Iodinated contrast media cause endothelial damage leading to vasoconstriction of human and rat vasa recta

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Contrast-induced acute kidney injury (CIAKI) is a complication caused by the use of iodinated contrast media (CM). CIAKI accounts for nearly 10% of all cases of hospital-acquired acute renal failure in Europe and the United States (4) and is associated with prolonged hospital stays and increased short- and long-term mortality (7, 11). In patients combining risk factors such as previous chronic kidney disease, diabetes mellitus, volume depletion, nephrotoxic drug use, and preprocedural hemodynamic instability, CIAKI incidence may be as high as 50% (17). Hydration and keeping the CM dose low, which are the only widely recommended preventive measures for CIAKI, only attenuate but do not eliminate the risk of CIAKI (2, 8).

The pathophysiology of CIAKI is not fully understood. Factors known to be involved include direct cell damage by CM, seen in several experimental models (23); renal hypoperfusion and hypoxia of the outer medulla, with increased local oxidative stress (13, 14, 22); and derangement of autocrine and paracrine factors, like endothelin, adenosine, and nitric oxide (NO) (14). The tissue damage may be increased by conditions like diabetes mellitus or hypotension, which may lead to vascular and tubular functional impairment marked enough to diminish the glomerular filtration rate, so that the worsening in renal function eventually becomes clinically detectable (13, 22, 23). Although all types of CM share a similar level of cytotoxicity (23), some properties of individual CM, such as extremes of viscosity and/or osmolarity, may potentiate the adverse effects of CM on renal perfusion and urine formation (22).

The medullary hypoperfusion seen in CIAKI is explained by the constriction of outer medullary descending vasa recta (DVR) caused by CM (25). DVR are long and thin microvessels (12–15 μm diameter) that constitute the sole source of blood supply to the renal medulla (19). Furthermore, DVR constriction by CM goes along with low NO production and enhanced response to ANG II (24). Such findings are suggestive of an impairment of DVR endothelial function (6). However, the mechanisms through which CM induce endothelial dysfunction in DVR have not yet been identified. In our present study, we hypothesize that: 1) the functional impairment of DVR is a consequence of cytotoxic damage to DVR endothelial cells by CM and that 2) preventing endothelial damage by CM will consequently avert DVR endothelial dysfunction and DVR constriction. To test our hypotheses, we investigate the vasoreactivity of isolated perfused human and rat DVR during perfusion with CM. Furthermore, we characterize the endothelial damage by electron microscopy and measured endothelial permeability after exposure to CM.

**MATERIALS AND METHODS**

**Isolation and perfusion of DVR and renal interlobar arteries.** Human nonmalignant renal tissue was obtained upon an internal review board-approved protocol from patients undergoing open or laparoscopic nephrectomy due to renal cell carcinoma. Tissue samples were obtained and processed immediately after kidney removal to
ensure viability of DVR. Kidney samples were kept on ice-cold solution, and DVR were manually isolated and perfused with concentric micropipettes (25). Because there are no reports of human DVR studied with this method, we observed carefully signs of DVR viability, like the typical appearance of pericytes, behavior after mechanical manipulation, onset of luminal flow, changes in temperature, and response to ANG II and compared them with the literature available about rat DVR [for an overview on the physiology of DVR, see Pallone et al. (19)]. After confirming that human DVR remain viable for a similar period of time as rat DVR, we proceeded with CM experiments. Figure 1 shows a perfused human DVR of our series. For obtaining DVR and renal interlobar arteries from rats, male Sprague-Dawley rats (weight 120 – 200 g) were sedated with isoflurane and killed. The kidneys were removed and sliced along the main axis of tubular and vascular structures deepening in the renal medulla. The solution used for dissection, perfusion, and bath solution of rat and human vessels consisted of (in mM) 140 NaCl, 10 sodium acetate, 5 KCl, 1.2 MgSO4, 1.2 Na2HPO4, 1 CaCl2, 5 HEPES, 5 alanine, 0.1 arginine, 5 glucose, and 0.08 albumin, and pH was adjusted to 7.4 at 37°C. After obtaining stable perfusion, DVR and/or renal interlobar arteries were warmed up to 37°C, and experimental protocols were initiated. All procedures involving human subjects (or tissue) and experimental animals were approved by the appropriate control organs of our institution and our state. All patients were informed about the procedures and experiments intended to be done with donated tissues (including the publication of our experiments’ results), and all patients signed a consent form before surgery.

