Cardiotrophin-1 Administration Prevents the Renal Toxicity of Iodinated Contrast Media in Rats

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Received November 7, 2012; accepted January 14, 2013

Although generally reversible, contrast media toxicity often induces contrast-induced nephropathy (CIN), which is associated with longer hospitalization time, the need for dialysis, and higher incidence of later cardiovascular events and higher mortality. Preventive cotreatments have been assayed at the preclinical and clinical levels, but recent meta-analysis has not demonstrated a beneficial effect, which supports the search for new nephroprotective strategies. We have assessed if the administration of cardiotrophin-1 (CT-1), an endogenous cytokine with protective properties on the heart and liver, might mitigate CIN in rats. We have developed a model of CIN induced by the administration of the contrast medium gastrographin iv (3.7 mg/kg) in rats sensitized by previous administration of subnephrotoxic doses of gentamicin (50 mg/kg/day, ip) for 6 days. The severity of CIN was assessed by the measurement of renal function; renal histological damage; urinary excretion of markers of tubular damage, including N-acetyl beta glucosaminidase (NAG), kidney injury molecule 1 (KIM-1), and plasminogen activator inhibitor 1; lipid peroxidation; and renal apoptosis. Treatment with CT-1 almost completely prevented the renal tissue damage, as evidenced by almost total prevention of tubular desepithelization and tubular obstruction, reduced caspase activation, and cell proliferation. Besides, CT-1 also prevented the increment in renal tissue levels of renal injury markers NAG, KIM-1, and neutrophil gelatinase–associated lipocalin. Oxidative stress, a hallmark of CIN, was also prevented by CT-1. Administration of CT-1 also prevented the derangement in kidney function induced by CIN. Renal hemodynamics, also impaired by the contrast medium, was normal in rats cotreated with CT-1. CT-1 administration significantly prevents the alterations in renal function and structure observed in a rat model of CIN.

Key Words: acute kidney injury; contrast-induced nephropathy; contrast nephrotoxicity; cardiotrophin-1; nephroprotection.

Iodinated contrast media (ICM) are a type of intravenous radiopaque used to facilitate vascular and organ visibility during X-ray based, radiographic diagnostic and interventional procedures. ICM have serious side effects, including anaphylactic reactions that occur rarely, and acute kidney injury (AKI) (Pasternak and Williamson, 2012). The incidence of ICM-induced AKI (contrast-induced nephropathy, CIN) has increased significantly in the past years due to (1) the growing number of therapeutic and diagnostic procedures involving ICM, (2) the higher number of high-risk patients (for instance, diabetic patients) (Calvin et al., 2010), and (3) the older age of patients subjected to these procedures (Laville and Juillard, 2010). For instance, CIN occurs in 2–25% of patients undergoing coronary interventions (Mehran et al., 2004; Solomon and Dauerman, 2010). Although generally reversible, CIN is not a trivial complication. It is associated to a longer hospitalization time, to the need of dialysis in some cases, and to a higher incidence of later cardiovascular events (Brown et al., 2008; Laville and Juillard, 2010), as well as higher incidence of chronic kidney disease (Maioni et al., 2012). In fact, CIN severity has recently been classified in three grades (0–2), depending on the increment in plasma creatinine concentration, which correlate with patients’ long-term prognosis (Harjai et al., 2008).

Knowledge of the underlying pathophysiological mechanisms may provide targets to prevent or to reduce CIN incidence and severity. Renal vasoconstriction, with the subsequent decrease in renal perfusion, plays a major role in CIN (Calvin et al., 2010). Increased oxidative stress has also been proposed as a major mechanism involved in CIN. N-acetylcysteine (NAC) is the most studied drug to prevent contrast-induced renal damage at the preclinical and clinical levels. Although NAC has proved efficacy in animal models, uncertain results have been obtained in the clinical setting (Trivedi et al., 2009). In fact, recent large, randomized trials have demonstrated that NAC does not reduce the risk of CIN or any other clinically relevant outcomes in at-risk patients undergoing coronary and peripheral vascular angiography (ACT Investigators, 2011; Amini et al., 2009).
Materials and Methods

Unless otherwise indicated, all reagents were purchased from Sigma (Madrid, Spain). Rat CT-1 (M.W. 21.5 kDa; batch 019R2500106543) was obtained from DRO Biosystems and stored at −80°C until use.

