity was expressed in pmol AMC released per minute of incubation time per mg of lysate protein. Lysate protein was measured by the Bradford method as described in the Bio-Rad protein assay kit with bovine serum albumin as standard.

### Statistical analysis

Experiments were performed in triplicate and repeated at least three times. All values are expressed as mean ± standard error (SE). Data were analyzed for significance using unpaired, two-tailed Student's *t* tests. A *p* value of <0.05 was considered statistically significant.

## Results

### Nuclear morphology of the PTs after cisplatin treatment

With the color combination, vehicle-treated PTs excluded PI staining and stained mostly with Hoechst 33342 in blue color (Figure 1A). In contrast, 50  $\mu$ M cisplatin-treated PTs showed extensive PI staining in red color, indicative of loss of membrane integrity and necrosis (Figure 1B). Those nuclei were smaller in size compared with controls which is indicative of homogenous masses of chromatin condensation in globular or crescent-shaped figures. Necrotic cells were quantitated in at least 200 randomly selected cells stained with Hoechst 33342 by fluorescent microscopy. Percentage of necrotic cells was highest in 50  $\mu$ M cisplatin-treated group ( $p < 0.05$ ,  $37.7 \pm 3.5$  vs.  $2.2 \pm 0.4\%$  in vehicle-treated group) (Figure 1D). PTs co-treated with 50  $\mu$ M cisplatin plus pan caspase inhibitor, QVD-OPH demonstrated significant reduction of necrotic cells to the level of vehicle-treated group ( $p < 0.05$ ,  $3.3 \pm 0.6$  vs.  $37.7 \pm 3.5\%$  in 50  $\mu$ M cisplatin group) (Figure 1C and D).

### LDH release from the PTs after cisplatin treatment

LDH release is a marker of cell membrane damage that occurs in necrosis. LDH release was increased by over 20% in 50  $\mu$ M

cisplatin-treated PTs ( $p < 0.05$ ,  $39.7 \pm 3.5$  vs.  $7.1 \pm 0.9$  in vehicle-treated group). Addition of QVD-OPH decreased LDH release almost to the levels of controls ( $p < 0.05$ ,  $13.5 \pm 1.8$  vs.  $39.7 \pm 3.5\%$  in 50  $\mu$ M cisplatin-treated group; Figure 2).

### Activation of caspase-1 *in vitro* in PTs after cisplatin treatment

Caspase-1 activity was increased significantly in 50  $\mu$ M cisplatin-treated PTs compared with vehicle-treated group ( $p < 0.05$ ,  $3.2 \pm 0.5$  vs.  $0.9 \pm 0.2$  nmol/mg/min in vehicle-treated group). There was a significant reduction in caspase-1 activity of cisplatin-treated cells in combination with QVD-OPH ( $p < 0.05$ ,  $1.1 \pm 0.2$  vs.  $3.2 \pm 0.5$  nmol/mg/min in 50  $\mu$ M cisplatin-treated group) (Figure 3A). The active form of caspase-1 (10 kDa) was increased significantly in 50  $\mu$ M cisplatin-treated groups ( $p < 0.05$  vs. vehicle-treated group), and decreased significantly in combination with QVD-OPH ( $p < 0.05$  vs. 50  $\mu$ M cisplatin-treated group; Figure 3B).

### The NLRP3 inflammasome and caspase-1 substrates (IL-1 $\beta$ , IL-1 $\alpha$ , BID) *in vitro* in PTs after cisplatin treatment

NLRP3 was strongly expressed in PTs, however there were no significant changes between groups (Figure 4A).