**Serial measurement of DVR diameter.** All DVR had basal diameter measurements made after stabilization of perfusion. Perfusate was then exchanged to either vehicle, CM, adrenomedullin (10^-6 M; Sigma-Aldrich, Munich, Germany), or CM + adrenomedullin. In all experiments, the CM used was iodoxanol, a dimeric, nonionic, isosmotic CM (GE Healthcare, Munich, Germany). We used a concentration of 23 mg iodine/ml, a nearly 13-fold dilution of the commercial solution comparable to what may be the plasmatic concentration during a coronary angiography (estimated from an iodine load of 60 g) (21). Where adrenomedullin was used in the perfusate, it was also applied in the bath solution simultaneously to the exchange of the perfusate. Serial digital pictures were acquired from perfused DVR for 20 min using a digital camera attached to the microscope. Vessel diameter was measured with the open-source software ImageJ (http://rsbweb.nih.gov/ij/) and expressed in Figs. 1–6 as percent change from the baseline measurement over time.

**Investigation of DVR reactivity to ANG II.** After treatment with either vehicle, CM, adrenomedullin, or CM + adrenomedullin, the effect of ANG II (Sigma-Aldrich) was tested at the concentration of 10^-10, 10^-9, and 10^-8 M, exchanged into the perfusion chamber at 5-min intervals. Diameter changes of DVR were expressed as percent from the measurement obtained at the minute before the first ANG II application.

**Scanning electron microscopy of renal interlobar artery endothelium.** For scanning electron microscopy (SEM), isolated segments of rat renal interlobar arteries were mounted on glass pipets of a perfusion-myograph (Danish Myo Technology, Aarhus, Denmark), tied with 11 nylon knots, warmed up to 37°C, and perfused for 20 min with either vehicle, CM, adrenomedullin, or adrenomedullin + CM (n = 3 for each group). The flow during perfusion was set to 16 μl/min. The CM concentration used was 23 mg iodine/ml, the same as in DVR experiments. Immediately after the end of each protocol, renal interlobar arteries were fixated by immersion in a solution of formaldehyde, glutaraldehyde, and methylene blue (3). The chamber was then rinsed two times with PBS solution, and vessels were dismounted and treated with OsO4 (at 4% for 1 min), rinsed again with PBS, opened lengthwise, and dehydrated in ethanol concentrations of 30% (2 h), 50% (2 h), and 75% (overnight). Samples were critical-point dried (CPD 30; Bal-Tec) and sputtered with ionized gold in a high-pressure argon atmosphere (CDC40). Images were obtained using an SEM system (Quanta 200; FEI, Kassel, Germany) (15).

**Transcellular electrical resistance.** Human umbilical cord vein endothelial cells (HUVEC) were isolated, grown on evaporated gold electrodes, and connected to an electrical cell-substrate impedance system (Applied Biophysics, Troy, NY) (18, 20, 10). Cells were exposed to CM alone or preincubated with adrenomedullin (10^-6 M) for 10 min as indicated. Resistance from each microelectrode was continuously monitored and corrected for the effect of agents used on microelectrodes without cells.

**Detection of myosin light chain phosphorylation.** Cells were harvested in lysis buffer containing phosphatase and protease inhibitors. Equal amounts of lysates were subjected to SDS-PAGE (12.5% gel) and blotted. Membranes were exposed to rabbit phosphospecific myosin light chain (MLC) [Thr18/Ser19]; Cell Signaling, Boston, MA) and goat antiactin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). After exposure of the membrane to secondary antibodies [Cy5.5-labeled anti-rabbit antibody, IRDye 800-labeled anti-goat antibody (Rockland, Gilbertsville, PA)], proteins were simultaneously visualized by infrared imaging (LI-COR Odyssey).