Experimental design. Male Wistar rats (weighing 225–250 g; Harlan Laboratories, Barcelona, Spain) were used according to the European Guide for Care and Use of Laboratory Animals (Directive 2003/65/CE) and Spanish regulation (Law 32/2007/Spain and RD 266/1998/Cyl). Rats were divided into six experimental groups (n = 6 each) that are mentioned subsequently.

1. Control group (C): rats received saline solution; (2) Gentamicin group (G): rats received gentamicin (50 mg/kg/day, ip) for 6 days (previous studies have demonstrated that this dosage does not produce any detectable changes in renal function but sensitize rats to CIN, whereas ICM per se do not induce CIN in rats; Quiros et al., 2010); (3) Cardiotrophin-1 group (CT-1): rats received CT-1 iv (100 ng/kg/day) for 5 days; (4) Contrast medium (gastrographin) group (Gg): rats received a single dose of gastrographin iv (3.7 mg/kg); (5) Gentamicin + Contrast medium group (G + Gg): rats received both drugs as single treatments in consecutive order: six doses of G and, the next day, the single Gg dose; (6) Gentamicin + Contrast medium + Cardiotrophin group (G + Gg + CT-1): rats received G and Gg as in group 5, and CT-1 the day before contrast administration and during the next 4 days. The second and fourth days after Gg or placebo (saline solution) administration, rats were allocated in metabolic cages with free access to food and water, for 24-h individual urine collection. Blood samples (150 µl) were obtained in heparinized capillaries from a cut in the tail tip every other day. Urine and plasma samples were stored at −80°C. At the end of the last metabolic cage period, acute clearance experiments were performed under anesthesia (as described subsequently). At the end of acute clearance experiments, both left and right kidneys were dissected and bisected in a transverse plane. One half of each kidney was fixed in 3.7% buffered paraformaldehyde for histological studies, and the rest were frozen in liquid nitrogen and stored at −80°C for lipid peroxidation measurement.

Renal function evaluation. Plasma and/or urine samples (as appropriate) were assayed for creatinine, urea, proteinuria, albuminuria, glycosuria, and renal injury markers. Urinary and plasma concentrations of urea and creatinine were measured with an automated analyzer (Reflotron, Roche Diagnostics, Barcelona, Spain). This method has a lower detection limit of 0.5 mg/dl for creatinine. From plasma (CrP) and urine (CrU) creatinine, glomerular filtration rate was estimated from endogenous creatinine clearance (CICr) using the standard formula: CICr = Cr × 24-h urine output × CrP-1. Proteinuria and glycosuria were determined according to the Bradford method and o-toluidine method (Morales et al., 2010), respectively.

Renal function was also measured by acute clearance methods. In short, animals were anesthetized, and their carotid artery, jugular vein, and urinary bladder were cannulated. The carotid artery catheter was connected to a pressure transducer, and arterial pressure was registered during the experiment (Mac Lab/4e, AD Instruments, Sydney, Australia). Tritium-labeled inulin (ARC, St Louis) and 14C-labeled para-aminohippuric acid (PAH) (PerkinElmer, MA) were infused throughout the jugular vein at 3 ml/h, and three consecutive (30 min) urine samples were collected from the urinary bladder. Every half hour, blood was collected from the carotid artery into heparinized capillary tubes. PAH was measured in plasma and urine using a two-channel Liquid Scintillation Counter (Wallac 1409 DSA, Turku, Finland). Glomerular filtration rate was also estimated from inulin clearance, and renal plasma flow (RPF) was measured as 3H PAH clearance. Renal blood flow (RBF) was calculated from RPF and hematocrit. Renal vascular resistance (RVR) was calculated from RBF and mean arterial pressure, as previously reported (Garcia-Criado et al., 1998; Morales et al., 2010; Rodriguez-Pena et al., 2004).

