CHAPTER

30 Pathophysiology of Nephrotoxic Cell Injury

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Nephrotoxic epithelial renal cell injury is induced by a variety of stimuli, including chemical exposure, which can lead to acute kidney injury (AKI). Chemicals can induce cell injury either directly or indirectly. Examples of chemicals that directly induce renal cell injury include chemotherapeutics, antibiotics, oxidants, metals, and cysteine conjugates. In contrast, indirect chemical insults are initiated at sites removed from renal epithelial cells. Processes that induce indirect renal epithelial cell injury include decreased renal blood flow, renal ischemia, and reperfusion-induced cell injury and death. Inflammatory cells also indirectly and secondarily induce renal epithelial cell injury in a number of models.[1–4]

A secondary effect of nephrotoxicant-induced cell death is the generation of "backleak." After injury, epithelial cells can be released from the basement membrane and adhere to each other via integrins.[5–8] These cellular aggregates form tubular casts that block the flow of filtrate and increase intraluminal pressure, decreasing the single nephron glomerular filtration rate.[5] In addition, the loss of epithelial cells leaves gaps in the basement membrane, allowing tubular filtrate to backleak into the circulation, further decreasing the single nephron glomerular filtration rate. Thus, backleak and the loss of epithelial cells contribute to decreased renal function (Fig. 30.1). Tubular cast formation can be induced by both direct and indirect chemical injury.

After either direct or indirect injury, renal epithelial cells can die or repair and regenerate. The processes involved in renal cell regeneration have been reviewed,[9] and they are categorized into four different mechanisms that include dedifferentiation, proliferation, migration, and redifferentiation—each having defined morphologic characteristics and activation of differential cell signaling pathways. Processes of renal cell regeneration are somewhat similar to epithelial-mesenchymal transition (EMT) in embryonic development and cancer[9] and EMT is hypothesized to mediate fibrosis during chronic kidney injury induced by multiple stimuli.[10,11]

Significant controversy exists concerning the source of postinjury regenerating renal epithelial cells. Although some researchers have suggested that the majority of regenerating epithelial cells are derived from stem cells, present in either the kidney or the bone marrow, most recent studies provide convincing evidence that extratubular cells do not appreciably contribute to epithelial repair and regeneration after AKI.[12] Furthermore, there was no evidence of intratubular "progenitor cells."

The process of renal cell repair begins when cells adjacent to the injured area dedifferentiate, proliferate, and migrate into the denuded areas. Ultimately, the cells differentiate, and tubular structure and function are restored. Of course, such renal cell regeneration is not applicable for all nephrotoxicants. For example, repair of renal proximal tubule cell necrosis induced by the aminoglycoside tobramycin is initiated 4 days after treatment, with cells resuming normal morphology after 14 days.[13] In contrast, a 4-day regimen of the anticancer agent cisplatin also resulted in renal dysfunction with proximal tubular necrosis; however, renal dysfunction persisted.[14]

Several chemicals mediate AKI by inhibiting repair via alteration of differentiation, migration, and proliferation or dedifferentiation. Inhibition of repair with these compounds occurs at concentrations that do not induce overt cell injury. For example, Counts and colleagues studied renal repair and regeneration in vitro in renal proximal tubular cells (RPTCs) using a model that involved mechanical injury[15] and showed that $HgCl_2$; the mycotoxin fumonisin B_1; and the haloalkene cysteine conjugate, S-(1,2)-dichlorovinyl-L-cysteine (DCVC), inhibited the normal proliferative and migratory renal cell responses in the absence of overt cytotoxicity. Thus, mechanisms involved in the pathophysiology of nephrotoxic-induced AKI are not always directly related to cell death.

The goal of this chapter is to review mechanisms by which chemicals produce renal epithelial cell injury and death. Other chapters in this volume, and several excellent reviews, discuss renal cell repair and regeneration, as well as mechanisms of renal cell death and AKI produced by specific chemicals.

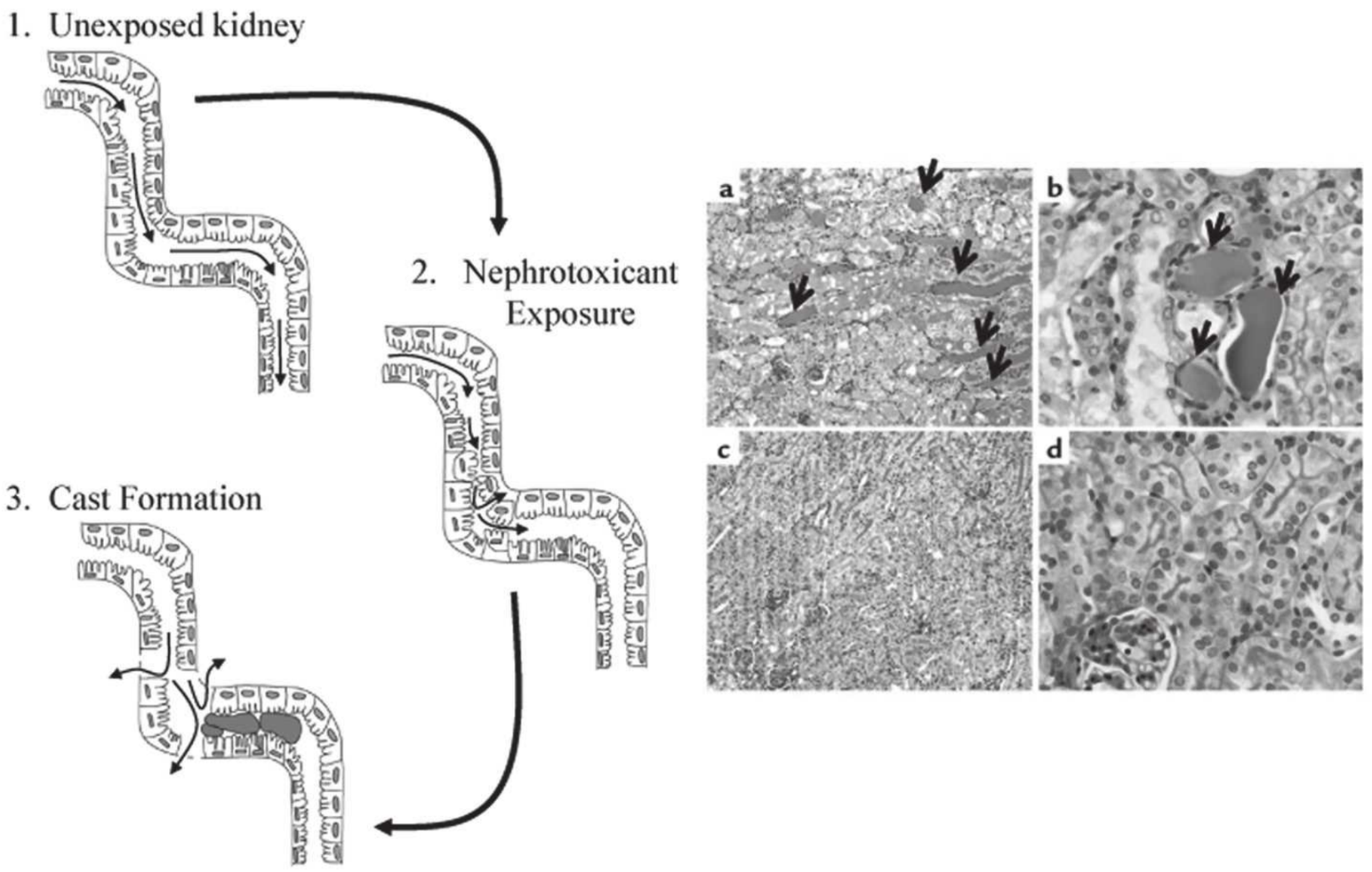

FIGURE 30.1 Cast formation in the nephron. **Left:** *1.* Filtrate flow (as represented by the *small arrows*) through the tubules is constant and unobstructed in unexposed kidneys. *2.* Exposure of the kidney to nephrotoxicants results in cell injury and death, and can induce detachment of the cells from the basement membrane. *3.* Detached cells can adhere to each other and form casts (*pink*), which obstruct filtrate flow and increase intraluminal pressure. This increases permeability in the basement membrane and back leak of filtrate into the interstitium. **Right:** Cisplatin-induced cast formation in wild-type mice (*a* and *b*) and mice mutant for tumor necrosis factor (TNF)-α (*c* and *d*) as determined by PAS staining. The magnification in panels *a* and *c* is ×100, and that in panels *b* and *d* is ×400. Cast formation is visible in panels *a* and *b* as indicated by the *pink/purple* aggregates between the tubules (*arrows*). In contrast, little cast formation can be seen in TNF-α knockout mice (panels *c* and *d*). (Adapted from Ramesh G, Reeves WB. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *J Clin Invest*. 2002;110(6):835–842, with permission.) (See Color Plate.)

SUSCEPTIBILITY OF THE KIDNEY TO INJURY

The kidney is highly susceptible to numerous agents because of several functional properties of this organ. These include: (1) receiving 20% to 25% of the cardiac output, ensuring high levels of toxicant delivery over a period of time; (2) extensive reabsorptive capacity with specialized transporters promoting cellular uptake of the toxicant; (3) concentrating abilities resulting in high concentrations of toxicants in the medullary lumen and interstitium; (4) biotransformation enzymes for the formation of toxic metabolites and reactive intermediates; (5) high metabolic rate and workload of renal cells causing increased sensitivity to toxicants; and (6) sensitivity of the kidney to vasoactive agents.

Nephrotoxicants can target specific nephron segments. The proximal tubule epithelial cell is typically the primary target; however, other parts of the nephron can also be affected by chemicals with a specificity that is concentration-dependent. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) and acetaminophen target the collecting ducts at low concentrations, but also induce damage to the proximal tubules at higher concentrations.[16,17] Furthermore, different segments of the proximal tubule (S_1, S_2, and S_3) are targets for different nephrotoxicants. For example, aminoglycoside antibiotics, chromate, cadmium chloride, and the mycotoxin citrinin primarily target the S_1 and S_2 segments, whereas cyclosporine, $HgCl_2$, uranyl nitrate, cisplatin, bromobenzene, and cysteine conjugates of halogenated hydrocarbons target the S_3 segment.[17,18] Interferon-α, gold, and penicillamine can target cells in the glomeruli, whereas angiotensin-converting enzyme (ACE) inhibitors can target cells in the renal vasculature.[17] Clostridium perfringens B and D and trichloroethylene can target distal tubules, and radiocontrast media, triethanolamine, amphotericin, and nystatin tend to target the loop of Henle. Studies suggesting that trichloroethylene targets the distal tubules are derived from in vitro models only at high doses,[19] whereas agents that target the loop of Henle also can also affect the proximal tubules.[16]

These segmental differences in chemical sensitivity may be attributed to: (1) differences in toxicant delivery to a given segment, (2) differences in transport and uptake among segments, and (3) differences in biotransformation enzymes among segments. Once again, concentration may be a deciding factor.

NEPHROTOXICANT TRANSPORT

Many nephrotoxicants require transport into epithelial cells to induce injury, either by passive diffusion or by active or facilitated transport. Increased accumulation typically correlates to increased injury and decreased cellular function, which leads to AKI.

Several transporters are expressed in the kidney for the purpose of ensuring renal cell homeostatic functions, such as reabsorption and secretion; however, these proteins can also transport nephrotoxicants.[20] Major transporters found in renal cells include, but are not limited to, the organic cation transporters (OCTs),[21,22] organic anion transporters (OATs),[23,24] the organic anion transporting polypeptide (OATP) family,[25,26] and transporters involved in multidrug resistance (MDR) such as P-glycoprotein.[27]

Organic Anion Transporters and Organic Cation Transporters

OATs and OCTs are members of the solute carrier superfamily group 22A (SLC22A: human nomenclature) as assigned by the human genome organization (HUGO) nomenclature committee.[28] Several members in the SLC22A family have homologs with human, mouse, rat, and rabbit kidneys, in addition to having overlapping substrate specificity with each other and with other transporter families. Other than physiologic substrates,[28,29] these proteins also transport drugs, natural products, industrial chemicals, and pollutants.[20,23,30–32]

The OCT family of proteins typically transports small, hydrophobic, positively charged chemicals. Major isoforms include OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), OCT6 (SLC33A16), OCTN1 (SLC22A4), OCTN2 (SLC22A5), and OCTN3 (SLC22A21).[28] Nephrotoxicants transported by OCTs include the chemotherapeutic cisplatin, which is a substrate for OCT2 in humans and rats.[28,33–35] OCT2 may also mediate proximal tubule cell death induced by paraquat, a commonly used herbicide known to induce AKI. OCT1 mediates the toxicity of platinum compounds including cisplatin, oxaliplatin, and carboplatin in Madin-Darby canine kidney (MDCK) cells,[22] and both OCT1 and OCT2 mediate the transport of 1-methyl-4-phenyl-pyridinium, disopyramide, and chlorpheniramine into renal cells.[36] OCT2 was also demonstrated to mediate the nephrotoxicity of ifosfamide both in vitro and in vivo.[37]

OCT1 and OTC2 were recently reported to mediate the transport and toxicity of several antiretroviral drugs used to treat human immunodeficiency virus (HIV) in human embryonic kidney 293 (HEK293) cells.[38] Furthermore, OCT1, OCT2, and OCT3 were reported to mediate the transport of tyrosine kinase inhibitors (TKIs), such as imatinib, in HEK293 cells.[39] More in vivo studies are needed to fully determine the role of OCTs in nephrotoxicity induced by both antiretroviral drugs and TKI.

The OAT families of proteins typically transport small organic anions into cells. Major isoforms include OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), OAT4 (SLC22A11), OAT5 (SLC22A19), OAT6 (SLC22A20), OAT7 (SLC22A9), OAT8 (SLC22A25), OAT10 (SLC22A13), and URAT1 (SLC22A12).[28] Nephrotoxicants reported to be transported by OATs include the mycotoxin ochratoxin A, which is transported into renal tubular cells by OAT1, OAT 3, and OAT 5.[30,31,40] Ochratoxin A transport into renal cells is inhibited by probenecid,[41] an inhibitor of most OAT proteins. Recent studies also suggest that aristolochic acid, an inducer of both acute renal failure (ARF) and cancer, is transported into HEK293 cells via OAT1, OAT3, and OAT4.[42] Other nephrotoxicants whose toxicity is mediated by OCTs include methotrexate (OAT1, OAT2, and OAT3), uremic toxins such as hippuric acid and indoleacetic acid (OAT1 and OAT3), and NSAIDs (OAT1, OAT2, OAT3, and OAT4).[28]

The ability of Hg^{+2} and its cysteine conjugates to induce cell death in vivo and in MDCK cells is altered by inhibitors or substrates of OAT proteins, suggesting that the nephrotoxicity of this environmental contaminant is regulated by these transporters.[23,24] This hypothesis was confirmed by studies demonstrating that overexpression of human OAT1 in MDCK cells altered the nephrotoxicity of these compounds.[43] Other compounds for which toxicity is suggested to be mediated by OATs include the trichloroethylene metabolite DCVC, some chlorinated phenoxyacetate-based herbicides, antiviral drugs, and β-lactam-based antibiotics.[28,44]

Organic Anion Transporting Polypeptides

OATPs are members of the solute carrier O family (SLCO, formerly referred to as the SLC21 family[45]). Endogenous substrates for these proteins include bile acids, hormones, and eicosanoids.[26] Currently, genes for 11 human OATPs, 15 rat OATPs, and 15 mice have been identified.[25,45] Not all of these are expressed in kidney. Furthermore, several OATPs expressed in humans are not expressed in rodents, such as OATP1A2, OATP1B1, and OATP1B3.[45] Additionally, there are several rodent OATPs that do not have a human homolog. Such differences should be taken into account when assessing the role of OATPs in nephrotoxicity.