**Statistics and analysis.** Results of DVR diameter measurements were expressed as means ± SE. The size of each treatment group is given together with the group’s results. DVR data were compared using Brunner’s test (a nonparametric ANOVA-like test for repeated measurements and multiple comparisons) (5). To perform this test, we used noncommercial, freely obtainable software (the program “R-environment for statistical computing” may be obtained at www.r-project.org, and the functions for running Brunner’s test may be obtained at www.ams.med.uni-goettingen.de/amsneu/longit-de.shtml). P < 0.05 was used to reject the null hypothesis.

**RESULTS**

**CM induces constriction of perfused human DVR.** Up to our knowledge, the present investigation is unique in using isolated perfused human DVR. The results of experiments with human DVR are shown in Fig. 2. CM led to constriction of human DVR: after a 20-min period of perfusion, human DVR of the control group remained at 99.5 ± 4% of initial diameter (n =
7), whereas the diameter of DVR perfused with CM fell to 54.3 ± 9.2% of initial diameter (n = 7). We used adrenomedullin, an endogenous peptide with several antiapoptotic and protective effects on endothelial cells (18), to test the role of endothelial damage for the changes in tone and vasoreactivity. Treatment with human adrenomedullin blunted the DVR constriction caused by CM: the diameter of DVR treated with adrenomedullin and perfused with CM was 77.7 ± 7 of initial diameter (n = 6). Diameter of DVR that received adrenomedullin alone did not differ from controls (98.4 ± 2.9% of initial diameter, n = 5).

**CM induces constriction of perfused rat DVR.** Perfusion with CM led to constriction of rat DVR, as shown in Fig. 3. After 20 min of perfusion, DVR from the control group remained at 99.7 ± 2.4% of initial diameter (n = 8), whereas DVR from the CM group significantly constricted to 50.9 ± 7.5% of initial diameter (n = 8). Treatment with rat adrenomedullin blunted the DVR constriction caused by CM: luminal diameter of DVR treated with adrenomedullin and perfused with CM was 77.1 ± 5.5 of initial diameter (n = 8). Diameter of DVR that received adrenomedullin alone did not differ from controls (101.5 ± 5.3 of initial diameter, n = 4).

**Enhanced response to ANG II in CM-perfused rat DVR.** ANG II is a main modulator of DVR tone (19). In our study, DVR perfused with CM had an augmented response to ANG II that was prevented by adrenomedullin (Fig. 4). At the ANG II concentration of 10^{-8} M, the luminal diameter of DVR was 35 ± 7.3 of initial diameter for controls (n = 8) and 13.6 ± 8% for the CM group (n = 8), whereas for DVR perfused with CM and treated with adrenomedullin the diameter was 67.5 ± 11.7% of the initial value (n = 5). For DVR receiving vehicle plus adrenomedullin, the diameter at 10^{-8} M ANG II was 53.3 ± 15.4% of the initial value (n = 4).

**Influence of CM on the morphology of endothelial cells.** We wanted to know whether CM, in the same concentration and length of exposition that causes constriction of DVR, cause damage of endothelial cells. Therefore, we used a model of isolated perfused renal interlobar arteries because DVR are too small to be recovered after each perfusion experiment. We perfused interlobar arteries with vehicle, CM, or adrenomedullin, alone or in combination (Fig. 5). At control conditions, the vessel surface was smooth, and endothelial cells lining the control vessels were aligned in the direction of flow and showed a slight, physiological bulging of the perinuclear area. Cell borders were outlined, generally tight, and small periph-
eral endocytic pits and/or microvilli occurred, as have been described in similar studies for healthy vascular endothelia from different organs (1, 12). Following perfusion with CM, the endothelial lining showed a ragged, irregular picture, with sharply protruding intimal folds. Endothelial cells acquired a spindle-like shape and bulged in the vessel lumen, with protrusions affecting the whole cell body. Interendothelial contacts were extended and bridged by cytoplasmic extensions. In some areas, cellular desquamations exposed or denudated the underlying smooth muscle layer. At higher magnifications, a large number of microvilli, blebs, and small protrusions could be seen at the cellular surface. Adrenomedullin alone did not change endothelial morphology. However, it was able to limit the endothelial damage seen with CM alone: taking into account that SEM is not a quantitative method, cellular bulging was reduced, the number of foci of desquamated endothelial cells was lower, and fewer blebs were seen at the cellular surface in vessels perfused with adrenomedullin and CM, indicating a reduction in endothelial cell damage by CM (Fig. 5).