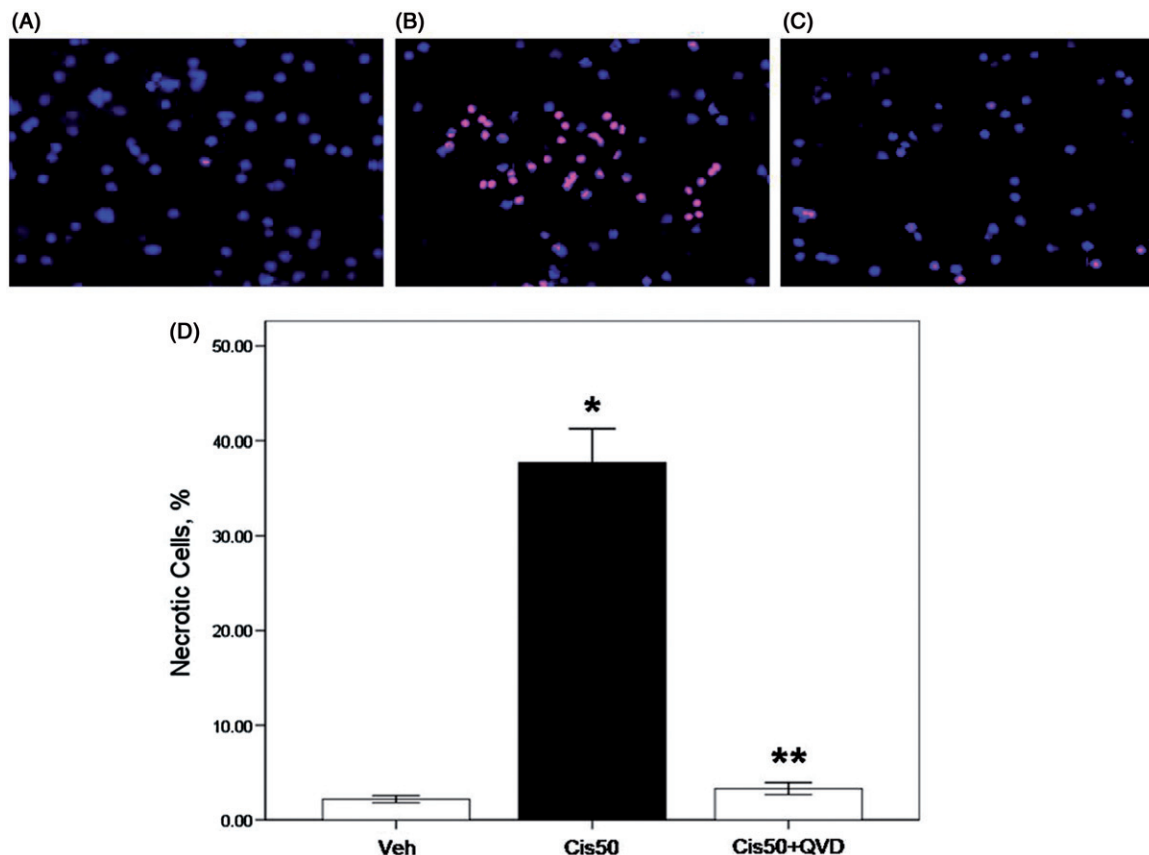


Figure 1. Propidium iodide staining of nuclei in freshly isolated proximal tubule cells is increased by cisplatin and decreased by the caspase inhibitor. The cells were stained with color combination of DNA-specific dyes Hoechst 33342 and propidium iodide (PI). Vehicle-treated proximal tubule cells (Veh) demonstrated an intact structure; they excluded PI and stained mostly with Hoechst in blue color (A). Cells treated with 50  $\mu$ M cisplatin (Cis50) showed extensive PI staining in red color, indicative of loss of membrane integrity and necrosis. Nuclear morphology was disrupted and those cells were more brightly stained indicative of chromatin condensation in globular or crescent-shaped figures (B). Cells treated with 50  $\mu$ M cisplatin in combination with pan caspase inhibitor, 50  $\mu$ M QVD-OPH (Cis50+QVD) demonstrated a normal morphology compared with the cells treated with 50  $\mu$ M cisplatin only (C,D). (\* $p < 0.05$  vs. Veh, \*\* $p < 0.05$  vs. Cis50).

Immunoblotting of BID (22 kDa) showed no difference between the groups (Figure 4B). IL-1 $\alpha$  activity was significantly increased in 50  $\mu$ M cisplatin-treated groups ( $p < 0.05$ ,  $3.1 \pm 0.7$  vs.  $0.1 \pm 0.0$  nmol/mg/min in vehicle-treated group), and was decreased significantly in combination with QVD-OPH ( $p < 0.05$ ,  $0.8 \pm 0.2$  vs.  $3.1 \pm 0.7$  nmol/mg/min in 50  $\mu$ M cisplatin-treated group; Figure 4C).

IL-1 $\beta$  activity was significantly increased in 50  $\mu$ M cisplatin-treated groups ( $p < 0.05$ ,  $13.2 \pm 0.4$  vs.  $7.6 \pm 0.9$  nmol/mg/min in vehicle-treated group). Co-treatment

of QVD-OPH reduced IL-1 $\beta$  activity significantly ( $p < 0.05$ ,  $8.8 \pm 0.5$  vs.  $13.2 \pm 0.4$  nmol/mg/min in 50  $\mu$ M cisplatin-treated group; Figure 4D).