Renal albumin excretion was measured with a Rat Albumin ELISA kit (Bethyl Laboratories, Montgomery) according to the manufacturer’s instructions. Urinary excretion of tubular toxicity markers such as N-acetyl beta glucosaminidase (NAG), kidney injury molecule 1 (KIM-1), or plasminogen activator inhibitor 1 (PAI-1) was also measured. NAG enzymatic activity (E.C. 3.2.1.30) was measured in urine samples using an automatic analyzer (Reflotron). Urinary levels of KIM-1 and PAI-1 were measured by Western blot, as previously reported (Morales et al., 2006; Quiros et al., 2010), with goat anti-KIM-1 (R&D Systems, Minneapolis) and mouse anti-PAI-1 (BD Biosciences, Madrid, Spain). Western blot quantification was performed with image quant software (GE-Healthcare, Madrid, Spain) after scanning the films on an Office jet 8500 scanner (Hewlett-Packard, Madrid, Spain).

Lipid peroxidation. As an index of oxidative stress, lipid peroxidation was measured as thiobarbituric acid–reacting substances (TBARS) using malonaldehyde (MDA) as control (Morales et al., 2010).

Histological studies. Kidney sections of 3 µm were obtained from parafin-embedded samples and stained with hematoxylin-eosin (HE). Renal injury was determined by means of a “total severity score,” which was calculated semiquantitatively as described (Asaga et al., 2008). Briefly, 10 fields per renal slice were examined under light microscopy (x400). Each field was divided in six experimental groups (n = 6 each) that are mentioned subsequently.

1. Control group (C): rats received saline solution; (2) Gentamicin group (G): rats received gentamicin (50 mg/kg/day, ip) for 6 days (previous studies have demonstrated that this dosage does not produce any detectable changes in renal function but sensitize rats to CIN, whereas ICM per se do not induce CIN in rats; Quiros et al., 2010); (3) Cardiotrophin-1 group (CT-1): rats received CT-1 iv (100 ng/kg/day) for 5 days; (4) Contrast medium (gastrographin) group (Gg):
into 10 sections. A score of 0–3 was assigned to each section, according to the following criteria: 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, or loss of nuclei in up to one third of the tubular perimeter; 2, same as for 1, but from one third to two third of nuclei loss; and 3, over one third of nuclei loss. Section scores were added to give a field score (maximal score per field = 30). The average score of 10 fields was used for each kidney sample.

Immunostaining was performed in 3-µm kidney sections as described previously (Sanchez-Gonzalez et al., 2011). Primary antibodies used were anti-rat Ki-67 (Abcam, Cambridge); and cleaved caspase-3 (Cell signaling-Life Technologies, Madrid, Spain) as an index of apoptosis. In addition, the number of positive nuclei (Ki-67) was also determined in 10 randomly selected fields (×400) per sample as an index of proliferation. The area stained for active (cleaved) caspase-3 was quantified in 10 randomly selected fields (×400) per sample using the Image-Pro Plus Software (Media cybernetics, Bethesda, MD).

Data analysis and statistics. Statistical analysis was performed using the NCSS software. Values from data with a normal distribution were expressed as mean ± SD. After ANOVA analysis, the post hoc Scheffe’s test was used for multiple comparisons. *p < 0.05 was considered statistically significant.

RESULTS

CT-1 Prevents CIN-Associated Renal Dysfunction

The administration of G, CT-1, or Gg alone did not change plasma creatinine and urea concentration or creatinine clearance. However, in the Gg + G group, a significant increase in plasma creatinine (Fig. 1A) and urea (Fig. 1B) and a decrease in creatinine clearance (as an index of glomerular filtration rate, GFR) were observed, which was completely prevented by coadministration of CT-1 (G + Gg + CT-1 group; Fig. 1C). Inulin clearance, another index of GFR, produced similar results (Fig. 2A). These data indicate that the reduction in glomerular filtration characteristic of acute kidney dysfunction (in this case provoked by contrast medium administration in sensitized rats) was prevented by CT-1. RBF, calculated from RPF measured as PAH clearance and hematocrit, follows a similar pattern to that of GFR (Fig. 2C). RVR was significantly higher in the group G + Gg than in the control group. In the group that received also CT-1 (G + Gg + CT-1), this increase in RVR was not observed (Fig. 2B). Consistent with tubular dysfunction resulting from tubular necrosis (as described subsequently), a marked increase in proteinuria and albuminuria was evident in the G + Gg group. These parameters were markedly lower in the group that also received CT-1 although the reversal in proteinuria and albuminuria was only partial (Figs. 3A and 3B).