**Effect of CM on endothelial permeability and MLC phosphorylation.** We wanted to know whether the endothelial damage by CM seen in our experiments with SEM goes along with functional impairment of endothelial cells. We investigated the influence of CM on the integrity of the endothelial barrier function in HUVEC monolayers. Transcellular electrical resistance was measured as an indicator of endothelial barrier integrity. Exposure of HUVEC to CM caused an increase in the permeability of HUVEC monolayers, expressed by a long-term decrease in transcellular electrical resistance compared with vehicle (Fig. 6A). Such changes were consistently prevented by adrenomedullin (Fig. 6A).

Phosphorylation of the MLC is necessary for the retraction of endothelial cells and further loss of endothelial barrier function (28). MLC phosphorylation may also take part on initiation of apoptosis in nonmuscle cells (9). Our results show that CM caused a time-dependent increase in the phosphorylation of the MLC in HUVEC in the 60 min following CM application (Fig. 6B). Adrenomedullin prevented the increases in MLC phosphorylation by CM (Fig. 6C).

**DISCUSSION**

An important aspect from our results is that the degree of constriction caused by CM in human isolated perfused DVR is similar to the constriction observed in rat DVR. Most data on the physiology of DVR come from studies using rats, and rarely, mice (19). Whereas most physiological data obtained using animals prove valid when extended to humans, exposure...
to CM is not a physiological condition, and it has been never investigated whether DVR of rats could have a different level of sensitivity to CM than human DVR. Our results confirm that conclusions drawn from the effect from CM in DVR from rats can be extended to humans, providing a direct explanation for the hypoperfusion of the renal medulla, which is a typical feature of CIAKI in humans as well as in several in vivo experimental models (14, 22).

Our results also show that CM, in the same concentration and length of exposure that led to constriction of human and rat DVR, causes structural damage of endothelial cells from rat renal interlobar arteries. The concentration we used is calculated to represent values reached after a coronary angiography (21), and it has been shown that all main types of CM commercially available cause DVR constriction in this concentration, independent of CM properties (25). Our present findings show that DVR contraction caused by CM is not simply a functional derangement but goes along with an evident structural damage of endothelial cells due to CM.

The endothelial cell damage seen in our SEM experiments is severe: it is typical from endothelial cells treated with chemotherapeutic agents that effectively disrupt tumor vascularization (2, 16). There is a common pattern of endothelial cell damage, irrespective of the toxic substance used, that correlates with vascular function in different models of endothelial cell dysfunction (atherosclerosis, endotoxin toxicity, acute liquid nitrogen-induced damage, ischemia-reperfusion injury) (1). This pattern of damage includes endothelial cell lesion and desquamation, “bleb” formation, and the extension of intercellular spaces. SEM studies investigating the in vivo effects of CM on aortic endothelial cells from rats reported similar findings previously (1, 12). We found a similar severe pattern of endothelial damage in our present study, and, importantly, in the same CM concentration and length of perfusion that causes constriction of DVR in our microperfusion studies.

Our results show that CM causes increased MLC phosphorylation and increased permeability of HUVEC monolayers (Fig. 6). Increased MLC phosphorylation is a necessary step for endothelial retraction and consequent increased endothelial permeability (28). Increased permeability of HUVEC monolayers is a functional consequence of endothelial cell retraction and represents a loss of endothelial barrier function (28). Importantly, these events take place in our series in the same time span in which DVR constriction and endothelial damage detected by SEM did (Fig. 6B).

Taken together, our SEM and HUVEC experiments help understand how CM induces constriction (see Figs. 2 and 3) and increased the ANG II response in DVR (see Fig. 4). It is known that the regulation of diameter in DVR is markedly more dependent on endothelial NO production than in other vessels (6). Indeed, total blockade of NO production constricts isolated perfused DVR to a similar level as CM (24). Furthermore, lowering endothelial NO production causes increased DVR response to ANG II (19) to a similar extent as CM does (24). This explains why the renal medulla in CIAKI is more susceptible to hypoperfusion and hypoxia than other parts of the kidney (14). Increased reactivity to ANG II (Fig. 4) and increased local superoxide concentration in DVR perfused with CM (24) may further aggravate medullary hypoperfusion in CIAKI. However, concomitant DVR treatment with CM and N\textsuperscript{G}-nitro-L-arginine methyl ester (l-NAME) causes an even more pronounced constriction of DVR than with either CM or l-NAME alone (6, 24). This suggests that other events than functional inhibition of the NO synthase are involved in CM-induced DVR constriction.