## Discussion

Cells in culture undergo a variety of phenotypic changes driven in large part by the adaptations necessary to survive *in vitro* as well as by a shift to a more proliferative mode that characterizes that setting. Cultured tubular cells also undergo considerable structural changes that include simplification of both their apical and basolateral membrane compartments<sup>20</sup> that affect cellular characteristics prominently involved in the behavior of the tubules during AKI *in vivo*. For these reasons, freshly isolated PTs have been favored by a number of investigators for studies of the cellular biology of PT injury.<sup>21–27</sup> *In vitro* preparations of freshly isolated PTs have been valuable for both metabolic and structural investigations, because the PTs retain the biochemical properties of the *in vivo* state as well as a high degree of structural integrity – highly polarized and fully differentiated features – of the normal proximal tubular epithelium. Thus we chose freshly isolated PTs rather than cultured cells for the present study.

Previous studies in cultured PTs<sup>7,8</sup> and the present study in freshly isolated PTs demonstrate that caspase inhibitors prevent cisplatin-induced PT injury. Our published data demonstrate that caspase-1  $-/-$  mice are protected against cisplatin-induced AKI<sup>28</sup> and ischemic AKI.<sup>29</sup> The caspase inhibitor, OPH 001, protects against ischemic AKI in mice.<sup>30</sup> However, there are no *in vivo* studies that demonstrate that caspase inhibitors protect against cisplatin-induced AKI. In view of the dramatic protection against cisplatin-induced PT injury by caspase inhibitors *in vitro*, it would be

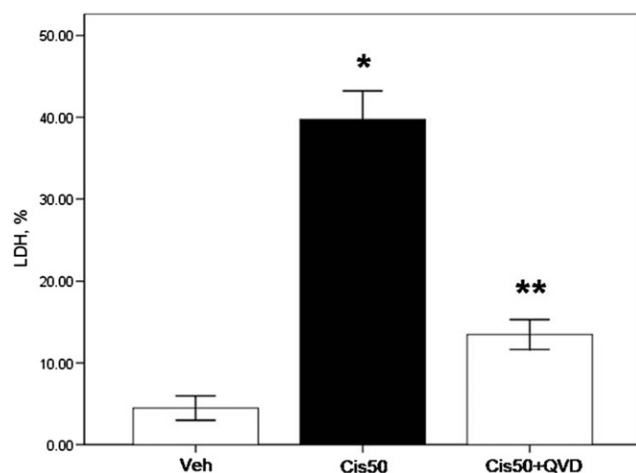


Figure 2. LDH release (%) from freshly isolated proximal tubule cells is increased by cisplatin and decreased by the caspase inhibitor. LDH release was under 10% in vehicle-treated proximal tubule cells (Veh). Cells treated with 50  $\mu$ M cisplatin (Cis50) showed increased LDH release. Co-treatment of QVD-OPH (Cis50 + QVD) decreased LDH release significantly. (\* $p < 0.05$  vs. Veh, \*\* $p < 0.05$  vs. Cis50).

