Abstract

Objectives. Radiocontrast agents are one of the most common causes of acute renal failure in the world. These agents are required for both diagnostic and therapeutic modalities of medical intervention, including computed tomography (CT), angiography and cardiac catheterization. Publications over the past 40 years support three potential mechanisms of toxicity: oxidative stress, haemodynamics and hyperosmolar effects. An in vitro model provides a rapid evaluation of cellular toxicity without the complications of haemodynamics. This study evaluated the renal toxicity of radiocontrast agents at clinically relevant concentrations.

Methods. This study investigated the toxicity of two radiocontrast agents, diatrizoic acid (DA) and iohalamic acid (IA), using an in vitro model. Renal cortical slices isolated from F344 rats were incubated with 0–111 mg I/ml DA or IA.

Results. Renal slices exposed to DA and IA showed toxicity as measured by increased lactate dehydrogenase (LDH) leakage at concentrations lower than previously published using isolated cell models. These data indicate that DA and IA are toxic to renal cortical slices, and this is a more sensitive model than previously used cell culture systems. DA and IA treatment failed to cause a significant decrease in total cellular glutathione or increase in percent glutathione disulphide (GSSG), implying that oxidative stress may not be an initial mechanism of toxicity. Finally, the addition of exogenous glutathione did provide complete protection from DA- and IA-induced LDH leakage.

Conclusion. These data validate the renal cortical slice in vitro model for investigation of radiocontrast nephrotoxicity. These studies further showed that glutathione was cytoprotective. Future research using this model is aimed at further characterization of radiocontrast nephrotoxicity, which may allow for improved prevention and treatment of radiocontrast-induced acute renal failure.

Keywords: glutathione; nephrotoxicity; radiocontrast; rat cortical model

Introduction

Nephropathy secondary to iodine-based radiocontrast agent exposure is one of the most common causes of acute renal failure in hospital settings [1]. Exposure to these agents occurs in the clinical setting of angiography, including coronary angiography and intervention, as well as intravenous contrast with computed tomography (CT). Contrast nephropathy (CN) is commonly defined as a 25% or >0.5 mg/dl increase in serum creatinine over baseline values within 48 h after radiocontrast administration and in the absence of alternative aetiology. However, as creatinine has been shown to peak 72–96 h after contrast exposure [2,3], CN may be overlooked clinically and underestimated in studies measuring creatinine concentrations at 48 h or less. CN is associated with significant morbidity and mortality. In a study of patients who had undergone percutaneous intervention, CN was associated with increased in-hospital mortality as well as higher mortality measured at 6 months, 1 and 5 years. The presence of diabetes mellitus and/or pre-existing renal insufficiency increases the risk of CN [4]. Other reported risk factors for CN include advanced age, repeated contrast administration, CHF (EF <50% or NYHA class III or IV heart failure), dehydration and high contrast load, along with medications that are nephrotoxic or otherwise affect renal function, such as aminoglycosides, diuretics, and NSAIDS [5]. Patients undergoing coronary angiography frequently have multiple cardiovascular disease risk factors and are therefore at an increased risk for the development of renal disease [6].

Despite the advances in knowledge of patient predisposition to CN and potential preventative measures, CN continues to be one of the most common global causes of acute renal insufficiency and renal failure in hospitalized patients [1]. The mechanism of toxicity of radiocontrast agents has been the subject of much research over the past 40 years.
Much of this research has focused on one of three mechanisms of toxicity: oxidative stress, hyperosmolarity and haemodynamic effects. Investigators have used a variety of models ranging from in vivo animal models to single cellular immortalized in vitro systems [7–10]. Despite the efforts of this research, there has been no consensus for the mechanism of CN as there are conflicting results between the various models. Each of these models possesses attributes that allow investigation of specific subsets of mechanistic investigation; however, these models are also limited. Specifically, in vivo models are an excellent source for understanding the effects of an agent on the organism as a whole. However, mechanistic investigations with the in vivo model are limited due to the myriad interactions within the organism. These models characterize terminal events and cannot identify the inciting event that initiates toxicity. Conversely, immortalized cell lines allow investigators the ideal system to determine the first events of mechanistic toxicity; however, these cell lines may behave very differently from freshly isolated cells. Specifically, immortalized cells possess genetic mutations that predispose them to survival and may change the behavior of these cells when exposed to a toxicant. Thus, correlation with a more natural model is required to confirm the model’s validity.