Although the renal medulla in CIAKI is more susceptible to hypoperfusion than other parts of the kidney, reduced blood flow after CM application in vivo can also be observed in the renal cortex (14, 22). Our data show functional impairment and anatomical damage of endothelial cells due to exposition to CM in different models. It is thus reasonable to suppose that endothelial cells of kidney resistance vessels like the afferent arterioles could be similarly compromised. This could explain the reduction in cortical blood flow seen in in vivo models of CIAKI. Additionally, the combination of afferent arteriolar constriction of juxtaglomerular glomeruli and of vasa recta, which originate mainly from these glomeruli, might be a
CONTRAST MEDIA CAUSE ENDOTHELIAL DAMAGE

ACKNOWLEDGMENTS

We tested in our study whether preventing endothelial cell damage by CM could influence the functional disturbances of both endothelial cells and perfused DVR by CM. To blunt the cytotoxicity of CM, we used adrenomedullin, an endogenous peptide shown to have antiapoptotic effects on endothelial cells in conditions associated with impaired endothelial barrier and endothelial damage (e.g., several models of sepsis and septic shock) (26). A reason why we used adrenomedullin instead of other possibly protective measures like antioxidants is that oxidative stress in CIAKI may be a consequence of CM cytotoxicity rather than its cause (23, 29). Adrenomedullin’s antiapoptotic effect might take place in a more upstream step of the tissue damage cascade by CM than antioxidant measures, primarily blunting direct cell damage by CM and secondarily diminishing oxidative stress due to cell damage. Our results show that adrenomedullin prevented the anatomical damage of endothelial cells by CM seen in SEM (Fig. 5) and that, by preventing endothelial cell damage, it also blunted HUVEC dysfunction and MLC phosphorylation (Fig. 6). Importantly, prevention of endothelial damage and dysfunction goes along with a less pronounced CM-induced constriction in both human and rat perfused DVR (Figs. 2 and 3).

A limitation of our study is that, because of the complexity of our methods, we did not repeat our experiments with other types of CM. We used ioxaglate, a modern type of CM, nonionic and dimeric, isoosmolar compared with plasma. Ioxaglate is currently recommended for patients at high risk for CIAKI by both the American College of Radiology and the European Society of Urogenital Radiology (2, 8). However, it has been shown that the four classes of CM currently available (nonionic dimeric and monomeric and ionic dimeric and monomeric), including ioxaglate, cause similar constriction of perfused DVR (25). Because our results suggest that CM cytotoxicity leads to DVR constriction via endothelial damage with consequent endothelial dysfunction, and knowing that all types of CM currently available share a similar level of cytotoxicity (23), it seems reasonable to suppose that repeating our experiments with other types of CM would provide similar results.

Our findings of MLC phosphorylation are similar to the patterns described for actinomycin D-induced apoptosis (9), a biphasic evolution with an initially increased MLC phosphorylation during the first 2 h followed by MLC dephosphorylation. Preventing apoptosis in that model, by blocking the activation of caspase 3, does not affect the phosphorylation pattern of MLC, pointing out that MLC phosphorylation is an upstream event in relation to the caspase 3 activation (9). This similarity of findings supports the notion that adrenomedullin blunts constriction of DVR by means of preventing endothelial damage by CM and not because of a possible direct vasoactive effect. Accordingly, our results show no signs of DVR dilation by adrenomedullin alone (Figs. 2 and 3), and our SEM findings show structural changes on endothelial cells that are indicative of cell damage (Fig. 5).

In conclusion, our results show that the functional effects of CM in human and rat vasa recta are a result of cytotoxic endothelial damage by CM and resulting endothelial cell dysfunction. Our data suggest that the hyperperfusion and hypoxia of the outer renal medulla observed in CIAKI may largely be explained by the cytotoxicity of CM alone, which may have clear clinical importance in the development of prophylactic measures for patients at high risk for CIAKI.

REFERENCES