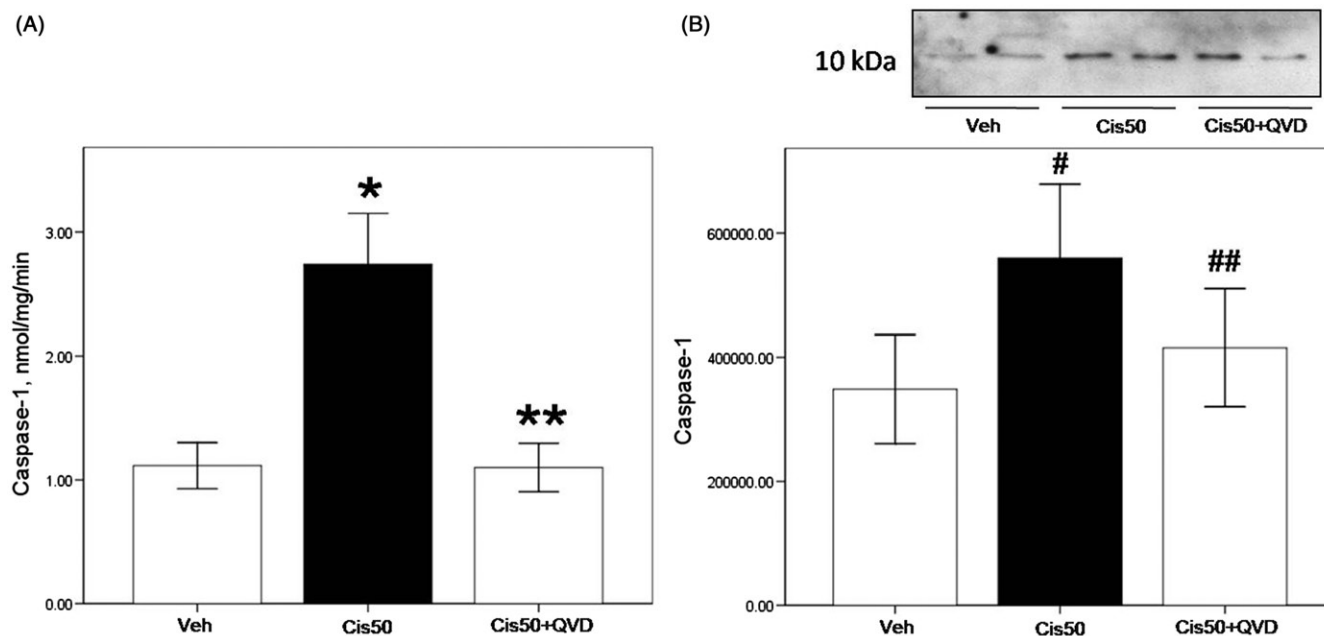


Figure 3. Caspase-1 activity and protein of freshly isolated proximal tubule cells treated with cisplatin is reduced by the caspase inhibitor. (A) Caspase-1 activity was increased significantly in 50  $\mu$ M cisplatin-treated proximal tubule cells (Cis50) compared with vehicle-treated group (Veh) (\* $p < 0.05$  vs. Veh). Co-treatment of QVD-OPH (Cis50 + QVD) decreased caspase-1 activity significantly (\*\* $p < 0.05$  vs. Cis50). (B) Representative immunoblot of caspase-1 showed active caspase-1 (10 kDa) was increased significantly in Cis50 compared with Veh (# $p < 0.05$  vs. Veh). Cis50 + QVD decreased the expression of active caspase-1 (## $p < 0.05$  vs. Cis50 + Veh). Densitometry of caspase-1 immunoblots is shown.