An ideal model would incorporate the beneficial attributes of each of these systems, and exclude the confounding factors. The use of the renal cortical slice model is an attempt to illustrate the best possible model for this study. This model provides a heterogeneous cell population that maintains the organization of the cortical nephron. Renal slices allow cells to maintain communication and metabolism. This model thus allows for specific mechanistic investigation using minimally altered living renal tissue. If toxicity is observed with this model, all required elements for the induction of cellular injury are contained within the renal cortex. As opposed to the in vivo system, this model also allows for manipulation of the experimental conditions, thus allowing for further mechanistic characterization. Furthermore, the in vitro model avoids confounding factors such as haemodynamic events that would complicate mechanistic evaluations at the cellular level. The goal of this research was to characterize the toxicity of radiocontrast agents in renal cortical slices as a novel model for the investigation of CN. This model provides unique attributes that will allow for further mechanistic characterization of CN. Characterization of the mechanistic of this toxicity may lead to specific preventative and/or therapeutic interventions to combat this all too common cause of renal injury.

**Methods**

**Chemicals**

Diatrizoic acid (DA) and iothalamic acid (IA) were obtained from Mallinkrodt Inc., (Hazelwood, MO, USA). Reduced glutathione (GSH) and glutathione disulfide (GSSG) were obtained from Sigma Chemical Company (St Louis, MO, USA). DMSO was HPLC grade. All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich Chemical Company.

**Animals**

Male Fischer 344 (F344) rats (200–250 g) were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA, USA). All rats were given a minimum 5-day acclimation period prior to initiation of any experiments. The University Committee on Animal Care and Use reviewed and approved the protocol for animal use. This investigation conforms to the Guide for the Care and Use of Laboratory Animals in an AALAC accredited facility. Animals were maintained under a controlled ambient temperature (21–23°C), humidity (40–55%) and 12-h light cycle (lights on 0600–1800 h). Animals were provided free access to tap water and Purina Rat Chow.

**Incubation of renal slices**

Animals were anaesthetized with diethyl ether, and the abdominal aorta was cut to exsanguinate the animals. The kidneys were excised, decapsulated, quartered and immediately placed in 5 ml ice-cold Krebs Ringer buffer. The composition of the Krebs buffer was 126.4 mM NaCl, 6 mM Na₂HPO₄, 5.2 mM KCl, 1.3 mM MgSO₄ and 1 mM CaCl₂. Renal cortical slices were prepared as described previously [11–12] and placed in 10 ml ice-cold Krebs buffer. The slices from one animal were transferred to 5 ml oxygenated Krebs buffer in a 30 ml beaker. The slices were rinsed 2 times in 5 ml oxygenated Krebs buffer each for 3 min at 25°C in a Dubnoff metabolic shaker under an oxygen environment with constant shaking (100 cycles/min). The tissue (50–100 mg) was transferred to 2 ml oxygenated Krebs in designated Erlenmeyer flasks and equilibrated for 10 min at 37°C under oxygen and constant shaking (100 cycles/min Dubnoff Incubator). Renal tissue was incubated for 60–120 min with DA or IA at a final concentration of 0, 9.25, 18.5, 37.0, 74.0 or 111.0 mg I/ml, which was added as a concentrated solution in oxygenated Krebs buffer to a total incubation volume of 3 ml. The concentrations selected are clinically relevant as some patients are administered in excess of 400 mg I/ml [13].

**LDH**

Media and tissue samples were collected to measure LDH leakage. Tissues were blotted, weighed and added to 10% Triton X-100 to release residual tissue LDH. LDH was determined using a kinetic assay. LDH release into the media was expressed as percentage of total LDH.

**GSH and GSSG determination**

Tissues were homogenized in 0.5% sulfosalicylic acid and adjusted to a 1 ml volume. Total GSH was determined using a GSH reductase and NADPH coupled reaction with 5,5′-dithiobis(2-nitrobenzoic acid) [14]. GSSG was measured following 2-vinylpyridine derivatization [15].

**Incubation with GSH**

A series of studies examined the impact of the thiol-containing agent, GSH on DA and IA toxicity. Slices were prepared as described above and pre-incubated for 30 min.
Characterization of a novel model for investigation of radiocontrast nephrotoxicity

Diatrizoic Acid (mg I/mL)
LDH Leakage (% of total)
0 1 2 3 4 5 6 7

B A C

Fig. 1. Concentration-dependent effects of DA on LDH leakage at 60 and 120 min. Renal cortical slices were incubated with varying concentrations of DA for 60 (upper panel) or 120 min (lower panel). LDH leakage was expressed as percentage of total. Values represent mean ± SEM with n = 4 animals/group. Different superscripts indicate a statistical difference (P < 0.05) between groups at a designated time period.

with 1 mM GSH at 37°C and constant shaking. DA or IA was added, and the tissues were incubated for an additional 60 min. At the end of the incubation period, LDH leakage was monitored.