OATPs demonstrated to be expressed in human kidneys include OATP1A2, OATP2A1, OATP2B1, OATP3A1, OATP4A1, and OATP4C1.[45] Rat and mouse kidneys are reported to express Oatp1a1, Oatp1a6, Oatp2a1, Oatp2b1, Oatp3a1, Oatp4a1, and 4c1[29,46,47] (the lowercase denotes rodent genes). Oatp1a3 is reported to be a rat specific isoform.[29] The expression of several mouse kidney oatp, such as Oatp1a1, Oatp3a1, and Oatp4c1, are reported to differ depending on gender,[47] but it is not known if this trend is replicated in human kidneys.

Several studies demonstrate that OATPs mediate the transport of nephrotoxicants. For example, ochratoxin A (OATP1A2, Oatp1a1), methotrexate (OATP1B1, Oatp1a3), and digoxin (OATP4C1) are known substrates.[29] Studies also suggest that the expressions of OATPs are altered by nephrotoxicants. For example, treatment of mice with nephrotoxic doses of cisplatin for 4 days increases the expression of Oatp2a1 and Oatp2b1 mRNA.[48] Future studies are needed to fully understand the role of OATPs in the pathophysiology of nephrotoxic renal cell injury.

Maillard Reaction Products

Maillard reaction products (MRPs) are members of the ATP-binding cassette super family (ABCC).[29] Substrates for these proteins include hydrophobic molecules, such as

the chemotherapeutics vincristine and doxorubicin. At least six different MRP genes have been identified (designated MRP1-6, and MDR1) and all are expressed in the kidney.[49]

P-glycoprotein is perhaps the most well known MRP. Localized to the apical membrane of proximal tubule cells, MRP is believed to mediate the efflux of organic anions from the kidney. Known substrates for P-glycoproteins include methotrexate and cisplatin.[49,50] The nephrotoxicity of cisplatin is altered by overexpression of P-glcyoprotein.[50] P-glycoprotein may also mediate the nephrotoxicity of diallyl disulfide and S-allyl-cysteine, $HgCl_2$, calcineurin, and cyclosporine.[51–54]

CELL DEATH

The mechanisms by which chemicals induce epithelial cell death are as varied as the chemicals themselves; nevertheless, some commonalities do exist. For example, many chemicals require transport into cells to induce death. Furthermore, regardless, of how nephrotoxicants gain entry into cells, cell death is thought to occur through one of three mechanisms: apoptosis (type I cell death), autophagy (type II cell death), or necrosis (type III cell death).[55,56] Other commonalities that exist in injured and dying cells include activation of proteases, increases in cytosolic Ca^{2+}, changes in mitochondrial function and morphology, and changes in nuclear morphology and chromatin/DNA structure.

Necrosis, apoptosis, and autophagy can be identified by assessing differences in cellular and nuclear morphology. In fact, some suggest that morphology is standard for delineating mechanisms of cell death; however, it is becoming evident that morphology alone is not the best way to identify the mechanisms of cell death.[55] This reflects the fact that the mechanism of death induced by a given chemical is dependent on multiple factors such as the cell type being injured, the time of compound exposure, and the compound dose. Such multiple dependencies are typified by arsenic, which can induce all three types of cell death in a given cell.[55] Additionally, the cell death mechanism may change midway through any series of postinsult events (Fig. 30.2). Thus, a chemical may initiate autophagy, but this pathway may switch to apoptosis as the dose and time of exposure increases, or if p53 is released to the cytosol. Furthermore, apoptosis may switch to necrosis as ATP decreases or if cytosolic Ca^{2+} increases high enough to activate select proteases or induce membrane rupture. Thus, a particular mechanism of cell death cannot always be directly linked to a specific morphology and, possibly, multiple cell death pathways may be activated in a single cell.[55]

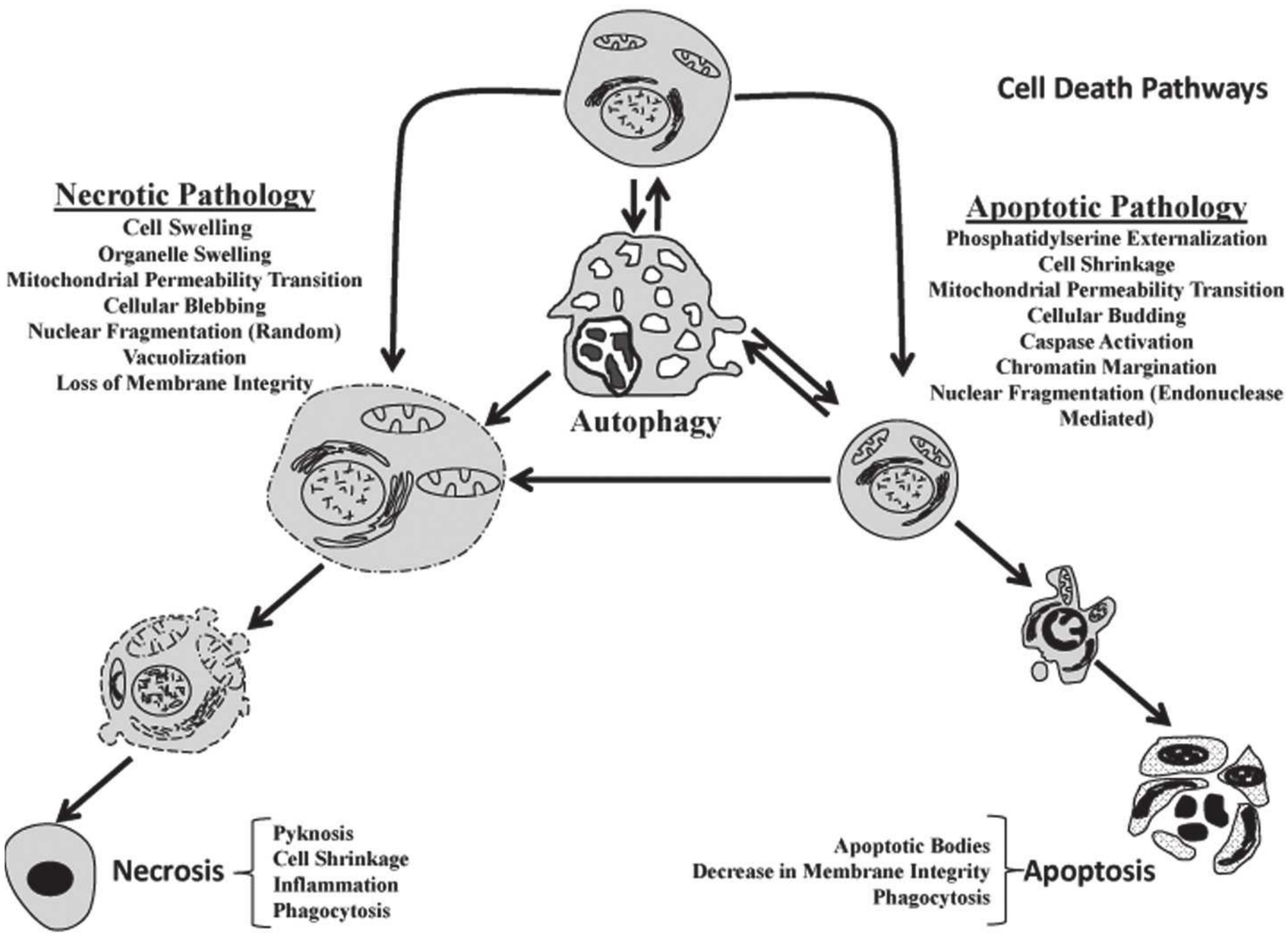

FIGURE 30.2 Schematic comparing the pathologic and morphologic features of necrosis, apoptosis, and autophagy. At the top middle, a normal cell is shown; below the normal cell is an autophagic cell demonstrating mass vacuolization; and autophagosomes (not shown). **Left:** Cell and organelle swelling, followed by vacuolization, blebbing, and increased membrane permeability (lysis) and finally necrotic changes (i.e., coagulation, shrinkage, and karyolysis). **Right:** Cell shrinkage followed by budding and karyorrhexis and finally necrotic changes (i.e., breakup into cluster of apoptotic bodies). The pathologies necrosis and apoptosis are listed. *Double arrows* represent the hypothesis that select pathways can switch. For example, autophagy can lead to cell survival and also progress to apoptosis.

Necrosis

Necrosis affects masses of contiguous cells and is characterized by swelling of organelles and increases in cell volume, after which the cell membrane becomes more permeable and ruptures with the release of cellular contents, followed by inflammation. Historically, necrosis has been used to describe drastic tissue changes occurring after cell death. These include karyorrhexis, karyolysis, pyknosis, condensation of the cytoplasm, and intense eosinophilia.

Morphologic markers for cellular necrosis include a loss of membrane and organelle integrity, cell swelling, and swelling of the endoplasmic reticulum (ER) and mitochondria (Figs. 30.2 and 30.3). Nuclear morphology in necrotic cells is usually typified by pyknosis (nuclear condensation without fragmentation); however, DNA fragmentation can occur in some cases, especially when agents that target the DNA are used. This can give rise to chromatin margination. Cellular blebs also form, but unlike apoptosis, necrotic cell blebs do not typically contain organelles. Necrosis usually induces inflammation, often with the infiltration of neutrophils and inflammatory cells in vivo (Fig. 30.3).

Biochemical markers for necrosis include a drastic and rapid loss of ATP (greater than 70%–80%), rapid and sustained increases in cytosolic Ca^{2+}, leakage of intracellular constituents such as lactate dehydrogenase, DNA fragmentation, and protease activation. A hallmark of necrosis is that it does not require ATP, separating it from both apoptosis and autophagy. DNA fragmentation also occurs in apoptosis, but DNA fragmentation that occurs during necrosis is random and not usually inhibited by caspases. Proteases activated during necrosis include select types of calpains, which are usually activated due to high concentrations of cytosolic Ca^{2+}. Evidence exists that calpains can also be activated during apoptosis (see below); thus, calpain activation alone is not a valid marker for necrosis.

Apoptosis

Apoptosis usually affects scattered individual cells and, morphologically, the cell shrinks whereas organelle integrity is initially retained (Figs. 30.2 and 30.3). Next, chromatin become pyknotic and marginate against the nuclear membrane and, ultimately, the cell shrinks to a dense, round mass

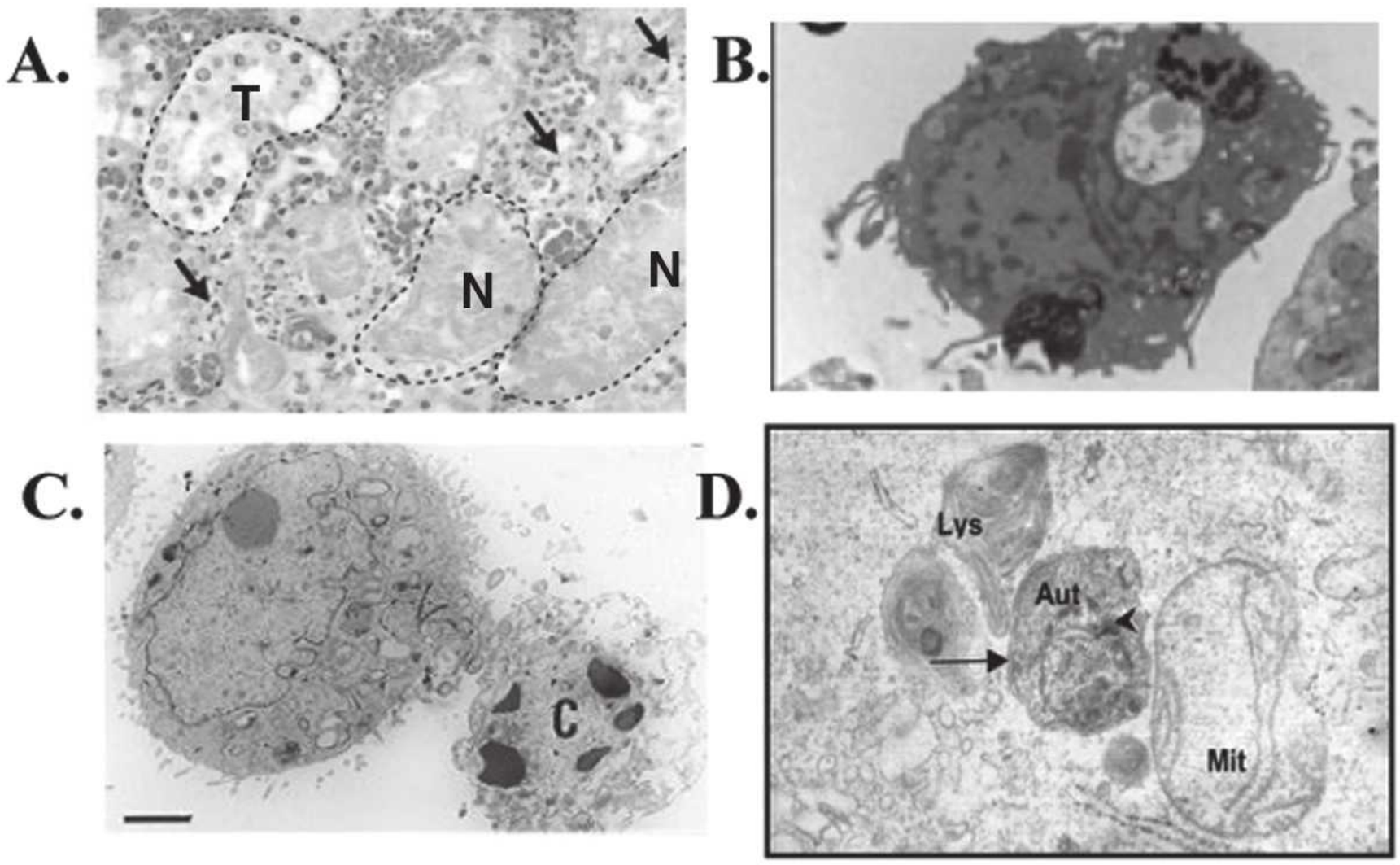

FIGURE 30.3 Comparison of the morphologic features of necrosis, apoptosis, and autophagy in tissues and cells. **A:** Hematoxylin and eosin staining of human kidney tissue after arterial embolization for treatment of renal cancer demonstrating intact tubules (*T*) and necrotic tubules (*N*). Arrows represent neutrophils and mononuclear inflammatory cells. (Modified from Hotchkiss RS, Strasser A, McDunn JE, et al. Cell death. *N Engl J Med.* 2009;361:1570, with permission.) **B:** Transmission electron microscopy (TEM) of necrotic human embryonic stem cells showing loss of membrane integrity without chromatin margination, cytosolic vacuolization, and spilling out of intracellular constituents. (Modified from Heng BC, Vinoth KJ, Lu K, et al. Prolonged exposure of human embryonic stem cells to heat shock induces necrotic cell death. *Biocell.* 2007;31(3):405, with permission.) **C:** TEM human HEK293 cells undergoing lysosomal mediated apoptosis. The cell on the right is relatively healthy whereas the cell on the left has shrunken and exhibits chromatin and nuclear condensation and is beginning to lose the membrane integrity (late apoptosis). (Modified from Heng BC, Vinoth KJ, Lu K, et al. Prolonged exposure of human embryonic stem cells to heat shock induces necrotic cell death. *Biocell.* 2007;31(3):405, with permission.) **D:** TEM of autophagic primary cultures of normal human renal cells exposed to cyclosporine demonstrating formation of the autophagosomes (*Aut*) with a double membrane (*long arrow*), next to a lysosomes (*Lys*) and a mitochondria (*Mit*). The *arrowhead* represents a cytoplasmic organelle. (Pallet N, Bouvier N, Legendre C, et al. Autophagy protects renal tubular cells against cyclosporine toxicity. *Autophagy.* 2008;4(6):72, with permission.)