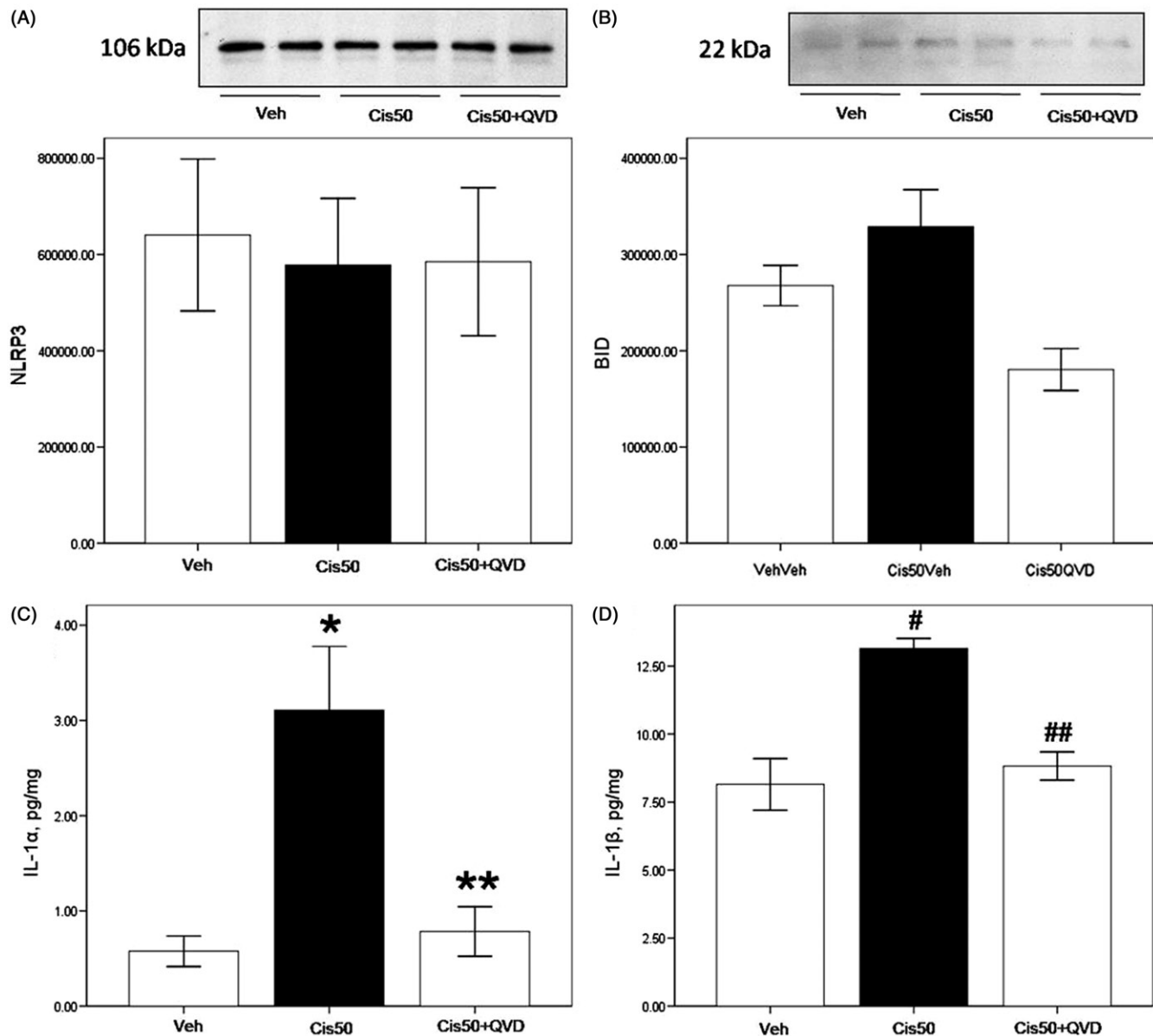


Figure 4. Activation of caspase-1 substrates IL-1 $\alpha$  and IL-1 $\beta$  in cisplatin-treated freshly isolated proximal tubule cells. (A) On immunoblot, NLRP3 was strongly expressed in PTs with no significant changes between groups on densitometry. (B) On immunoblot, BID (22 kDa) was unchanged between the groups. (C) IL-1 $\alpha$  activity was significantly increased in Cis50 (\* $p < 0.05$  vs. Veh), and was decreased significantly in combination with QVD-OPH (\*\* $p < 0.05$  vs. Cis50). (D) IL-1 $\beta$  activity showed increases in Cis50 (# $p < 0.05$  vs. Veh), and was decreased significantly in combination with QVD-OPH (## $p < 0.05$  vs. Cis50).

expected that caspase inhibitors would protect against cisplatin-induced AKI *in vivo*. However, a recent study demonstrated that the caspase inhibitor, zVAD-fmk, worsened renal function in cisplatin-induced AKI *in vivo*.<sup>31</sup> The caspase inhibitor prevented cisplatin-induced cleavage of autophagy proteins, but impaired autophagic flux and worsened renal function. Autophagy has been shown to be protective in ischemic AKI.<sup>32</sup> Thus differences in autophagy induced by cisplatin treatment *in vivo* versus *in vitro* may explain the protection against cisplatin-induced AKI *in vitro* but not *in vivo*.

The inflammasome is a molecular complex which contains NLRP (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing) proteins and an adaptor protein called ASC (Apoptosis-associated Speck-like protein containing a Caspase recruitment domain).<sup>33,34</sup>

The most fully characterized inflammasome is the NLRP3 inflammasome which contains the NLRP3 protein.<sup>35</sup>

Caspase-1 is activated in the inflammasome. The inflammasome is activated mostly in inflammatory cells rather than epithelial cells and plays an important part in the innate immune response.<sup>34,35</sup> The inflammasome plays an important role in causing tissue inflammation. In the present study, NLRP3 and inflammasome proteins were determined in isolated PTs, a system devoid of inflammatory cells.