Statistical analysis
Values represent mean ± SEM with n = 4 animals/group. Differences between groups were analyzed using an analysis of variance (ANOVA) followed by a Newman–Keuls test (Sigma Stat, SPSS Inc., Chicago, IL, USA). Differences within a treatment group were determined using a repeated measures ANOVA followed by a Newman–Keuls test. All statistical analyses were conducted using a 95% confidence interval.

Results
DA concentration and time-dependent effects on LDH leakage and GSH status
LDH leakage was used as an indicator of a loss of cellular membrane integrity. DA concentrations of 18.5 and 37 mg I/ml increased LDH leakage within 60 min (Figure 1, upper panel). This increase was concentration dependent as there was a significant difference (P < 0.05) between 18.5 and 37 mg I/ml. At 120 min, DA treatment caused an increase in LDH leakage at concentrations as low as 9.25 mg I/ml (Figure 1, lower panel). This increase was also concentration dependent with a graded increase in LDH leakage as the concentration of DA increased. The maximal LDH leakage occurred following a 2-h incubation with 74 mg I/ml DA.

DA did not cause a decrease in cellular total GSH or an increase in GSSG
There was no significant decrease in cellular GSH levels following a 60-min exposure to DA (Figure 2, upper panel). There was, however, an increase in total GSH at 60 min with 18.5 mg I/ml DA. There were no significant changes in total glutathione at 120 min (data not shown). These data indicate that loss of membrane integrity occurs without depletion of total cellular glutathione. DA exposure did not cause an increase in percent GSSG (%GSSG) following a 60- or 120-min incubation (data not shown). These data indicate that there is not a significant change in %GSSG,

Fig. 2. Time-dependent effects of DA and IA on total glutathione. Renal cortical slices were incubated with varying concentrations of DA or IA for 60 min as described in the methods. Total glutathione was expressed as nmol/g tissue. Values represent mean ± SEM with n = 4 animals/group. An asterisk (*) indicates a significant difference (P < 0.05) from control.
an indicator of oxidative stress, prior to loss of cellular membrane integrity.

GSH pretreatment prevented DA-induced LDH leakage

Pretreatment of renal cortical slices for 30 min with 1 mM GSH prior to DA exposure provided complete protection from DA-induced increases in LDH leakage. GSH protected slices exposed to 18.5 and 38 mg I/ml DA (Figure 3, upper panel). These data indicate that GSH plays a protective role in DA toxicity.

IA concentration and time-dependent effects on LDH leakage and GSH status

LDH leakage was induced within 60 min by 37 and 74 mg I/ml IA (Figure 4, upper panel). This increase was concentration dependent as reported by a significant difference between 37 and 74 mg I/ml. At 120 min, IA treatment caused an increase in LDH leakage at concentrations as low as 37 mg I/ml (Figure 4, lower panel). The concentrations of IA required to induce LDH leakage were higher than those of DA.

IA did not cause a decrease in cellular total GSH

There was no significant difference in cellular total GSH at 60 min for all evaluated concentrations of IA (Figure 2, bottom panel). These data indicate that there is not a significant change in cellular total GSH prior to or during the loss of cellular membrane integrity. IA treatment did not increase the percent of GSSG following a 60-min incubation (data not shown). These data indicate that there is no significant increase in %GSSG, an indicator of oxidative stress, prior to or during the loss of cellular membrane integrity.

GSH pretreatment prevented IA-induced LDH leakage

A 30-min pre-incubation with 1 mM GSH provided complete protection from IA-induced increased LDH leakage (Figure 3, lower panel). GSH protected renal slices exposed to 37 and 74 mg I/ml. These data indicate that GSH plays a protective role in IA toxicity.

Discussion

Nephropathy secondary to iodine-based radiocontrast agent exposure is one of the most common causes of acute renal
failure in hospital settings. The mechanism of toxicity of radiocontrast agents has been the subject of much research over the past 40 years, focusing on three mechanisms of toxicity, including oxidative stress, hyperosmolality and haemodynamic effects. These investigations have used a variety of models ranging from in vivo animal models to single cellular immortalized in vitro systems. Despite the efforts of this research, there has been no consensus for the mechanism of CN as there are conflicting results both within and between the various models used. Each of these models possesses attributes that allow them to investigate specific subsets of mechanistic investigation; however, they are also limited.

This study utilized the renal cortical slice model for investigation of CN. The renal slices provided a heterogeneous cell population that maintains the organization of the cortical nephron. Renal slices allow cells to maintain communication and metabolism. Furthermore, the in vitro model avoids confounding factors such as haemodynamic events that would complicate mechanistic evaluations at the cellular level.