(apoptotic body) or forms pseudopodia (i.e., buds) containing nuclear fragments and/or organelles that break off into small fragments (apoptotic bodies). In either case, adjacent cells or macrophages phagocytize the apoptotic bodies, and inflammation typically does not occur.

A key difference between necrosis and apoptosis is the activation of caspases in the latter. Caspases are cysteinyl aspartate-specific proteases that belong to an 18-member family.[57–59] Caspases can be divided into three groups based on structural differences and substrate preferences: initiator caspases (caspase -2, -8, -9, -10, and possibly -12), executioner caspases (caspases -3, -6, and -7), and cytokine processors (caspases -1, -4, -5, -13, and -14). Caspases-15 to 18 have been identified in numerous mammals, but not in humans, with the exception of caspase 16.[59]

Initiator caspases are activated by numerous processes including receptor-directed mechanisms and chemical exposure. They mediate chemical-induced apoptosis in numerous cell types, including proximal tubular cells,[60–62] glomerular cells,[63] medullary cells,[64,65] and cells present in the collecting ducts.[66–68] Activation of initiator caspases results in the activation of executioner caspases, which leads to several of the biochemical characteristics of apoptosis. Initiator caspases can also be substrates for executioner caspases.[69]

Caspase-8 is an initiator caspase that plays an integral role in receptor-mediated apoptosis.[69–71] It is activated by membrane receptors such as Fas-ligand and tumor necrosis factor (TNF)-α receptors[71,72] and, in turn, cleaves the Bcl-2 family protein Bid to form tBid.[70] tBid acts on mitochondria to cause the release of pro-apoptotic proteins and results in the activation of caspase-9 and caspase-3. In contrast, caspase-8 can directly activate caspase-9 or caspase-3, independently of the mitochondria (Fig. 30.4).

Caspase-8 can be activated by nephrotoxicants independent of receptor-mediated mechanisms. For example, cisplatin and etoposide activate caspases-8, -9, and -3 in LLC-PK1 cells in the absence of receptor-stimulation.[73] In contrast, cisplatin and cyclosporine activate caspase-3 in the absence of caspase-8 in mouse and rabbit RPTC.[74–76] Thus, the role of caspase-8 in chemical-induced renal cell apoptosis is variable.

Executioner caspases cleave numerous substrates that ultimately result in the morphologic features of apoptosis. Perhaps the most important substrates are proteins that control DNA degradation (DNAase). Caspases are known to mediate the activation of the nuclease DNA fragmentation factor (DFF). DFF is composed of two subunits: a 40-kDa DNAase subunit (CAD/DFF40) and a 45-kDa inhibitor of caspase-activated deoxyribonuclease (ICAD/DFF45).[69] Caspase-3 cleaves ICAD/DFF45 during apoptosis, which results in the release and activation of CAD/DFF40. Active CAD/DFF40 results in double-stranded DNA breaks in chromosomes, giving rise to the characteristic nonrandom DNA-ladderlike pattern seen with apoptosis on agarose gels.[69]

Caspases can also mediate DNA degradation by cleaving poly(ADP-ribose) polymerase (PARP). PARP is involved in DNA repair and maintenance of stability, and regulates DFF40 activity.[69] Caspases-3 and -7 can cleave and inactivate PARP.[77] PARP cleavage is used as a marker for apoptosis in renal cells, including apoptosis induced by antimycin A and DCVC.[78,79] Cleavage of DNA repair enzymes (such as PARP) by caspases is thought to prevent cells from making a futile repair attempt.

Caspases have numerous other substrates other than DNAases. Initiator caspase substrates include other caspases, the pro-apoptotic protein Bid, α-tubulin, and vinculin.[58] Cytokine caspase substrates include inflammatory mediators such as such Il-18, Pro-IL-1B, and Il-17, whereas executioner caspase substrates include protein kinase C (PKC), focal adhesion kinases (FAK), and the cell cycle regulator p21.[58] Cleavage of these proteins is believed to inhibit futile repair attempts, facilitate apoptosis signaling cascades, and allow for cytoskeleton reorganization and packaging of cell constituents into apoptotic bodies.[80]

Caspase-3, perhaps the best studied executioner caspase, can also cleave receptors, such as type 1 inositol(1,4,5) P4 receptor, Ca^{2+}-ATPase, the Na^{+}/Ca^{+} exchanger, and the Na^{+}/K^{+}-ATPase pump.[55] The Na^{+}/K^{+} ATPase may also be cleaved by initiator caspases, such as caspase-8 and -9.[81] Cleavage of these receptors is believed to alter ion homeostasis and facilitate decreases in intracellular K^{+}, which further promotes caspase activation. Cleavage of these receptors also leads to cell size alterations, such as cell shrinkage, early during apoptosis after cleavage of Na^{+}/K^{+} ATPase, or cell rupture due to swelling after Ca^{2+}-ATPase inactivation during late-stage apoptosis/secondary necrosis.

Role of Mitochondria in Apoptosis

The role of mitochondria in cell death cannot be understated, especially for apoptosis. Mitochondria regulate apoptosis by at least two major processes: maintenance of ATP production and release of pro-apoptotic proteins, such as cytochrome c, Bcl-2 family proteins, and DNAases. In addition, mitochondria regulate apoptosis by participating in Ca^{2+} signaling cascades and mediating protease activation.[55]

ATP is considered to be a requirement for both the initiation and execution of apoptosis.[55] It is required for formation of an apoptosome protein complex (see later), which facilitates the activation of caspase-9. It may also be required for transport of pro-apoptotic proteins into the nucleus.[55] ATP may also represent an important switch point between apoptosis or necrosis; depleting ATP below 30% transforms apoptotic liver cell death to necrotic death patterns.[82] In addition, ATP is needed to maintain Na^{+}/K^{+} ATPase pumps on the plasma membrane, and pump inactivation will eventually lead to cellular swelling, pathologic increases in intracellular Ca^{2+}, and cellular lysis, which is typical of necrosis.

Cytochrome c is a heme protein bound to the inner mitochondrial membrane, transferring electrons between complexes III and IV of the electron transport chain. Release of cytochrome c from mitochondria activates the intrinsic pathway of apoptosis. Cytosolic cytochrome c will bind

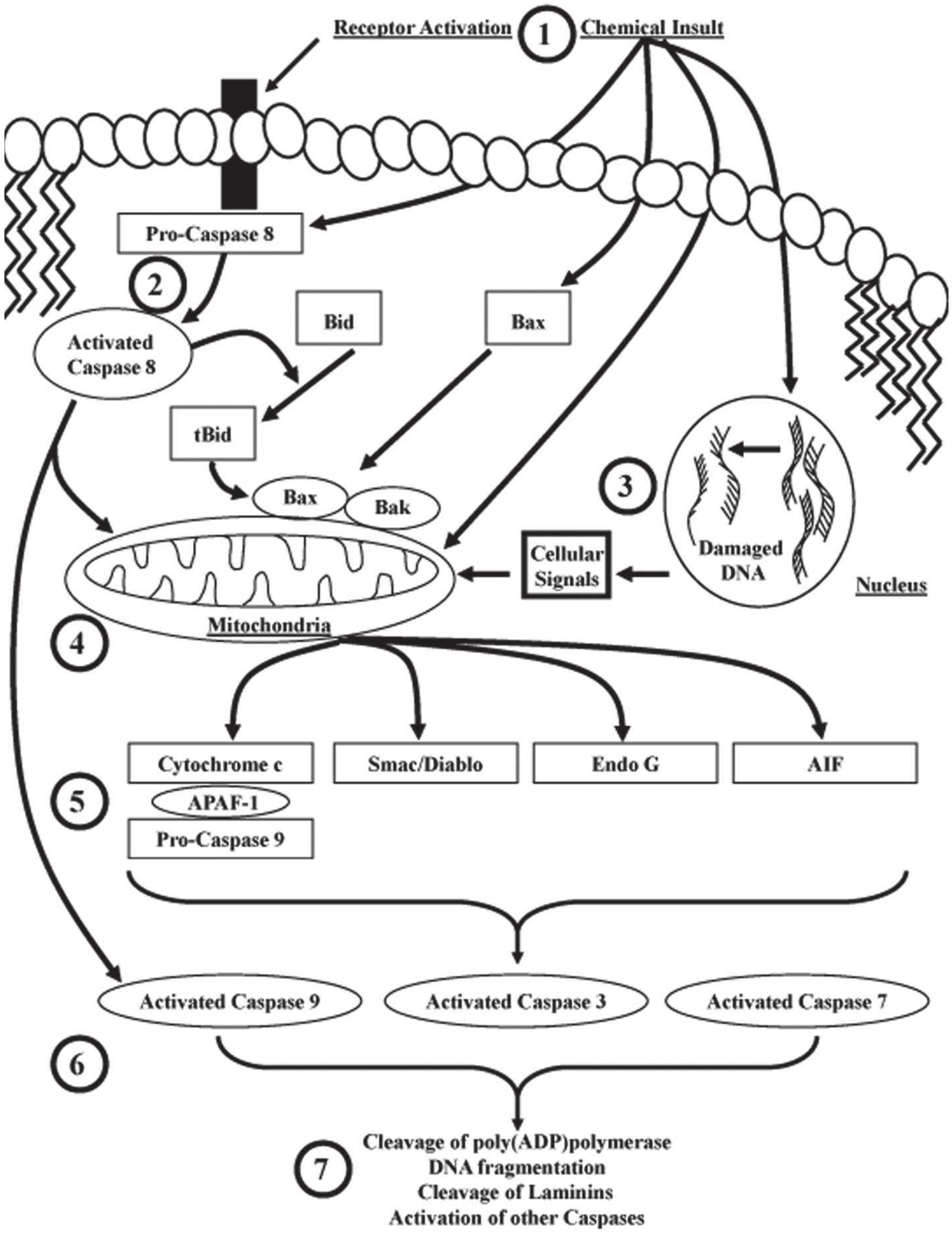

FIGURE 30.4 Cell signaling cascades involved in the activation of caspases and apoptosis. **1:** Receptor-mediated death signals or chemicals can initiate apoptosis through multiple mechanisms. **2:** Pro-caspase 8 is activated by receptor-mediated signals at the cellular membrane or directly by chemicals. Once activated, caspase-8 cleaves Bid to t-Bid, which interacts with Bax/Bak to induce mitochondrial-mediated apoptosis or directly activates caspase-9 and other caspases. **3:** Some chemicals cause DNA damage that signals the release of pro-apoptotic proteins from the mitochondria. **4:** Receptor-mediated signals, direct chemical injury, or signals resulting from DNA damage can all cause cytochrome c, Smac/Diablo, Endo G, and AIF release from the mitochondria. **5:** Released cytochrome c forms a complex with APAF-1 and pro-caspase 9, resulting in caspase-9 activation. **6:** Activated caspase-9 cleaves and activates pro-caspase-3 and -7, which can also be activated by caspase-8 independently of cytochrome c. **7:** Activated caspases (e.g., 3 and 7), AIF, and Endo G cause the classical markers of apoptosis such as cleavage and activation of poly(ADP)polymerase, inactivation of inhibitors of DNases leading to DNA fragmentation, cleaved laminins, and the activation of other caspases.

to apoptotic protease activating factor 1 (APAF-1), which promotes the binding and proteolytic cleavage of pro-caspase-9 to caspase-9 (the apoptosome),[83] and then activated caspase-9 cleaves and activates executioner caspases (i.e., caspases-3, -6, and -7) (Fig. 30.4). Nephrotoxicants known to induce cytochrome c release in correlation with apoptosis include cisplatin and DCVC.[71,84] Cytochrome c release from the mitochondria is associated with a decrease in the mitochondrial inner membrane potential and the accumulation of several pro-apoptotic proteins such as Bad, Bax, and Bax at the mitochondria (Fig. 30.4). Other pro-apoptotic proteins released from the mitochondria include apoptosis-inducing factor (AIF), Smac/Diablo, Omi, and Endo G (Fig. 30.4).[70,71,85–92]

Bad, Bak, Bax, and Bid belong to the Bcl-2 family of pro-apoptotic proteins, which are characterized by specific regions of homology, termed Bcl-2 homology domains.[93] Under nonstressed conditions, these proteins exist bound to proteins in the mitochondria and cytosol.[70] After toxicant exposure, Bax, Bid, or Bak can dissociate and translocate to the mitochondria which initiates the formation of a pore complex that causes membrane rupture[55] and subsequent loss of mitochondrial membrane potential, facilitating the release of cytochrome c, Endo G, Smac/Diablo, Omi, and AIF (Fig. 30.4).[68,70,74] Bid mediates apoptosis induced by hypoxia and ATP depletion in cultures of rat RPTC[94]; Bax mediates proximal tubular apoptosis in mice treated with cisplatin in vivo[65]; and Bak is elevated during apoptosis in primary bovine glomerular endothelial cells induced by TNF-α or lipopolysaccharide (LPS[95]) or during ischemia-reperfusion-induced renal cell apoptosis in mice.[96]

In contrast to Bax, Bid, and Bak, Bcl-2 is an anti-apoptotic protein.[60] Increased Bcl-2 prior to toxicant exposure protected numerous cells, including renal cells,[96] from toxicant-induced apoptosis.[96] The protective effect of Bcl-2 may be the result of its ability to bind Bax, Bid, and Bak, preventing them from inducing mitochondrial pore formation, altering mitochondrial membrane permeability, initiating the release of mitochondrial pro-apoptotic proteins, and activating caspases.[97] Overexpression of Bcl-2 protected against ATP-depletion-induced apoptosis in cultures of rat RPTC,[94] and upregulation of Bcl-2 protected kidney epithelial cells both in vitro and in vivo against apoptosis induced by hypoxia, azide, cisplatin, and staurosporine.[98]

AIF is released from mitochondria in response to decreases in the mitochondrial membrane potential induced by ATP depletion[86,99]; ischemia-reperfusion; anti-fas antibodies[100]; or exposure to high concentrations of Ca^{2+},[101] t-butyl hydroperoxide,[101] or atractyloside.[101] Cellular pathologies associated with AIF release are similar to those seen with caspases (chromatin condensation and oligonucleosomal DNA fragmentation).[100] Recent studies suggest that increases in cytosolic Ca^{2+} and calpain activation also facilitate the release of AIF from mitochondria[102]; and studies in LLC-PK1 cells support this hypothesis.[103]