CT-1 Prevents CIN-Associated Renal Tissue Injury

Urinary markers of tubular damage including NAG, KIM-1, and PAI-1 were significantly higher in the G + Gg group than in the C, G, Gg, and CT-1 groups. NAG, a lysosomal enzyme excreted in the urine after renal tubular damage, was also markedly and significantly higher in the Gg + G group than in the C, G, Gg, and CT-1 groups. This increase was slightly (but not significantly) prevented by coadministration of CT-1 (G + Gg + CT-1 group; Figs. 4A–C). KIM-1 is a type I membrane

FIG. 1. Values of plasma creatinine (A), plasma urea (B), and creatinine clearance (C) 2 and 4 days after gastrographin administration. Data represent the average ± SEM of n = 5. *p < 0.05 with respect to the control group; *p < 0.5 with respect to the G + Gg group. C, control; G, gentamicin; Gg, gastrographin; and CT-1, cardiotrophin-1.
protein that has been demonstrated to be a sensitive and early marker of tubular damage. In these circumstances, its extracellular domain is cleaved and shed into the urine (Ferreira et al., 2011). In the G + Gg group, there was a marked increase in urinary KIM-1 concentration compared with C, whereas separate administration of G or Gg did not induce any relevant change. In the group that received CT-1 with Gg and G (G + Gg + CT-1), the urinary excretion of KIM-1 was markedly and significantly lower than in the G + Gg group (Fig. 4B). PAI-1 excretion has also been used as a marker of tubular damage (Ferreira et al., 2011). Urinary PAI-1 excretion showed a urinary excretion profile similar to that of KIM-1 (Fig. 4C).

HE staining revealed that C, G, Gg, and CT-1 groups had normal renal parenchyma features (Supplementary fig. 1). However, in the G + Gg group, significant tubular necrosis was evident in the cortex, and considerable tubular obstruction with

![FIG. 2. Values of inulin clearance (A), renal vascular resistance (B), and renal blood flow (C) 4 days after gastrographin administration. Data represent the average ± SEM of n = 5. *p < 0.05 with respect to the control group; &p < 0.5 with respect to the G + Gg group. C, control; G, gentamicin; Gg, gastrographin; and CT-1, cardiotrophin-1.](image)

![FIG. 3. Values of urinary protein excretion (A) and urinary albumin excretion (B) 4 days after gastrographin administration. Data represent the average ± SEM of n = 5. *p < 0.05 with respect to the control group; &p < 0.5 with respect to the G + Gg group. C, control; G, gentamicin; Gg, gastrographin; and CT-1, cardiotrophin-1.](image)
hyaline material was observed in the medulla (Fig. 5). Tubular necrosis was characterized by loss of cell integrity and the accumulation of cell debris in the tubular lumen. CT-1 administration significantly prevented all these aspects of tubular damage produced by the administration of G + Gg (Fig. 5).

Renal cell proliferation was assessed by the nuclear staining of Ki-67 that is expressed only in proliferating cell nuclei. The kidneys from the C, G, Gg, and CT-1 groups show almost no proliferating cells (Supplementary fig. 2). In the kidneys from the G + Gg group, many proliferating cells were detected, which were mainly tubular cells. The number of proliferating cells was markedly lower in the kidneys from the Gg + G + CT-1 group (Fig. 6). Apoptosis was assessed by the staining with antibodies against activated caspase-3. Our results show a great number of cells stained for cleaved caspase-3 in both the cortex and medulla of kidneys from the G + Gg group. This staining was markedly lower in the group that also received CT-1 (G + Gg + CT-1).