The current study showed that both DA and IA are directly toxic to renal cortical slices in a concentration- and time-dependent manner. The in vitro toxicity within 1–2 h shows that any components needed for activation or metabolism to generate toxicity by DA and IA are present within the renal cortex. Thus, exogenous activation from extrarenal sites is not required for DA- or IA-induced toxicity. These data also indicate that toxicity occurs in the absence of haemodynamic events, as this model is independent of these effects. This implies that toxicity of both DA and IA cannot be explained by haemodynamic changes that may contribute in vivo. The observed haemodynamic changes by other researchers may be secondary events. The present findings suggest that there is a non-haemodynamic component to DA and IA toxicity. Further studies are necessary to determine the relative contribution of the haemodynamic and cellular mechanisms.

DA and IA toxicity in renal cortical slices (Figure 1 and 4) occurred with lower concentrations and shorter time periods than other studies utilizing cell culture systems [16,17] indicating that the renal cortical slice model is more sensitive than cell lines. For example, LLC-PK1 cells showed no loss of cell dysfunction when incubated for 5 h with 150 mg I/ml [16]. Further, LLC-PK1 cells required a 24-h exposure to 37 mg I/ml in order for viability to be diminished [16]. Another study observed that concentrations of 50 mg I/ml or higher were needed to induce apoptosis after 1-h exposure of NRK-52E cells [17]. In the present studies, renal cortical slices exhibited increased membrane leakage within 1-h exposure to 18.5 mg I/ml DA and 37 mg I/ml and this increased sensitivity should provide a model suitable for mechanistic investigation of CN.

As noted the toxicities of DA and IA were concentration dependent, thus as higher doses of DA or IA are used, more toxicities will occur. These data are consistent with previous results using other models showing a concentration-dependent toxicity. This also correlates with the observation that dye load is a risk factor and a prognostic indicator of CN [1,13].

This research also showed that DA and IA toxicities were time dependent. These results indicate that the duration of radiocontrast exposure contributes to renal toxicity. When this finding is applied to the in vivo system, a better understanding of CN may be obtained. If the kidney is exposed to radiocontrast dye for a prolonged duration of time either through continued IV administration or decreased renal clearance, it is more likely to develop renal toxicity. This finding correlates well with the observation that pre-existing renal disease or dysfunction predisposes to CN. With these conditions in addition to having reduced renal function reserve, an individual is less able to clear their dye load, leading to prolonged exposures to these agents. This prolonged exposure to the renal toxicant may allow for increased injury in a kidney that is already predisposed to dysfunction.

Previous literature has supported the role of glutathione as a cytoprotective agent, but the mechanism is not yet understood for this protection. Previous studies have shown that exogenous glutathione treatment of renal cortical slices increased cellular glutathione concentrations [18]. This increase is likely mediated by uptake from the glutathione transporter located on the basolateral membrane of the proximal tubular epithelial cells [19]. The protective role of GSH was evident for DA and IA (Figure 3). There are two possible pathways for GSH cytoprotection (Figure 5). GSH may detoxify radicals through glutathione peroxidase or GSH could scavenge reactive electrophiles. Further studies are necessary in order to examine the mechanism of GSH cytoprotection for DA and IA toxicity.

DA and IA did not induce a significant decrease in cellular total glutathione as would be expected by an oxidative mechanism of toxicity. These data imply that glutathione is not being aggressively depleted in the presence of DA/IA toxicity. Additionally, there was not an increase in %GSSG, implying that there was no significant oxidative stress due to DA and IA. The absence of GSH depletion may be dependent on the short incubation time of 1 h. It is possible that studies for a longer duration may show more dramatic changes in GSH, but additional studies need to examine longer duration in order to prove or disprove this hypothesis. An alternative mechanism of
protection would be through scavenging reactive intermediates that are independent of oxidative stress (Figure 5). In this setting, total glutathione is depleted through conjugation with reactive electrophiles without concomitant increases in %GSSG. This depletion may be much less dramatic in that this mechanism may require metabolic activation to the reactive electrophile; also this conjugation is not driven by an enzymatic process and is thus competitive with cellular macromolecules such as proteins. Further research is needed to characterize the mechanism of GSH-mediated cytoprotection.

In summary, DA and IA are directly toxic to the renal cortical slice model. This model is more sensitive than previously published studies using immortalized cells. This study also demonstrates that at clinically relevant concentrations, the radiocontrast dyes DA and IA are toxic in a model that obviates confounding haemodynamic factors. Further, this is the first study to show that GSH reduces the toxicity of DA and IA in renal cortical slices. Additional studies are needed to better characterize the mechanism of DA and IA toxicity as well as the protection afforded by exogenous GSH. This renal cortical slice model will likely play an important role in the mechanistic investigation of CN.

Acknowledgements. The authors wish to acknowledge Drs Paulette Wehner and Dr Mark Studeny for their support and guidance through this study. This project was funded by internal funding from Cardiovascular Services.

Conflict of interest Statement. None declared.

References


Received for publication: 10.3.08
Accepted in revised form: 3.9.08