AIF is a protease with properties similar to caspases, including being inhibited by N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), a commonly used broad spectrum caspase inhibitor.[86] Thus, the decrease in renal cell death observed in the presence of Z-VAD-fmk may be a result of AIF or caspase inhibition. AIF can induce DNA fragmentation independently of caspases.[69] AIF is released in opossum kidney (OK) cells after ATP depletion-induced by sodium cyanide and 2-deoxy-D-glucose.[87,99] AIF is also activated in HEK293 cells after exposure to cadmium,[104] in LLC-PK1 cells after exposure to cisplatin,[105] and in OK cells after exposure to the peroxisome proliferator-activated receptor agonist ciglitazone.[106]

Smac/Diablo is a pro-apoptotic protein released from the mitochondria to the cytosol during apoptosis. It blocks anti-apoptotic activity of inhibitors of apoptosis proteins (IAP), which increase apoptosis.[89] The ability of Smac/Diablo to promote apoptosis is not exclusively a result of its ability to bind IAP.[107] Smac/Diablo functions at the same level of executioner caspases, but downstream of the Bcl-2 family of proteins.[90]

Smac/Diablo is expressed in the mouse kidney and in several renal cell models.[108] It mediates apoptosis in vivo in mice after treatment with high concentrations of folic acid or after exposure of cultures of renal epithelial cells to TNF-α.[89] Increased expression of Smac/Diablo potentiates TNF-α- and etoposide-induced apoptosis in HEK293 cells[107]; however, similar to several other pro-apoptotic proteins, expression of Smac/Diablo is not essential for apoptosis in kidney cells. For example, acetaminophen-induced renal cell apoptosis proceeds in a caspase-dependent manner in the absence of Smac/Diablo activity.[109]

Omi is a mammalian serine protease homologous to bacterial HtrA endoprotease.[110] Omi localizes to the mitochondria and is expressed ubiquitously in a number of cell types including RPTC.[91] Omi is released from the mitochondria after exposure to apoptotic stimuli and binds to, and cleaves, IAP.[91] Omi-directed degradation of IAP facilitates caspase activation and the subsequent biochemical and morphologic features of apoptosis. In addition, Omi can translocate to the nucleus and activate the transcription factor p73, which induces pro-apoptotic proteins such as bax.[69] Omi participates in both caspase-independent and caspase-dependent cell death,[69,111] an event that has been proven using either siRNA against Omi, or a synthetic inhibitor, called ucf-101, in in vitro and in vivo models, including primary cultures of mouse RPTC.[91,111] More work is needed to determine if Omi can mediate cell death induced by other nephrotoxicants.

Autophagy

Autophagy is essentially "a cell eating itself."[112,113] This process was originally thought to be a cell survival pathway activated to produce energy during times of metabolic stress, such as starvation.[113] Some cells undergoing autophagy can recover; however, ample evidence exists that autophagy itself leads to cell death, specifically referred to as type II cell death.[55,112,113]

Significant evidence shows that autophagy mediates renal cell death.[56,114,115] The role of autophagy in renal cell death differs depending on the experimental conditions.[56] Further, it is difficult to determine if autophagic cells are a result of a cell death mechanism, or a failure in repair or survival mechanism.[56] Complicating this issue is that induction of autophagy is cell- and toxicant-dependent. Nevertheless, clear correlations exist between nephrotoxicity and autophagy. Autophagic cells are present in vivo in rat renal cells after ischemia-reperfusion and after treatment of mice with tunicamycin, a stimulant of ER Ca^{2+} release.[56] In vitro, autophagy was identified in HK-2 cells after exposure to H_2O_2, in RPTC cultures after exposure to cisplatin, and in primary cultures of human renal cells treated with cyclosporine A.[56,116]

Morphologic markers for autophagy include the presence of autophagic vacuolization of the cytoplasm and the autophagosome, which forms near the lysosome and can contain cytosolic organelles (Figs. 30.2 and 30.3).[55] This occurs in the absence of chromatin condensation. Double-membrane vesicles, autophagosomes, are formed and fuse with lysosomes to facilitate protein degradation[112] and other morphologic changes that are best identified using transmission electron microscopy.

Biochemical markers of autophagy include expression of microtubule-associated protein-1 light chain 3 (LC3), and degradation of the cell signaling adaptor p62.[112] LC3 is only considered an autophagic cell marker when it is cleaved to a lower molecular weight protein called LC3II. Cleavage allows LC3 to bind to phosphatidylethanolamine, which facilitates the formation of autophagosomes. Autophagosome formation is also facilitated by two kinases: autophagy-specific phosphatidylinositol 3-kinase (PI3K) Vps34 (also called human class III PI3K) and target of rapamycin (TOR) kinase.[56,112]

Beclin-1 is another protein whose expression is critical for autophagy. Beclin-1 facilitates formation of autophagosomes by regulating Vps34 (human class III PI3K[56]). It contains a BH-3 only domain, and is inhibited by other BH-3 only domain containing proteins called Bcl-2 and Bcl-X_L.[55] Proteins containing BH3-only domains are typically pro-apoptotic; however, beclin-1 does not induce apoptosis. In fact, beclin-1 is cleaved by caspases. Cleavage of beclin-1 by caspases is believed to be an important switch point used by cells to inhibit autophagy and stimulate apoptosis.[55]

p53, another regulator of autophagy, is a tumor suppressor protein found in the cytosol of living cells in an inactivated state and bound to the co-repressor Mdm2 (see later). The release of p53 from Mdm2 is stimulated by ionization radiation, DNA damage, oxidative stress, and several other death-inducing stimuli. Released p53 can translocate to the nucleus and induce apoptosis, cell cycle alterations, and the transcription of several proteins, including those that mediate autophagy.[55] Interestingly, cytosolic p53 (unbound to Mdm2) appears to inhibit autophagy in nonrenal cells.[117] It is not known if p53 can regulate autophagy in renal cells using similar mechanisms.

INITIATORS OF CELLULAR INJURY

Nephrotoxicants initiate renal cell injury by a variety of mechanisms. Some initiate toxicity directly because of their reactivity with selected cellular macromolecules, such as observed with the antifungal drug amphotericin B, which increases the permeability of the plasma membrane to cations,[118] the mycotoxin fumonisin B_1 that inhibits sphinganine (sphingosine) N-acyltransferase,[119] and aminoglycosides that bind initially to cellular anionic phospholipids.[120] Other nephrotoxicants initiate toxicity following biotransformation to a reactive intermediate or a stable metabolite, and nephrotoxicants can initiate toxicity indirectly through the production of reactive oxygen species.

Role of Biotransformation

Renal xenobiotic metabolism contributes significantly to whole-body metabolism and/or renal toxicity of numerous chemicals because of the role of the kidney as a primary route of xenobiotic excretion. Some chemicals require metabolism or biotransformation to a toxic reactive intermediate for cellular injury to occur (Fig. 30.5). Then the reactive intermediate binds covalently to critical cellular macromolecules, which are thought to interfere with the normal functioning of the macromolecules and thereby initiate cellular injury. Often, these reactive intermediates or "alkylating" agents are electrophiles that bind to cellular nucleophiles. The renal xenobiotic-metabolizing enzymes found in experimental animals and humans have been reviewed by Lock[121] and are summarized in Table 30.1. These include cytochrome P-450, flavin containing monooxygenase (FMO), and glutathione S-transferase (GST).

Cytochrome P-450

The kidney contains many of the xenobiotic-metabolizing enzymes found in the liver; however, in general, their concentration within the kidney is lower. For example, renal cytochrome P-450 ranges between 0.1 and 0.2 nmol per mg microsomal protein across a variety of species, which represents approximately 10% of hepatic cytochrome P-450.[121] The distribution of cytochrome P-450 also varies along the nephron, with the highest levels typically found in the S_2 segment, followed by the S_3 and S_1 segments, with the other tubular segments having less than 10% of that of the S_1 segment.[121]

The renal cytochrome P-450 system is active against a variety of endogenous and exogenous compounds, and numerous cytochrome P-450 isoforms have been identified in renal tissue. For example, cytochromes P-450 1A1, 1A2,1B1, 2A, 2B1, 2B2, 2B6, 2B9, 2B10, 2C2, 2C11, 2E1, 2J2, 2J3, 2J5, 2J9, 3A1, 3A4, 4A1, 4A2, 4A3, 4A5, 4A6, 4A8, 4A11, 4F1, 4F4, 4F5, 4F6, 4F11, and 4F12 have been identified in renal cells of the human, mouse, rat, and rabbit kidney.[121–125]

Cytochrome P-450 expression depends on the species and sex being studied, as well as the site along the nephron. For example, cytochrome P-450 2A, 2C, and 2E are present in male mouse kidneys but are barely detectable in female mouse kidneys.[121] Several studies suggest differences in the expression of cytochrome P-450 isoforms between human and rodent kidneys. An important example is the expression of cytochrome P-450 2E1, which has been detected in renal proximal and distal tubular cells of mice and rats, but not human kidneys.[121,122,126] In contrast, both human and rodent kidneys express high amounts of cytochrome P-450 4A isoforms. However, rat kidneys express 4A1, 4A2, and 4A3, whereas the human kidney appears to express 4A11.[122,127] Such differences in xenobiotic expression must be considered when assessing the role of biotransformation in chemical-induced nephrotoxicity.

In contrast to the liver, fewer compounds are documented to produce nephrotoxicity through renal cytochrome P-450

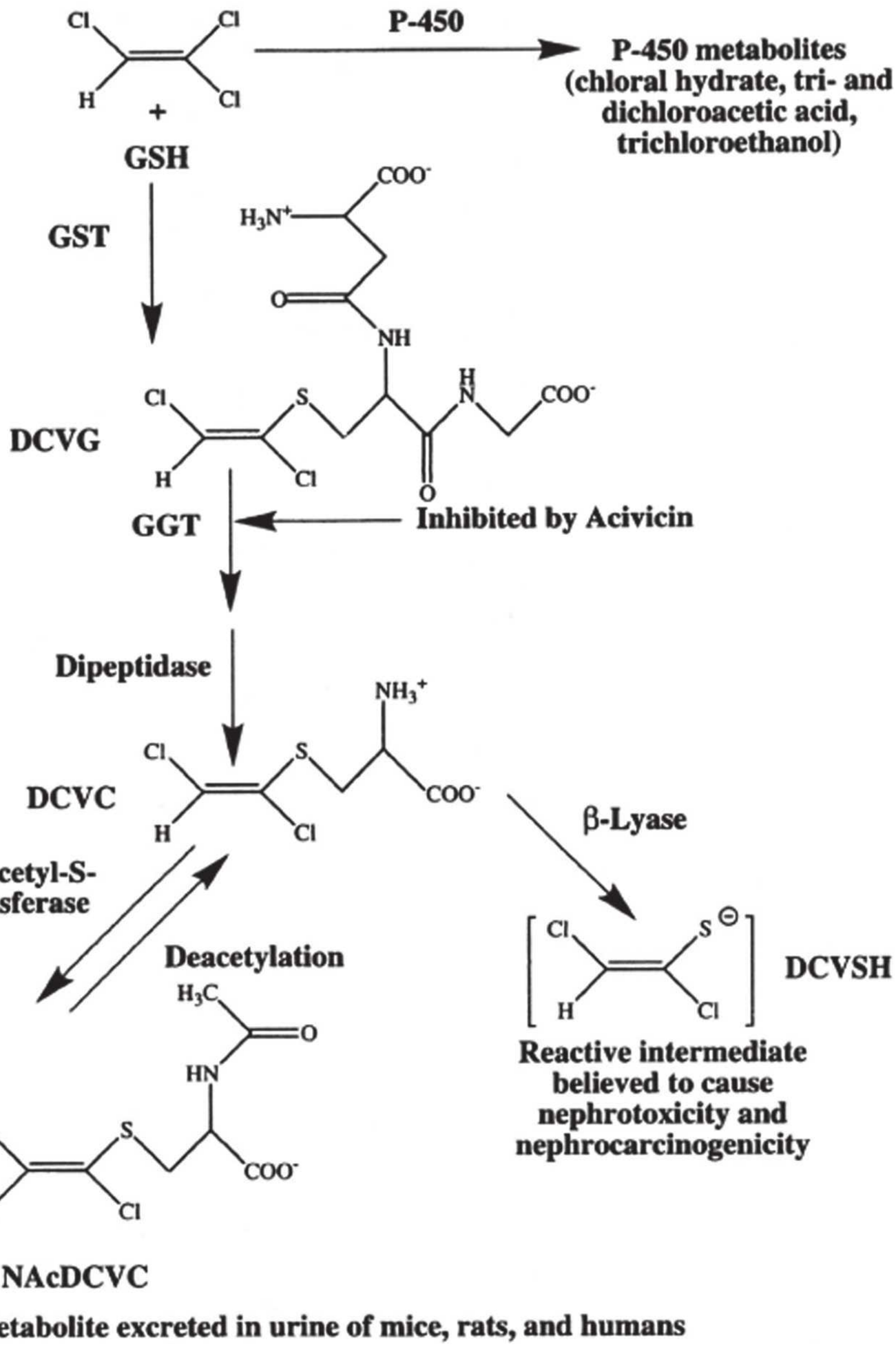

FIGURE 30.5 The bioactivation of trichloroethylene by the glutathione-(GSH-) conjugation pathway. Trichloroethylene (*top left*) can be metabolized by either cytochrome P-450 to the compound listed (*top right*) or be conjugated to GSH by the glutathione S-transferase (GST) to form S-(1,2)-dichlorovinyl-glutathione (DCVG). These reactions can occur either in the liver or in the kidney. DCVG formed in the liver is delivered to the kidney via the bile or the blood where the high concentrations of γ-glutamyltransferase (GGT) and dipeptidase in the kidney results in the cleavage of the GSH moiety and the formation of S-(1,2)-dichlorovinyl-L cysteine (DCVC). Metabolism of DCVC by *N*-acetyl-S-transferase produces *N*-acetyl-S-(1,2)-dichlorovinyl-L-cysteine (NAcDCVC), which is excreted in the urine of mice, rats, and humans exposed to trichloroethylene. NAcDCVC also can be deacetylated back to DCVC. Metabolism of DCVC by cysteine-conjugate β-lyase results in the formation of a reactive thiol that can rearrange to form a protein acylating species. (From Cummings BS, Parker JC, Lash LH. Role of cytochrome P450 and glutathione S-transferase alpha in the metabolism and cytotoxicity of trichloroethylene in rat kidney. *Biochem Pharmacol.* 2000;59:531, with permission.)

bioactivation, although renal cytochrome P-450 contributes to the nephrotoxicity of chloroform[128,129] by metabolizing it to the unstable trichloroethanol, which releases HCl to form phosgene. Phosgene reacts with: (1) two molecules of glutathione to produce diglutathionyl dithiocarbonate, (2) water to produce two molecules of HCl and CO_2, (3) cysteine to produce oxothizolidine-4-carboxylic acid, or (4) cellular macromolecules to initiate toxicity.[128,130,131]

Chloroform bioactivation by renal cytochrome P-450 is sex- and species-dependent. The marked sex difference in the nephrotoxicity of chloroform is reversed by castration of males or treatment of females with testosterone, suggesting that the renal cytochrome P-450 responsible for chloroform bioactivation is under androgenic control.[130,132] Because cytochrome P-450 isozymes 2B1 and 2E1 are present in male mice and are expressed in female mice treated with testosterone, these isozymes may be responsible for renal chloroform bioactivation.[131]

Acetaminophen is metabolized in the mouse kidney by cytochrome P-450 2E1 to the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQ), which binds to cellular proteins.[132,133] In the liver, NAPQ binds to a selenium binding protein (58 kDa),[134,135] microsomal glutamine synthetase (44 kDa),[136] cytosolic N-10-formyl tetrahydrofolate dehydrogenase (100 kDa),[135,137] and mitochondrial glutamate dehydrogenase (50 kDa).[137] It is possible that similar protein binding may occur in renal cells.