There have been few studies of the inflammasome in PT epithelial cells. In human and mouse primary renal tubular epithelial cells, NLRP3 expression was increased in response to TGF- $\beta$ 1 stimulation. TGF- $\beta$ 1-induced effects were significantly decreased in mouse NLRP3  $-/-$  renal tubular epithelial cells, suggesting a role for NLRP3 in TGF- $\beta$ -dependent signaling. In response to TGF- $\beta$ 1, the effect

of NLRP3 was inflammasome independent, as IL-1 $\beta$ , IL-18, and caspase-1 were not involved.<sup>36</sup> In the present study, in cisplatin-treated PTs, there was activation of caspase-1, IL-1 $\beta$  and IL-1 $\alpha$  suggesting activation of the inflammasome. However, while NLRP3 protein was abundantly present in PTs, there was no increase in NLRP3 arguing against activation of the NLRP3 inflammasome. In addition to the NLRP3 inflammasome, caspase-1 is also activated in the NLRP1, NLRC4 or AIM2 inflammasomes. Our previous study showed NLRP1 protein was increased in cisplatin-induced AKI suggesting a role of NLRP1 inflammasome in cisplatin-induced AKI.<sup>37</sup> In the present study, it is possible that caspase-1 is activated in the NLRP1, NLRC4 or AIM2 inflammasome. In support of the lack of a role of the NLRP3 inflammasome in cisplatin-induced PT injury is our recent study that despite activation of caspase-1 in cisplatin-induced AKI, NLRP3  $-/-$  mice are not protected against cisplatin-induced AKI *in vivo*.<sup>37</sup>

Activation of caspase-1 in the inflammasome results in secretion of “leaderless” proteins via direct or indirect physical interaction.<sup>38</sup> Active caspase-1 in the inflammasome activates IL-1 $\beta$ , IL-18 and IL-1 $\alpha$ . In the present study, there was an increase in caspase-1 protein and activity in cisplatin-treated PTs. IL-1 $\beta$  and IL-1 $\alpha$  that are activated by caspase-1 in the inflammasome were also increased in cisplatin-treated PTs. BID, that was originally thought to be activated by caspase-1 but is now thought to be activated by caspase-8,<sup>39</sup> was not increased by cisplatin in PTs. NLRP3, the major component of the NLRP3 inflammasome was present in the isolated PTs but unchanged between the groups. The present results demonstrate the novel finding of activation of caspase-1-dependent proteins, IL-1 $\beta$  and IL-1 $\alpha$  in isolated PTs. The activation of caspase-1, IL-1 $\beta$  and IL-1 $\alpha$  was independent of an increase in NLRP3.

Caspase-1 is a mediator of hypoxic PT injury *in vitro* and ischemic AKI *in vivo*. PTs isolated from caspase-1  $-/-$  mice are protected against hypoxic injury.<sup>40</sup> Caspase-1  $-/-$  mice are protected against ischemic AKI *in vivo*.<sup>29</sup> Based on these studies, the question arose as to whether caspase-1 activated in the inflammasome was the mediator of the hypoxic and ischemic PT injury. Our recent study demonstrating that NLRP3  $-/-$  mice are protected against ischemic AKI suggests a role for caspase-1 activation in the NLRP3 inflammasome as a mediator of ischemic AKI.

While the present study suggests that caspase inhibitors offer promise in the prevention of cisplatin-induced PT injury, a recent study showing that caspase inhibition worsens AKI by preventing autophagy,<sup>31</sup> dampens the enthusiasm to use caspase inhibitors to prevent cisplatin-induced AKI. Little is known of the expression of inflammasome-related genes in renal cells or in kidney diseases. The human kidney expresses much lower mRNA levels of most inflammasome-related molecules as compared with the human spleen except for NLRP2 and NLRP10. In contrast, kidneys from C57BL/6 mice express much higher levels of most NLR genes compared with the spleen, with the exception of neuronal apoptosis inhibitory protein (NIAP) and NLRP3.<sup>41</sup> Although NLRP1 and NLRP3 expression more or less overlaps in blood immune cells, expression in epithelial cells is quite different. NLRP1 is localized mainly in the nucleus, whereas NLRP3 is

predominantly cytoplasmic. A difference in subcellular distribution between NLRP1 and NLRP3 suggests site-specific roles of NLRPs in the inflammation. Which cells express functional inflammasomes in the kidney is largely unknown. However, some studies suggested the potential of tubular epithelial cells to secrete IL-1 $\beta$  and IL-18;<sup>42</sup> hence, we should harbor all necessary elements of the inflammasome-caspase-1-IL-1 $\beta$ -18 axis.

In summary, the present study demonstrates the following novel findings: (1) NLRP3 was expressed in PTs, but not increased by cisplatin. (2) Caspase-1, IL-1 $\alpha$ , and IL-1 $\beta$  were activated in PTs. (3) Pan Caspase inhibitor, QVD-OPH protected against necrosis, and the protection was associated with a decrease in caspase-1 as well as IL-1 $\alpha$ , and IL-1 $\beta$ .

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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