**FIG. 4.** Values of urinary NAG excretion (A), urinary KIM-1 excretion (B), and urinary PAI-1 excretion (C) 4 days after gastrographin administration. Data represent the average ± SEM of n = 5. Representative images of Western blot of KIM-1 and PAI-1 are shown in B and C, which were obtained with urine samples from three unselected rats per group. *p < 0.05 with respect to the control group; &p < 0.5 with respect to the G + Gg group. AU, arbitrary units (Materials and Methods section). C, control; G, gentamicin; Gg, gastrographin; and CT-1, cardiotrophin-1.

**FIG. 5.** Representative images of renal cortex and medulla sections stained with HE from the G + Gg and G + Gg + CT-1 groups at sacrifice (A) and quantification of injury score (B). Data represent the average ± SEM of n = 3. *p < 0.05 with respect to the control group; &p < 0.5 with respect to the G + Gg group. AU, arbitrary units (Materials and Methods section). C, control; G, gentamicin; Gg, gastrographin; and CT-1, cardiotrophin-1.
CT-1 Prevents CIN-Associated Lipid Peroxidation

Renal tissue oxidative stress was evaluated through the level of lipid peroxidation (Morales et al., 2002). Renal MDA was similarly low in C, G, Gg, and CT-1 groups. In the G + Gg group, MDA level was significantly higher than in control groups, whereas coadministration of CT-1 (G + Gg + CT-1 group) returned MDA to a level similar to that in the control groups (Fig. 8).

DISCUSSION

Despite being transitory in most cases, CIN is not a minor complication: (1) CIN poses the third most frequent cause of acute renal failure in hospitalized patients, with an estimated
12% incidence (Nash et al., 2002); (2) about 10% of CIN patients need permanent dialysis, and many others will retain some degree of renal insufficiency (Maioli et al., 2012); (3) CIN increases the risk of chronic renal disease and nonrenal complications, extends hospitalization time, and increases immediate, 30-day, 1-year, and 5-year mortality (Best et al., 2002; Gupta et al., 2005; McCullough et al., 2006; Rihal et al., 2002; Sinning et al., 2010); (4) extended hospital stay derived from an episode of AKI significantly increases sanitary costs. It was estimated that, in 2003 in the United States, a 0.3-mg/dl increment in Cr originated an extra cost of $8902 per patient, whereas an increment of 2.0 mg/dl cost an additional $33,162, which, furthermore, consumed 5% of the whole hospital budget (Chertow et al., 2005). For these reasons, both early detection and, especially, prevention are two critical aspects for improving patient prognosis and health expenditure control. In this sense, most renoprotective strategies assayed hitherto to prevent or minimize the incidence and severity of CIN have only afforded, in the best case, a limited benefit.

For its alleged physiological and endogenous role in tissue repair in several organs, we hypothesized that exogenous administration of CT-1 might efficiently prevent CIN-associated renal damage and, as a consequence, renal dysfunction. To this purpose, we set up a model of contrast nephropathy in which the kidneys are predisposed to suffer a toxic damage by the previous administration of another toxin (in this case gentamicin) at subnephrotoxic dosage. This is because contrast media do not exert a clear renal injury per se in rats. However, as we have demonstrated previously (Quiros et al., 2010), a subnephrotoxic regime of gentamicin makes rats more susceptible to the subsequent effect of other nephrotoxins, including contrast media. Although it is a different approach, it has been also demonstrated that a 5-day gentamicin regime (100 mg/kg) that produced a mild AKI, followed by an iodinated contrast, induced a massive AKI (Hsu et al., 2011). Our results clearly demonstrate that prophylactic treatment with CT-1 significantly prevented kidney damage and renal dysfunction in this model, as evidenced by the preserved tissue integrity, absence of injury markers, and normal or near normal values of kidney function parameters.

The renal protection afforded by CT-1 might be based on the combination of several effects, which might make it a promising preventive therapy for CIN. First, contrast nephropathy is characterized by an increased renal vasoconstriction and the subsequent decrease in RPF and GFR (Heyman et al., 2010; Sendeski, 2011; Tumlin et al., 2006), a feature clearly reproduced in our experimental model (Figs. 1D–E). As such, vaso-dilator drugs such as dopamine, fenoldopam, and theophylline have been proposed for CIN prevention (Calvin et al., 2010), which needs further exploration. Our results show that CT-1 efficiently prevents contrast-induced renal vasoconstriction and the reduction in RPF and GFR. This effect might be ascribed to its known vaso-dilator properties, previously described elsewhere (Jin et al., 1996), which are mediated, at least in part, by stimulating NO synthesis (Nomura et al., 2003).