Studies also suggest that acetaminophen mediates renal cell death in mouse RPTC by inducing ER stress.[109] In this model, acetaminophen treatment increased the expression of GADD153, an ER stress protein, and induced caspase-12 cleavage and apoptosis—independently of caspase-3, -9,

30.1 Expression of Selected Xenobiotic Biotransformation Enzymes in the Kidney

Enzyme	Cell Type	Species	References
Cytochrome P450 monooxygenases			
IA	Proximal tubules	Rat, mouse, human	390, 391
IA2	Proximal tubules	—	123
IIB	Proximal tubules	Rat and mouse	122
	Distal tubules	Rat and mouse	122
IIC2	Proximal tubules	—	
IIC9	Unknown[a]	Human but not rat	390
IIC11	Distal tubules	Male rat	122
IID	Proximal tubules	—	125
IIE1	Proximal tubules	Rat, mouse, not human	122, 127, 392
	Distal tubules	—	122
IIJ	Proximal tubules	Human, rat, mouse	123, 393–395
IIIA1	Glomerulus	Rat, mouse, not human	122, 390, 391
IIIA4	Proximal tubules	Human, not rat or mouse	390
IVA2	Proximal tubules	Rat, mouse, not human	122, 390
	Distal tubules	—	122, 390
IVA3	Proximal tubules	Rat, mouse, not human	122, 390
	Distal tubules	—	122, 390
IVA11	Proximal tubules	Human, not rat or mouse	126, 127
IVF	Proximal tubules	Human and mouse	124, 127
	Distal tubules	Mouse	124
Flavin-containing monooxygenases		—	
FMO1	Unknown[a]	Rat, mouse, and human	141, 390
FMO3	Unknown[a]	Rat, mouse, and human	141, 390
FMO5	Unknown[a]	Human	141
Glutathione S-transferases			
GST α	Proximal tubules	Rat, mouse, and human	19, 126, 148
	Distal tubules	—	
GST μ	Proximal tubules	Rat, mouse, not human[b]	19, 126, 148, 396
GST π	Proximal tubules	Rat, mouse, and human	19, 126
GST θ	Proximal tubules	Human	126

[a]Activity and expression have been measured in kidney microsomes only.
[b]GSTμ is expressed in some human kidney malignancies.

or the release of the mitochondrial pro-apoptotic protein Smac/Diablo.

Flavin-containing Monooxygenase

Flavin-containing monooxygenase (FMO) oxidizes the nucleophilic nitrogen, sulfur, and phosphorus moieties of a number of chemicals, including DCVC, tamoxifen, and cimetidine.[121,138,139] The role of FMO in nephrotoxicity has received less attention than cytochrome P-450, but several FMO are expressed in the kidney. Like cytochrome-P450, renal FMO expression and activity is species- and sex-dependent. For example, rabbit kidneys express FMO1, 2, 4, and 5, but not 3, and FMO1 is expressed in the female, but not the male kidney.[121] FMO3 activity is detected in the kidneys of rats, dogs, mice, rabbits, and humans.[140] Rat kidneys appears to have two- to sixfold greater activity levels (as determined by methionine S-oxidase activity) than other species, including humans.[141] Studies in human kidney microsomes demonstrate that FMO1, FMO3, and FMO5 are all expressed, but at different levels.[141] Furthermore, samples from African American patients had significantly more FMO1 activity compared to their Caucasian counterparts, suggesting that the expression of renal FMO isoforms may differ depending on race.[141] Studies in mice

suggest that sex- and age-dependent differences exist for the expression of FMO mRNA in the kidney[142]; however, no differences in the expression of FMO1, FMO3, or FMO5, and overall FMO activity were detected between human male and female kidney microsomes.[141] Thus, more work is needed to determine if FMO expression is sex-dependent in human kidneys.

In vitro, FMO1, FMO3, FMO4, and FM05 metabolize cysteine S-conjugated S-allyl cysteines, whereas FMO3 metabolizes DCVC.[141] However, little DCVC was metabolized in human kidney microsomes, even though FMO3 was expressed in these tissues, suggesting that FMO may not contribute to the nephrotoxicity of this compound in human renal cells. In contrast, treatment of human proximal tubular cells with the FMO inhibitor methimazole decreased DCVC-induced apoptosis.[143] Studies also suggest that FMO catalyzed sulfoxidation of the sevoflurane (a commonly used anesthetic) degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether mediates its renal toxicity.[144] Other nephrotoxicants suggested to be metabolized by FMO include 4-amino-2,6-dichlorophenol.[145] 4-amino-2,6-dichlorophenol is a metabolite of 3,4-dichloroanaline, a common industrial manufacturing intermediate. Finally, FMO may also mediate the nephrotoxicity of some pesticides such as organophosphate thioether compounds.[146]

Glutathione S-Transferase

The conjugation enzymes glucuronosyltransferases, sulfotransferases, and glutathione S-transferases (GST) are located in the kidney where they conjugate both endogenous and exogenous compounds. These enzymes increase the water solubility, excretion, and elimination of several nephrotoxicants.[121] Although this typically decreases renal cell injury, some nephrotoxicants are bioactivated by these enzymes.

GST mediates the conjugation of the tripeptide glutathione (GSH, γ-glutamylcysteinlyglycine) to compounds with electrophilic centers.[121] They are considered phase II biotransformation enzymes and are divided into cytosolic, membrane associated, and mitochondrial members. There are seven different cytosolic subfamilies (A, alpha; M, mu; P, pi; T, theta; O, omega; S, sigma; and Z, zeta). Microsomal GST is referred to as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), whereas mitochondrial GST is called K (kappa) GST.[121,147] All of these GSTs, with exception of GST S, are expressed in rat and human kidneys,[19,121,126,147] and GST expression in normal human RPTC appears to be similar to those observed in rat RPTC.[126]

GST expression can differ among nephron segments. For example, in the rat kidney, GST A is expressed primarily in proximal and distal tubules, whereas GST M and P are primarily expressed in the distal tubules.[19,121,126] These expression patterns differ from a previous study, especially with regard to GST A in distal tubules.[121,148] Thus, more research is needed to resolve this discrepancy.

As mentioned previously, conjugation of toxicants to GSH is normally a detoxification pathway in which electrophiles are neutralized and made more amenable for excretion. Unfortunately, numerous extrarenally formed glutathione conjugates are nephrotoxic. For example, the extrarenal conjugation of GSH is important for the nephrotoxicity of $HgCl_2$,[149] halogenated alkenes, and aromatics, and possibly acetaminophen.[131,150,151] The nephrotoxicity of the halogenated alkene trichloroethylene in rats and humans is believed to be a direct result of its conjugation with GSH to form S-(1,2)-dichlorovinyl-glutathione, and the subsequent processing of the glutathione-conjugate to DCVC in RPTC (Fig. 30.5).[19]

In vivo, trichloroethylene is conjugated with GSH in the liver and delivered via the bile or blood to the kidney. The expression of enzymes, such as γ-glutamyl transferase and dipeptidase in the RPTC and biliary and intestinal tract, results in the cleavage of the γ-glutamyl and glycyl moieties, respectively, and the formation of DCVC. Metabolism of DCVC by N-acetyl-S-transferase produces N-acetyl-S-(1,2)-dichlorovinyl-L-cysteine, which is excreted in the urine of mice, rats, and humans exposed to trichloroethylene.[152] N-acetyl-S-(1,2)-dichlorovinyl-L-cysteine also can be deacetylated back to DCVC. Metabolism of DCVC by cysteine-conjugate β-lyase results in the formation of a reactive thiol that can rearrange to form a protein acylating species. A strong correlation exists between increases in markers of renal injury (proteinuria, creatinine clearance, glucosuria) and GSH metabolites of trichloroethylene in the blood and urine of humans exposed to high amounts of trichloroethylene.[153] Key determinants in the nephrotoxicity of trichloroethylene and similar chemicals, such as sevoflurane, isoflurane, and desflurane, which utilize this common pathway of biotransformation,[144,154] appear to be dependent on γ-glutamyl transferase, dipeptidase, and cysteine-conjugate β-lyase activity found in the kidney.

Role of Reactive Oxygen Species

Reactive oxygen species (ROS) mediate cellular injury during inflammatory responses, ischemia-reperfusion, and after nephrotoxicant exposure. Cellular ROS are generated during the normal function of the mitochondrial and microsomal electron transport chains as a result of the incomplete reduction of O_2 to water (Fig. 30.6).[155] Superoxide anion free radical is produced by a one-electron reduction of O_2, and H_2O_2 is produced by a two-electron reduction of O_2. Superoxide anion can dismutate to form H_2O_2, or H_2O_2 can be formed directly. The hydroxyl radical is formed from H_2O_2, and the superoxide anion free radical is formed via the metal-catalyzed Haber-Weiss reaction or the superoxide-driven Fenton reaction. Ferrous iron (Fe^{2+}) appears to be the major intracellular initiator of the reaction, but cuprous ions may participate as well. The precise source and form (e.g., ferritin) of the ferrous iron is still unclear.

One source of Fe^{2+} may be the heme-moiety that resides in the active site of cytochrome P-450 isoforms.[156]

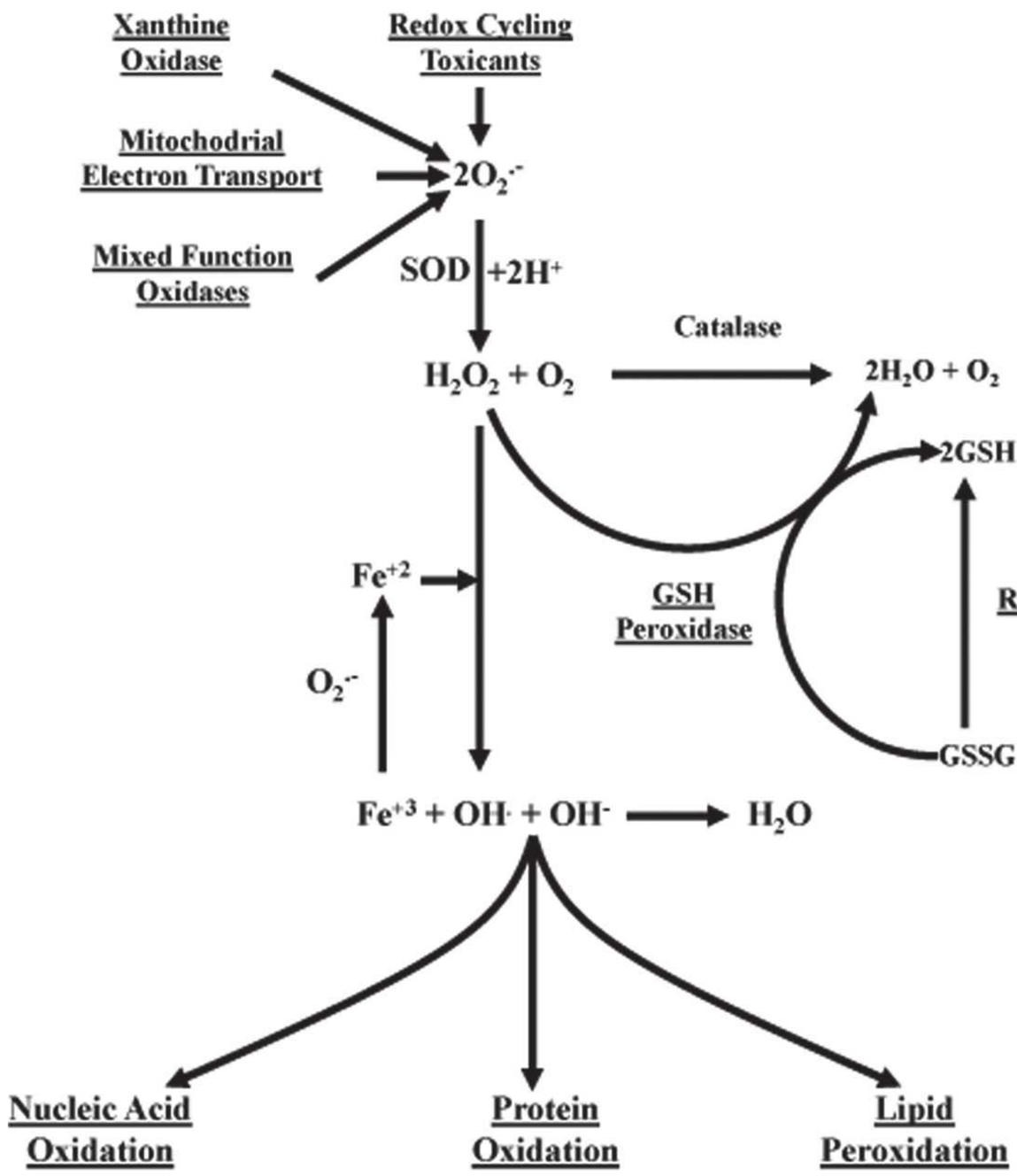

FIGURE 30.6 A schematic representation of the major pathways and possible intracellular targets and oxidants. Detoxification pathways and protective agents are also shown. See text for details. *GSH,* glutathione; *GSSG,* glutathione disulfide; *SOD,* superoxide dismutase.

This hypothesis is supported by the observation that rats treated intraperitoneally with cisplatin for 4 days had significantly lower renal cytochrome P-450 compared to control rats, and the decrease in P-450 correlated with increases in bleomycin-detectable iron content in the kidney. Piperonyl butoxide (a cytochrome P-450 inhibitor) decreased cisplatin-induced release of iron in the kidney and the functional and morphologic markers of kidney toxicity.[157] These same effects were observed in LLC-PK_1 cells; thus, P-450 may serve as one source of Fe^{2+} to initiate the formation of ROS.

Superoxide anion acts as a reductant for Fe^{3+}, and the Fe^{2+} generated reduces H_2O_2 to the hydroxyl radical, which reacts rapidly with adjacent molecules. Superoxide anion and H_2O_2 are less reactive, and H_2O_2 may diffuse away from the initial site of formation to produce injury at a distant site within the cell. Although H_2O_2 readily crosses cell membranes, superoxide anion and hydroxyl radicals do not. Because ROS production is a natural byproduct of metabolically active cells, such as the kidney, significant defenses exist against the normal production of ROS or those produced under pathologic conditions (Fig. 30.6).

The term "oxidative stress" is commonly used to describe conditions that lead to increased ROS formation. Chemicals may initiate oxidative stress indirectly by augmenting ROS production. For example, Walker and Shah[158] showed that gentamicin enhances H_2O_2 generation in isolated rat renal cortical mitochondria, and Lund and associates[159] showed that mitochondria isolated from rats treated with $HgCl_2$ have elevated H_2O_2 production.

Another mechanism by which chemicals produce oxidative stress is through "redox cycling." Certain compounds, especially quinones, can undergo a one-electron reduction to a semiquinone radical and a second one-electron reduction to the hydroquinone. The hydroquinone is oxidized to the quinone, and the cycle begins again, hence the term "redox cycling." During the reduction process, superoxide anion is formed from O_2, and oxidative stress ensues. For example, Brown and colleagues[160] demonstrated that menadione (2-methyl-1,4-naphthoquinone) produces toxicity in isolated rat renal epithelial cells through its ability to undergo redox cycling and cause oxidative stress. It should be recognized that the ability of quinones to undergo redox cycling varies with the quinone, and some quinones produce toxicity through their ability to arylate cellular macromolecules, particularly protein sulfhydryls.[160,161]

ROS can induce lipid peroxidation, inactivate enzymes by directly oxidizing protein sulfhydryl or amino groups, depolymerize polysaccharides, and induce DNA strand breaks. Lipid peroxidation results from the interaction of free radicals with polyunsaturated fatty acid side chains of membrane phospholipids to form free radicals and relatively stable lipid hydroperoxides.[162] Transition metals can catalyze the decomposition of lipid hydroperoxides, which results in the formation of alkoxyl and peroxyl free radicals that propagate the reaction. Lipid breakdown products such as hydroxylated fatty acids, 2-alkenyls, and 4-hydroxyalkenyls, are toxic, and may contribute to organelle and cellular dysfunction. Thus, ROS-induced degradation of membrane lipids

decrease cellular membrane integrity, alter enzymatic activity and transport properties, and induce isotropy.[163] The oxidation of protein sulfhydryl and amino groups by ROS can alter enzyme activity and membrane structure and function to the point that cell death will ensue. Finally, ROS can produce DNA strand breaks. Although H_2O_2 does not directly damage DNA, its stability and ability to diffuse throughout the cell allows it to come into contact with metal-based enzymes, like cytochrome P-450, which catalyze the formation of hydroxyl radicals, which can damage DNA.

A variety of structurally diverse nephrotoxicants produce renal cell injury by mechanisms involving oxidative stress, including $HgCl_2$,[159,162] haloalkene cysteine conjugates,[164,165] cyclosporine A,[165] and cisplatin.[76,166,167] The diversity of these nephrotoxicants highlights the critical and common roles that ROS play in the mechanism of renal cell death.

MEDIATORS OF CELL INJURY

Numerous common cellular pathways that mediate cell death have been identified. It is generally thought that upon initial exposure, nephrotoxicants will activate at least one, if not more, of these pathways. For necrosis, apoptosis, and possibly autophagy, a point exists along the sequence, yet to be identified, referred to as the point of no return. Here, the cell will die irrespective of any intervention. Also, along this sequence are switch points at which the cell death pathways may change from one mechanism to another. Investigators have tried to identify the sequence of deleterious events, the point of no return, and switch points for years and these efforts have led to the identification of numerous intracellular mediators critical in the generation of renal cell injury and death.

p53 and p21

The tumor suppressor protein p53 and the cell cycle inhibitor protein p21 can mediate renal cell death and acute renal failure.[76,168,169] Activation of p53 typically induces cell death and cell cycle arrest.[76,169] Activation of p21 is protective against numerous types of nephrotoxic events, including ischemia and cisplatin exposure.[168–171] p53 can induce p21 during renal cell injury, but p21 can also be activated independently of p53.[172] The mechanisms of p53-meidated activation of p21 are under study, but may involve increases in transcription. Transcription and signaling kinases are believed to mediate mechanisms by which p21 expression is increased independently of p53.[168,173]

p53 is activated in renal cells by agents that that induce DNA damage, like cisplatin,[172,174] and it is activated in renal cells after ischemia and oxidative stress subsequent to exposure to bromate, Fas, antimycin A, histone deacytelylase (HDAC) inhibitors, and aristolochic acid.[175,176] The mechanism of p53 activation involves its phosphorylation at numerous serine residues, followed by its release from the regulator protein Mdm2. Other regulators of p53 include ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia and Rad-3 related kinase (ATR), checkpoint kinase (ChK), and NF-kB.[169]

After activation, p53 can translocate to the nucleus or the mitochondria, or remain in the cytosol. In the nucleus, p53 induces the transcription of a number of genes, including p21,[172] and can activate several apoptotic pathways such as those involving caspases.[75] Translocation of p53 to the mitochondria results in its interaction with the outer mitochondrial membrane and binding with the anti-apoptotic proteins Bcl-2 and Bcl-xL.[169] This interaction releases the pro-apoptotic proteins Bax and Bak, which induce mitochondrial pore formation in mitochondria and facilitate the release of the pro-apoptotic proteins cytochrome c, Omi, Smac/Diablo, and Endo G.[55] Both nuclear and cytosolic p53 may regulate autophagy, and studies suggest that p53 localization represents a key switch point between autophagy and apoptosis.[55]

Pharmacologic and molecular studies demonstrate that inhibition of p53 decreases cisplatin-induced apoptosis in rabbit RPTC through a mechanism that includes caspase inhibition of.[75,76,169] In support of this hypothesis, others have demonstrated that inhibition of p53 nuclear translocation inhibits AKI and renal cell death in vivo after renal ischemia in rats.[177] Of note, p53 inhibition in these studies did not totally inhibit cell death; thus, quite possibly, p53-independent mechanisms contribute to nephrotoxicity induced by cisplatin, oxidative stress, ischemia, and other DNA damaging agents.

Induction of p21 occurs in response to DNA damage and p53-induced cell cycle arrest.[170] p21 is also activated independently of p53 by transcriptional-mediated mechanisms and by mechanisms involving mitogen activated protein kinases (MAPK).[168,173] p21 is a cyclin-dependent kinase (cdk) inhibitor that interacts with numerous cdk, such as cdk2, to control cell cycle.[168,170,178] Activation of p21 decreases renal cell death induced by cisplatin and ischemia reperfusion.[168] The mechanism of protection is linked to its ability to alter the cell cycle and allow for cell repair. In support of this hypothesis, knockout mice lacking p21 exhibited increased renal cell cycle activity and apoptosis, and were more susceptible to cisplatin and ischemia-induced ARF compared to wild type controls.[170] The majority of studies with p21 and renal cell death have focused on cisplatin and ischemia reperfusion. More work is needed to determine if p21 can mediate other forms of chemical-induced nephrotoxicity.

Signaling Kinases

Signaling kinases alter the activity, expression, or localization of another protein by altering its phosphorylation, including other signaling kinases. Signaling kinases differ in terms of the amino acids targeted for phosphorylation (serine/threonine/tyrosine), the location within a cell (membrane-bound or cytosolic), and the protein targeted for phosphorylation.

Table 30.2 lists several signaling kinases identified in the kidney, the site within the kidney or cell involved, the nephrotoxicant involved, and several references to studies that suggest critical roles for signaling kinases both in the development of renal cell death and in the recovery of renal cells after toxicant-induced injury.

Protein Kinase C

Protein kinase C (PKC) is a family of serine/threonine kinases, and at least 12 different mammalian isoforms have been identified. These are divided into conventional PKC (cPKC: α, $\beta_{1/2}$, and γ), novel PKC (nPKC: ϵ, ϵ', δ, η, θ, and μ), and atypical PKC (aPKC: τ, λ, and ζ).[179] The isoforms differ in terms of preferred substrates and mechanisms of action. Activation of cPKC is Ca^{2+}- and diacylglycerol-dependent, whereas activation of nPKC is Ca^{2+}-independent.[180] In contrast, activation of aPKC is independent of both Ca^{2+} and diacylglycerol. RPTC have been reported to express α, β_1, β_2, ζ, δ, λ, and ϵ,[181,182] and several other isoforms are expressed in the kidney of rats, mice, and humans.[183]

PKC is reported to mediate the toxicity of cisplatin, ischemia, oxidants, and TNF-α in multiple renal cell models.[184–189] The exact role of PKC in renal cell death depends on the toxicant and the specific isoform(s) involved.[190,191] For example, activation of PKC-α in rabbit RPTC during cisplatin treatment results in mitochondrial dysfunction and cell death,[189] and similar findings have been reported for PKC-ϵ in RPTC after oxidative stress.[192] Interestingly, PKC-ϵ targeted several mitochondrial proteins including complexes I and IV, and F(0)F(1)-ATPase in RPTC.[192] In contrast, activation of PKC-ζ after exposure to t-butylhdyroperoxide mediates cellular repair.[190,191]

MAPK (ERK1/2, p38 and JNK)

MAPK are serine-threonine kinases activated by a cascade of protein-protein interactions. They mediate cell growth, adhesion, differentiation, gene expression, and apoptosis. They can also mediate activation of p53.[193,194] ERK1/2 (p42/44MAPK), p38 MAPK, and c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) are three of the

30.2 Selected Signaling Kinases Involved in Renal Cell Injury, Survival, or Repair

Kinase	Location	Nephrotoxicant	Reference
Protein Kinase C (PKC)			
Conventional PKC			
PKCα	Proximal tubules	Cisplatin	189, 397
	DCVC[a]		
Novel PKC			
PKCϵ	Proximal tubules	Oxidative injury	192
Atypical PKC			
PKCζ	Proximal tubules	t-Butylhydroperoxide	191
Mitogen Activated Protein Kinase (MAPK)			
ERK1/2	Proximal tubules	Cisplatin	189, 198, 205,
		H_2O_2	208, 398
		TGHQ[c]	
JNK/SAPK[a]	Proximal tubules	Cisplatin	205
P38	Proximal tubules	Cisplatin	198, 205, 208
		H_2O_2	
		TGHG[c]	
Other Kinases			
Protein kinase B[b]	LLC-PK1	Cisplatin	398, 399
	Proximal tubules	Mechanical injury	
		H_2O_2	
Phosphoinositide-3-kinase	LLC-PK1	Cisplatin	399
	Proximal tubules	Mechanical injury	

[a]S-(1,2)-dichlorovinyl-L-cysteine.
[b]Also known as AKT.
[c]2,3,5-tris-(glutathion-s-yl)hydroquinone.

most studied MAPK. These kinases are activated by additional kinases: MAPK/extracellular signal-regulated kinase or MEK.[195]

ERK1/2, also referred to as extracellular regulated kinases,[196] are activated through epidermal growth factor receptor (EGFR), which is mediated by a small G-protein Ras.[197,198] Studies show that ERK1/2 is activated in renal cells after exposure to cisplatin, oxidants, bromate, and aminoglycosides.[115,189,198,199] ERK1/2 activation in these studies is reported to be protective, which correlates with the role of this kinase in survival and proliferation.[197,200,201] The ability of ERK1/2 to act as prosurvival signals is partially due to their ability to translocate to the nucleus and activate transcription factors such as AP-1, Elk-1, and c-Myc.[202] In addition, ERK1/2 can phosphorylate and inhibit caspase-9, which can inhibit apoptosis.[69]

The role or ERK1/2 in renal cell injury is toxicant- and cell-dependent, and some studies suggest that ERK1/2 can mediate apoptosis.[203,204] For example, Arany et al.[205] demonstrated that ERK activation mediated cisplatin-induced renal cell death in vivo and in vitro in mouse models. However, the same investigators observed that ERK activation protected against oxidant-induced (H_2O_2) cell death.[205] Interestingly, cisplatin-induced ERK activation and renal cell death was dependent on EGFR and c-Src activation. Studies by Zhuang and colleagues[206,207] also showed that exposure of RPTC to H_2O_2 activates Src, EGFR, and ERK1/2, as well as protein kinase B (Akt) and phosphoinositide-3-kinase (PI3K). ERK1/2 activation in this model was proposed to mediate apoptosis by activating caspase-3.[207] EGFR-induced activation of ERK1/2 is also believed to mediate 2,3,5-tris-(glutathione-S-yl)hydroquinone (TGHQ)-induced death in LLC-PK1 cells.[198,208] Thus, activation of ERK1/2 in renal cells after toxicant exposure may not always result in survival.

p38 MAPK and JNK/SAPK are activated in response to cellular stress, inflammation irradiation, heat shock, ROS, LPS, TNF-α, and IL-1.[209–211] These proteins are activated by Rac, a small G protein that activates distinct MEK rather than those involved in ERK1/2 activation.[210] p38 and JNK are generally thought to mediate cell death and cytostasis; however, like ERK1/2, the role of p38 and JNK in renal cell injury is toxicant- and cell-dependent. For example, activation of p38 by nephrotoxicants such as cisplatin and bromate in renal cell lines activates p53 and induces cell death.[169,199] In contrast, activation of p38 in primary cultures of RPTC after oxidant induced injury mediates dedifferentiation.[206] The detailed mechanisms of Src, EGF-R, PI3K, ERK1/2, and p38/SAPK activation, the targets of these kinases, and their role in cell death produced by diverse toxicants remain to be determined.

Altered Calcium Homeostasis

The role of Ca^{2+} in the pathophysiology of nephrotoxic cell injury cannot be understated. Intracellular Ca^{2+} homeostasis is necessary for cell viability because Ca^{2+} is a second messenger that plays a critical role in a variety of cellular functions.[55,212] With regard to cell death Ca^{2+} can mediate necrosis, apoptosis, and autophagy.[55,112] Initially, it was thought that only high, supraphysiologic increases in Ca^{2+} would induce cell death; however, it is now accepted that even small changes in Ca^{2+} signaling can significantly affect cell death, especially with regard to apoptosis.[55,213]

Ca^{2+} interacts with many key organelles known to participate in cell death, including the ER and the mitochondria. Ca^{2+} also mediates the activation of several proteins involved in cell death, such as calpains, endonuclease, phospholipase A_2, and protein kinases. Recent studies suggest that interaction of Ca^{2+} with calpains mediates activation of the pro-apoptotic protein AIF.[102] There is also a well-documented requirement of Ca^{2+} for autophagy[112] that directly links the release of ER Ca^{2+} stores and activation of AMP activated proteins kinases and calcium/calmodulin kinase β.[112] The ability of Ca^{2+} to stimulate autophagy in response to nephrotoxicant-induced renal cell exposure has not been heavily studied.

Cytosolic free Ca^{2+} is ~100 nM and is tightly regulated in the face of a large extracellular-intracellular gradient (10,000:1) by a series of pumps and channels located on the plasma membrane and ER. Mitochondria were not originally thought to participate in Ca^{2+}-mediated cell signaling processes under normal conditions. The advent of more advanced techniques for measuring low Ca^{2+} has changed this view, and strong evidence supports the idea that mitochondria are integral to Ca^{2+} signaling under both normal and pathologic conditions.[55]

Increases in intracellular/cytosolic free Ca^{2+} can induce cell injury and death after nephrotoxicant exposures. The source of this Ca^{2+} is typically the ER or the extracellular space (i.e., from the plasma membrane). Release of Ca^{2+} from the ER increases cytosolic free Ca^{2+} from ~100 nM to 300 nM within seconds.[214] Such increases are typically transient and buffered by transport of Ca^{2+} back into the ER, to the extracellular space or into the mitochondria. As mentioned above, release of ER Ca^{2+} can induce autophagy, and buffering of ER Ca^{2+} release by the mitochondria can induce apoptosis. Entry of Ca^{2+} from the extracellular space can increase the cytosolic free Ca^{2+} to μM levels—and even mM levels—if membrane integrity is lost. Such increases typically induce necrosis.