Second, increased oxidative stress has been also proposed as a major mechanism involved in contrast nephropathy (Heyman et al., 2010), which has prompted the use of the antioxidants, most notably NAC, as a preventive strategy (Trivedi et al., 2009). In our model, CIN is associated to oxidative stress, as demonstrated by the higher levels of renal TBA-reacting substances, which is prevented by CT-1. The effect of CT-1 on tubular cell apoptosis probably plays a major role in the kidney protection afforded by CT-1. In fact, tubular cell apoptosis, assessed as the staining for cleaved (activated) executioner caspase-3, was markedly reduced in animals receiving CT-1. The protection exerted by CT-1 on several cell types against apoptosis was already described (Bustos et al., 2003; Dolcet et al., 2001; Lopez et al., 2005; Peng et al., 2010). In vivo, the hepatoprotective effect of CT-1 on several models of hepatic failure is thought to be based on the inhibition of apoptosis (Bustos et al., 2003; Ho et al., 2006; Marques et al., 2007; Yang et al., 2008). Moreover, CT-1 pretreatment in rats with left coronary ligation decreased the number of apoptotic cardiomyocytes and the expression of proapoptotic molecules in ischemic myocardium (Ruixing et al., 2007). CT-1 administration also reduced the apoptosis in the ischemic cortex after cerebral ischemia (Dolcet et al., 2001). Although CT-1 administration induces cell proliferation in several organs, including the kidney (Jin et al., 1996), reduced proliferation after CT-1 addition in this model of contrast nephropathy is probably the consequence of a lower tubular damage, as tubular proliferation is a compensatory mechanism.

The results of this study have potential clinical application. CT-1 holds promise as an effective renoprotective agent due to its endogenous tissue-repairing properties targeting different mechanisms of injury. Interestingly, CT-1 has been recently approved for human use. CT-1 has been granted the status of orphan drug by the U.S. Food and Drug Administration, for the treatment of acute liver failure (designa\n
tion request 11-3507) and protection of the liver from the ischemia/reperfusion injury inherent in the transplantation procedure (designa\n
tion request 07-2449), and by the European Medicine Agency for the prevention of ischemic/reperfusion injury associated with solid organ transplantation (EU/3/06/396). The first clinical studies on the use of CT-1 for this purpose will start shortly, as a study on the use of CT-1 in kidney preservation for transplantation has been recently approved in Spain. In addition, a clinical trial with the goal of determining safety, tolerability, and early pharmacokinetics of CT-1 in early volunteers has been registered in U.S. Clinical trials.gov (identifier NCT01334697) (www.clinical\ntrials.gov, checked on July 15, 2012). However, some studies will be probably necessary in order to demonstrate the safety of CT-1 use, as it has been recently described in an in vitro study that CT-1 has arrhythmogenic effects in cultured myocardocytes (Ruiz-Hurtado et al., 2012).

In summary, our results demonstrate that the administration of CT-1 protects the kidneys against the functional and structural damage induced by the contrast medium graphin
in gentamicin-predisposed rats. This protection was observed in all the parameters studied (renal function, tubular damage, lipid peroxidation, tubular structure, proliferation, and apoptosis). For its endogenous antiapoptotic effect, these results pose exogenously administered CT-1 as a new and interesting candidate to prevent CIN, which might be especially relevant in high-risk patients.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

Digna Biotech S.L. to Bio-inRen S.L. (Spain); Instituto de Salud Carlos III (Retic 016/2006, RedinRen). The Renal and Cardiovascular Pathophysiology Unit holds the Excellence Group mention (GR-100) and awarded by the Junta de Castilla y León.

ACKNOWLEDGMENTS

F.J.L.-H. and J.M.L.-N. are minority shareholders of Bio-inRen S.L., and they serve as scientific advisors for this company. The authors thank Daniel Lopez-Montañes for his excellent technical assistance.

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