Decreasing the extracellular Ca^{2+} concentration or blocking extracellular Ca^{2+} influx will decrease cell death.[215–217] For example, increases in cytosolic free Ca^{2+} were observed in a hypoxia model using rat RPTC and in a mitochondrial inhibitor model using rabbit RPTC, and chelating intracellular Ca^{2+}, or decreasing the influx of extracellular Ca^{2+}, decreased cell death.[216–219] Nephrotoxicants that increase cytosolic free Ca^{2+} include $HgCl_2$,[220,221] pentachlorobutadienyl-glutathione,[167] pentachlorobutadienyl-L-cysteine,[222] tetrafluoroethyl-L-cysteine,[223] DCVC,[224–226] oxidants,[227,228] sevoflurane, miconazole,[229,230] cyclosporine A,[231] and gentamicin.[158]

Ca^{2+} is proposed to mediate apoptosis by both direct and indirect methods.[213] The direct pathway involves Ca^{2+}-mediated activation of calpains, which can induce caspases and.[55,232] Apoptosis may ensue, provided that ATP is maintained above 20% to 30%.[82] Furthermore, as mentioned above, the mitochondria can buffer ER Ca^{2+} release. Transport of Ca^{2+} into the mitochondria can activate Ca^{2+}-mediated matrix dehydrogenases, which can stimulate ATP and ROS production. Furthermore, Ca^{2+} uptake into the mitochondria can result in permeabilization of the outer mitochondrial membrane, which leads to opening of the permeability transition pore and release of pro-apoptotic proteins such as cytochrome c and AIF.[55,233,234]

The indirect pathway of Ca^{2+}-mediated apoptosis involves the activation of the phosphatase calcineurin,[213] which results in activation of the nuclear factor protein of activated T-cells (NFAT), which then increases the expression of pro-apoptotic Fas and TRAIL. Calcineurin activation may be mediated by calpain, which cleaves an endogenous inhibitor of calcineurin called cain/cabin 1.[235] The contribution of calcineurin to nephrotoxicant-induced epithelial renal cell injury has not received much attention; however, calcineurin inhibitors used during transplants surgeries induce significant renal proximal tubule injury,[236] suggesting that calcineurin acts to protect renal cells from damage.

Proteinases

Nonphysiologic activation of proteinases in the cytosol, organelles, or membranes can disrupt the normal function of these structures, leading to cell death and ARF. Proteases known to mediate nephrotoxic-induced renal cell injury include those found in the lysosomes (serine and cysteine proteases), along with calpains and caspases.

Lysosomal Proteases

Proteases found in lysosomes include serine and cysteine proteases that are acidic hydrolases. These require a lower pH to facilitate degradation (~pH 5) and thus will not typically function if released into the cytosol. They can mediate cell death during extreme cases of hypoxia and ischemia reperfusion, which induce lysosomal rupture in correlation with acidosis.[237] In contrast, less evidence exists that these proteases mediate nephrotoxicant-induced cell death.[238] In support of this hypothesis, studies with cysteine and serine proteinase inhibitors revealed these compounds to be ineffective in protecting rabbit RPTC segments from antimycin A, tetrafluoroethyl-L-cysteine, bromohydroquinone, and t-butylhydroperoxide.[239] Several inhibitors of lysosomal proteases, such as the cysteine proteinase inhibitor t-trans-epoxysuccinyl-leucylamido(4-guandino)butane (E64), have been shown to protect against RPTC injury induced by cyclosporine A[240]; however, E64 was only slightly protective, as was an aspartic acid proteinase inhibitor. These results suggest that lysosomal cysteine and aspartic acid proteinases do not play a significant role in RPTC death produced by these nephrotoxicants.

Calpains

Calpains, a group of least 15 isoforms of Ca^{2+}-activated neutral cysteine proteinases, comprise two groups. Group 1 calpains, typical calpains, contain a Ca^{2+}-binding domain, and include calpains 1, 2, 3, 8, 9, 11, 12, and 14.[241,242] Group 2, atypical calpains, lack a Ca^{2+}-binding domain, and include calpains 5, 6, 7, 10, 13, and 15.[241,242] Calpains 1, 2, 5 7, 10, 13, and 15 are reported to be expressed ubiquitously in the cytosol, whereas calpains 3, 6, 8, 9, 11, and 12 have more select tissue expression.[243]

With regard to renal cell death, the most heavily studied calpains are 1, 2, and 10, which have been implicated in cell death induced by numerous toxicants including bromohydroquinone, antimycin A, tetrafluoroethyl-L-cysteine, and t-butylhydroperoxide.[216,218,244,245] Evidence for roles of calpain in cell death in many of these studies was derived from the use of calpain inhibitors such as calpeptin. Calpeptin also inhibited increases in calpain activity induced by hypoxia in RPTC, and protected against renal dysfunction in rats subjected to ischemia reperfusion.[246,247] These studies suggest that calpains mediate cell death by enhancing extracellular Ca^{2+} influx and/or by cleaving cytoskeletal proteins.[218,241,244] Thus, calpains may play a critical role in cell death produced by a wide range of nephrotoxicants and renal dysfunction; however, calpains are also suggested to play a role in the pathology of several other diseases, including Alzheimer, Duchenne muscular dystrophy, and diabetes.[243]

Historically, calpains were thought to be primarily cytosolic, but several membrane-associated calpains are known to exist, including calpain 10. Calpain 10 is expressed in the mitochondrial fraction of the kidney of multiple species, and it appears to mediate mitochondrial dysfunction induced by oxidants, Ca^{2+} overload, and thapsigargin.[248,249] Calpain 10 appears to induce mitochondrial dysfunction by cleaving complex I of the electron transport chain.[250] Recently, calpain 10 has been suggested to mediate renal cell viability and aging in the kidney in vivo,[251] and mitochondrial biogenesis in the kidney through regulation of the peroxisomal proliferator activator receptor γ coactivator 1-α (PGC-1α,[252] see later).

Calpains are key mediators of apoptosis and necrosis, inhibiting apoptosis and inducing necrosis by cleaving and deactivating caspases, including caspases-3, -7, -8, and 9.[253,254] Originally it was thought that calpains only inhibited apoptosis; however, it is now accepted that calpains can actually participate in apoptosis signaling cascades.[55,234] The role of calpains in necrosis or apoptosis appears to be cell type–dependent, and it is unclear whether calpains can also mediate apoptosis, or autophagy for that matter, in renal cells exposed to nephrotoxicants.

Caspases

Caspases are cysteine proteases. The role of caspase activation in renal cell apoptosis has been discussed previously, but it is important to note that caspase activity is not always

needed for apoptosis. For example, caspase-2-directed permeabilization of the mitochondrial membrane results in the release of the pro-apoptotic proteins cytochrome c and Omi, which occurs in the absence of any caspase-2 catalytic activity. Similar results are seen with caspase-2 mediated release of AIF.[234,255–257] Our work demonstrated that apoptosis induced by four diverse toxicants (cisplatin, vincristine, staurosporine, and A23187) proceeded in rabbit RPTC in the presence of caspase inhibitors and the absence of caspase-3, -8, and -9 activity.[258] The mechanisms involved in caspase-independent apoptosis in renal cells appear to involve the activation of DNAases such as Endo G and AIF.[69] This is supported by studies in renal cells showing that cisplatin-induced apoptosis is mediated by Endo G as opposed to caspases.[259,260]

Phospholipase A_2

Phospholipase A_2 (PLA_2) cleaves glycerophospholipids at the sn-2 ester bond, releasing fatty acids and lysophospholipids.[261] They are classified into five different families: secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), platelet-activation factor (PAF) acetylhydrolases, lysosomal PLA_2, and calcium-independent PLA_2 ($iPLA_2$).[261,262] These proteins have different substrate preferences, Ca^{2+}-dependencies, and biochemical characteristics. Isoforms representing $cPLA_2$, $sPLA_2$, and $iPLA_2$ are all expressed in human, rat, rabbit, and mouse kidneys.[263–269]

PAF acetylhydrolase is expressed in microsomes and cytosol in rat and human kidneys,[270,271] but information about its expression in renal epithelial cells is lacking. Lysosomal PLA_2 is Ca^{2+}-independent and is an acidic hydrolases identical to peroxiredoxin 6.[272,273] Few studies exists assessing lysosomal PLA_2 expression in the kidney, but studies of peroxiredoxin 6 indicate that it is expressed in the proximal and distal tubules of rat kidneys.[274] The roles of PAF acetylhydrolase and lysosomal PLA_2 in nephrotoxicant-induced cellular injury are not well understood and deserve further study.

PLA_2 contribute to the mechanisms of cell injury and death by metabolizing glycerophospholipids and releasing fatty acids. The high concentrations of polyunsaturated fatty acids in plasma and organelle membranes make them prime targets for oxidants. Increase in lipid peroxidation facilitates PLA_2 activation and the production of fatty acids and lysophospholipids. This further enhances PLA_2 activity, which enhances phospholipid degradation to the point that membrane integrity is lost and intracellular Ca^{2+} increases which facilitates cell swelling and the activation of $sPLA_2$.

Recently, roles for $cPLA_2$ and $iPLA_2$ in nephrotoxicity have received much attention. Data from these studies demonstrate that PLA_2 mediates nephrotoxicant-induced cell injury in a cell- and toxicant-dependent manner. $cPLA_2$ or $iPLA_2$ is reported to mediate nephrotoxicity induced by oxalate, cisplatin, and isoflurane, as well as oxidants such as H_2O_2, t-butylhydroperoxide, and menadione.[174,267,275,276] $cPLA_2$ and $iPLA_2$ also mediate cell death induced by Fas and TNF-α, hypoxia, anoxia, and ischemia/reperfusion.[72,67,277–280]

PLA_2 are both initiators and executioners of nephrotoxicant-induced cellular death. They can act as executioners when they are activated after initial increases in cytosolic Ca^{2+}. This is especially true for $sPLA_2$, which is activated by mM concentrations of Ca^{2+}, which typically occur after loss of membrane integrity.[281,282] $cPLA_2$ is not directly activated by Ca^{2+}, but μM increases in Ca^{2+} facilitate translocation of $cPLA_2$ to membranes, which increases its activity.[281,283] Increase in Ca^{2+} also induce signaling kinases, such as ERK, that activate $cPLA_2$ and $iPLA_2$.[284–286] The metabolites of PLA_2 (fatty acids and lysophospholipids) can induce cell death by causing inflammation, mitochondrial dysfunction, and lipid peroxidation.[174,267,277]

PLA_2 can also initiate nephrotoxicant cell injury independently of Ca^{2+}. This is more the case for $iPLA_2$ than $cPLA_2$ or $sPLA_2$. $iPLA_2$ mediates cisplatin- and oxidant-induced nephrotoxicity in primary cultures of RPTC and several other renal cell lines.[174,267,287] The mechanisms involved also include cleavage of phospholipids and release of lipid mediators, except Ca^{2+} is not involved in the initial phases of injury.[277,287]

Most of our knowledge about the role of $iPLA_2$ in nephrotoxicant-induced cell injury is derived from studies using pharmacologic inhibitors, such as bromoenol lactone.[262] Studies using small interfering RNA also demonstrate that inhibition of $iPLA_2$ isoforms (groups VIA and B, commonly called $iPLA_2\beta$ and γ) induce cytostasis, apoptosis, and mitochondrial dysfunction in renal cell lines and primary cultures of RPTC.[277,288,289] These pathologies have been directly linked to changes in phospholipid metabolism and release of fatty acids.[277,290] Genetically modified mice lacking specific forms of $iPLA_2$ do exist,[291,292] but few studies have used these models to address roles of $iPLA_2$ in nephrotoxicant-induced cell injury.

Similar to $iPLA_2$, much of the knowledge about the role of $cPLA_2$ in nephrotoxicant-induced cell injury is derived from studies using inhibitors such as arachidonyl trifluoromethyl ketone or methyl arachidonyl fluorophosphonate.[262] Alteration of $cPLA_2$ activity using arachidonyl trifluoromethyl ketone alters oxidant-induced renal cell necrosis in multiple renal cell models.[275,293] Genetically altered mice lacking specific forms of $cPLA_2$ (group IV PLA_2 or $cPLA_2\alpha$) do exist, and suggest that these mice experience some alteration in kidney function.[294,295] Few studies exist using knockout mouse models to study the role of $cPLA_2$ in nephrotoxicant-induced cell death.

Compared to $iPLA_2$ and $cPLA_2$, less is known about the role of $sPLA_2$ in nephrotoxicity. Activation of $sPLA_2$ increased cell death in renal carcinoma cells in correlation with membrane hydrolysis,[296] and mediated cell death induced by IL-1-stimulated release of fatty acids, such as arachidonic acid, in HEK293 cells.[297] Thus, $sPLA_2$ may mediate renal cell injury after inflammation.[298,299] One role for $sPLA_2$ in nephrotoxicity is regulation of other PLA_2. This hypothesis is supported by studies in LLC-PK1 cells exposed to the oxidants H_2O_2 and menadione.[293] These studies suggest that

PLA_2 cross-talk can mediate certain types of nephrotoxicant-induced renal cell injury.

MITOCHONDRIA, ENDOPLASMIC RETICULUM, LYSOSOMES, AND THE CELL MEMBRANE

Mitochondria, ER, lysosomes, and the cell membrane all play roles in nephrotoxic cell injury as well as play roles in necrosis, apoptosis, and autophagy. As mentioned previously, these types of cell death do not proceed through mutually exclusive pathways consisting of single-event sequences. The interplay among necrosis, apoptosis, and autophagy is often mediated by cellular organelles, and key biochemical mediators of switches among necrosis, apoptosis, and autophagy include Ca^{2+} and ATP. These mediators are often controlled by the function of specific organelles, such as the ER (Ca^{2+}), the cell membrane (Ca^{2+}), and the mitochondria (ATP). In addition, lysosomes are critical mediators of autophagy.

Because of the presence of multiple cell death pathways and multiple targets, inhibition of one pathway may not block nephrotoxicant-induced cell death, but rather switch it from one type to another. This is often accompanied by an alteration in organelle function. For example, if a treatment blocks oxidative stress associated with DCVC, pentachlorobutadienyl-L-cysteine, or tetrafluoroethyl-L-cysteine exposure to RPTC, the rate of cell death is diminished, but the cells eventually die due to mitochondrial dysfunction (Fig. 30.7).[300] Thus, a given chemical can cause cell death by interacting with numerous organelles, and blocking interaction at one organelle may not decrease cell death. Rather it

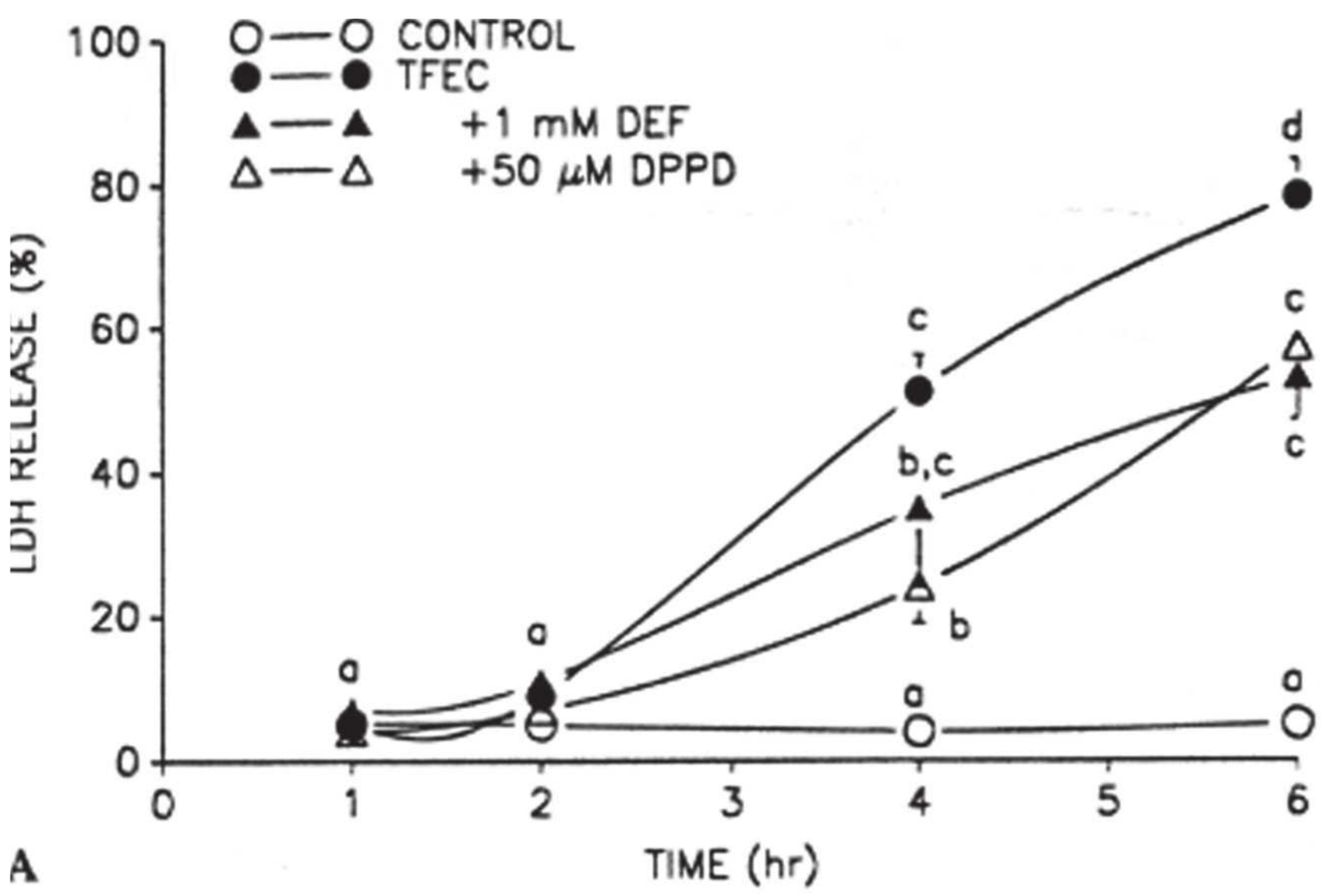

FIGURE 30.7 A: The time-dependent effects of deferoxamine (DEF) and N,N′-diphenyl-1,4-phenylenediamine (DPPD) on S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC)-induced cell death (lactate dehydrogenase [LDH] release) from rabbit renal proximal tubules. DEF and DPPD were added at the same time as TFEC (25 μM). Values are means ± SEM. Values at a given time point or within a given treatment with different superscripts are significantly different from one another ($P \leq 0.05$). **B:** The time-dependent effects of DEF and DPPD on TFEC-induced lipid peroxidation (malondialdehyde [MDA] formation) in rabbit renal proximal tubule suspensions. Values are means ± SEM. Only the TFEC alone is significantly different from controls ($P \leq 0.05$). (Adapted from Groves CE, Lock EA, Schnellmann RG. Role of lipid peroxidation in renal proximal tubule cell death induced by haloalkene cysteine conjugates. *Toxicol Appl Pharmacol.* 1991;107:54, with permission.)

may alter the organelle targeted, which can switch the mechanism of cell death.

Mitochondria

The renal tubular reabsorption of solutes and water requires a large expenditure of energy. Although ATP is generated by both oxidative phosphorylation and glycolysis, ~95% of renal ATP is formed by oxidative phosphorylation.[301] The amount of oxidative phosphorylation that occurs within a given cell varies along the nephron. Thus, toxicants that interfere with mitochondrial function and anoxia will produce cell injury and death, particularly in tubular cells that have limited glycolytic capabilities, such as the S_1 and S_2 segments of the proximal tubules.

Many nephrotoxicants cause mitochondrial dysfunction prior to cell death. For example, $HgCl_2$ altered mitochondrial function and mitochondria morphology in vivo in renal cortical mitochondria prior to proximal tubule necrosis.[302,303] When added to isolated rat renal cortical mitochondria, $HgCl_2$ produced similar changes in various respiratory parameters.[302,303] $HgCl_2$ also decreased mitochondrial function in rabbit RPTC prior to the onset of cell death.[304]

The mechanism of mitochondrial dysfunction induced by nephrotoxicants is toxicant-specific. Pentachlorobutadienyl-L-cysteine initially uncouples oxidative phosphorylation in RPTC cells by dissipating the proton gradient.[305–307] In contrast, tetrafluoroethyl-L-cysteine does not uncouple oxidative phosphorylation, but inhibits state-3 respiration by inhibiting sites I and II of the electron transport chain.[307] Other nephrotoxicants that have been shown to affect mitochondrial function include cisplatin,[308,309] citrinin,[310–313] ochratoxin A,[314,315] cephaloridine,[316,317] N-(3,5)-dichlorophenyl-succinimide,[318] DCVC,[319,320] and 2-bromohydroquinone.[321]

Mitochondria can act as primary or secondary mediators of necrosis, apoptosis, or autophagy.[55,322,323] When mitochondria are the primary target of nephrotoxicants, release of cytochrome c and other apoptotic inducing proteins (see previous) can occur early in the apoptotic process. If mitochondria are not a direct target of the nephrotoxicant, these proteins may still be released, but later in the apoptotic process. Central to the role of the mitochondrion in apoptosis is its ability to release pro-apoptotic proteins that activate caspases.[83] Mitochondria can also release caspase-independent DNAases such as Endo G and Omi (Fig. 30.4).

As previously described, a key difference in mitochondrial function during apoptosis and necrosis is the maintenance of ATP during apoptosis. Cellular ATP acts with the mitochondrial membrane potential as a switch that dictates whether a cell dies by apoptosis or necrosis.[82] If the mitochondrial membrane potential is lost quickly and cellular ATP is drastically decreased (below 20% to 10% of normal), then necrosis occurs. Events that result in the rapid loss of mitochondrial membrane potential include a rapid influx of Ca^{2+} into the mitochondria and the rupture of the inner and/or outer mitochondrial membranes.[82] In contrast, if the loss of membrane potential is slower and ATP is maintained through oxidative phosphorylation or glycolysis, then the cell is more likely to die through apoptosis. It should be noted that the majority of cells in culture derive their energy from glycolysis and can maintain ATP levels in the presence of mitochondrial dysfunction. Consequently, cultured cells are generally more susceptible to apoptosis than cells in vivo.

Mitochondria can mediate autophagy by mediating the activation of p53 and caspases.[55] As mentioned previously, cytosolic p53 can inhibit autophagy,[117] whereas caspases can cleave the pro-autophagic protein beclin-1.[55] Cleavage of beclin-1 by caspases is believed to be a primary signal by which cells switch from autophagy to apoptosis. However, more studies are needed to prove that such mechanism occurs during nephrotoxicant-induced renal cell death.

Studies are focusing on the exact protein targets within the mitochondria. For example, experiments seeking to determine the effect of hypoxia on mitochondrial electron transport chain constituents in rabbit RPTC reveal that complex I may be particularly sensitive.[324,325] Other studies have revealed that cisplatin-induced changes in oxidative phosphorylation, membrane potential, and ATP levels in rabbit RPTC are all preceded by inhibition of F(0)F(1)-ATPase (complex IV).[189]

Recent studies in renal cortical mitochondria suggest that an increase in Ca^{2+} influx activates mitochondrial calpain 10, which induces mitochondrial dysfunction by cleaving proteins in complex I of the electron transport chain (see previous text).[248,249,326] Such studies are critical to the understanding of the pathology of mitochondrial-mediated renal cell death and to identifying novel therapeutic targets for inhibition of renal cell death and possibly AKI.

The mechanisms by which toxicants induce release of mitochondrial cytochrome c have also received recent attention. Cytochrome c is normally bound to the inner mitochondrial membrane in association with cardiolipin, a mitochondrial-specific phospholipid. Oxidation of cardiolipin results in cytochrome c release,[55] and oxidant-induced release of cytochrome c is reported to be preceded by cardiolipin peroxidation.[327] These data may explain the protective effect of mitochondrial antioxidants[55] and may explain why inhibition of mitochondrial PLA_2 ($iPLA_2\gamma$)-induced apoptosis in renal cells, such as this enzyme, is suggested to aid in repair of phospholipid oxidation.[55,277] Once released from cardiolipin, cytochrome c may gain entry to the cytosol through a pore formed in the outer mitochondrial membrane by the pro-apoptotic proteins Bax, Bid, or Bak (see previous).

Endoplasmic Reticulum

The ER is the site of protein synthesis and processing as well as bioactivation and detoxification pathways, including those involving cytochrome P-450 and FMO. The ER is also a key regulator of cellular Ca^{2+} homeostasis. Under physiologic conditions, ER Ca^{2+} is typically released after receptor activation through the binding of inositol triphosphate (IP_3) to IP_3 receptors on the ER. Cytosolic free Ca^{2+} increases as a consequence of the ER Ca^{2+} release and is subsequently

decreased by ER uptake via the smooth ER Ca^{2+}-ATPases (SERCA) or extrusion via the plasma membrane Ca^{2+}-ATPase. Similar to the mitochondria, the ER can mediate necrosis, apoptosis, and autophagy.

Schnellmann and colleagues demonstrated that ER Ca^{2+} release is an important signaling pathway in RPTC necrosis.[214,216] Specifically, depletion of ER Ca^{2+} stores with the SERCA inhibitors thapsigargin or cyclopiazonic acid prior to antimycin A or hypoxia exposure inhibited necrosis.[214,216] Also, Ca^{2+} release from the ER activated calpains (calpain 1 and 2), which led to further disruption of ion homeostasis, cleavage of cytoskeleton proteins, and cell swelling, which ultimately resulted in necrosis.[214,218,219,244]

The cytoprotective effects of some stress proteins may be mediated through their ability to regulate ER Ca^{2+}. For example, iodoacetamide and DCVC can activate heat shock proteins (HSPs), calreticulin, and glucose related protein 78 (GRP78) in LLC-PK1 cells.[328] HSPs are typically ER localized proteins that are critical mediators of protein folding. Glucose-related protein 78 and calreticulin are Ca^{2+} binding proteins that aid in the sequestering of Ca^{2+} during toxic stress. Sequestering of Ca^{2+} by these proteins may protect renal cells by preventing cellular oxidative stress induced by Ca^{2+}-mediated mitochondrial injury.[329,330] The increased expression of Ca^{2+}-sequestering HSPs after injury is meant to condition the cell to withstand further necrotic injury.

The ER also mediates apoptosis induced by numerous nephrotoxicants, including acetaminophen, tunicamycin, Fas, and TNF-α.[109] A role for Ca^{2+} in calpain activation and subsequent activation of caspase has been described, as has a role for Ca^{2+} in induction of mitochondrial pore formation. It is suggested that the source of this Ca^{2+} is the ER.[55] ER Ca^{2+} release is also known to mediate the activation of the murine caspase-12 in mouse RPTC.[331] Mice in which caspase-12 had been genetically deleted were resistant to renal cell apoptosis induced by the ER stress agents tunicaymycin, brefeldin A, and thapsigargin, compared to wild-type animals. In contrast, kidneys from mice null for caspase-12 underwent similar degrees of apoptosis caused by the Fas antibody, TNF-α plus cycloheximide, or staurosporine—both agents that cause apoptosis by mechanisms other than ER stress. The key to the activation of caspases-12 in contrast to other caspases may be perturbations in the ER membrane and/or Ca^{2+} levels.

ER Ca^{2+} release is also hypothesized to mediate apoptosis by receptor-mediated mechanisms. Studies in nonrenal cells suggest that an ER Ca^{2+} receptor, type 3 inositol-1,4,5-trisphosphate (Ins[1,4,5]P_3), was upregulated in lymphocytes undergoing dexamethasone-induced apoptosis,[55,332] and that inhibition of Ins(1,4,5)P_3 receptors made T-lymphocytes resistant to apoptosis.[333] Interestingly, sensitivity to apoptosis in T-lymphocytes was restored after artificially increasing cytosolic Ca^{2+}. It is possible that Ca^{2+} released by the Ins(1,4,5) P3 receptor may induce apoptosis by mechanisms described previously (caspase activation, mitochondrial-mediated mechanisms, etc.). It is unknown whether Ins(1,4,5)P3 mediates nephrotoxicant-induced renal cell apoptosis.

Several studies have shown that autophagy is induced by ER stress and ER Ca^{2+} release,[56,115,116,334] and some of these studies have been performed in kidney tissue and renal cells.[56,116] For example, cyclosporine and thapsigargin, agents known to induce ER stress, increase autophagy in primary cultures of human renal cells and in rat kidneys after cyclosporine exposure in vivo.[116] Additionally, tunicamycin or brefeldin A induced autophagy in immortalized rat proximal tubular cells.[115] Mechanisms involved in autophagy induced by ER Ca^{2+} release have been discussed.[112]

Plasma and Organelle Membranes

Some nephrotoxicants can interact with the plasma membrane directly, increase ion permeability, and disrupt ion homeostasis. For example, amphotericin B is an antifungal polyene that binds to cholesterol in the plasma membrane and forms a pore that increases potassium and proton permeabilities.[118,335] Several heavy metals such as silver, gold, mercury, and copper can also react with the plasma membrane and increase potassium permeability.[336,337] It remains to be determined how changes in potassium and proton permeability ultimately lead to cell death.

Toxicants can disrupt cell volume and ion homeostasis by directly or indirectly inhibiting energy production. The loss of ATP inhibits the activity of membrane transporters that maintain differential ion gradients across the plasma membrane. The Na^+-K^+-ATPase is responsible for maintaining the normal Na^+ and K^+ gradients and the secondary ion transport processes. As ATP levels decrease, Na^+-K^+-ATPase activity decreases, resulting in K^+ efflux and Na^+ influx and a decrease in the normally negative membrane potential.[338] The decrease in the negative membrane potential allows Cl^-, as well as additional Na^+, to enter down a concentration gradient resulting in water influx and cellular swelling. Similar mechanisms can occur in kidney cells. For example, treatment of rabbit RPTC suspensions with the mitochondrial inhibitor antimycin A inhibits respiration within 1 minute, followed by ATP depletion, and the loss of the sodium and potassium gradients and transport over the next 5 to 10 minutes (Fig. 30.8).[219,339] Subsequent studies demonstrated that increases in Cl^- influx occurred between 15 and 30 minutes, during the late stages of cellular injury, followed by cellular rupture.[340] Decreasing extracellular NaCl concentrations by 50% with iso-osmotic substitution of mannitol decreased Cl^- influx, cellular swelling, and cellular rupture.[341] Furthermore, hyperosmotic incubation buffer decreased the cellular swelling and cellular lysis but not the increased Cl^- influx.[341] Thus, the delayed increase in Cl^- influx may be the trigger for the water influx and additional Na^+ influx that provides the osmotic force for cellular swelling and rupture.

Increased Cl^- influx occurs during the late stages of cell injury in RPTC and LLC-PK1 cells exposed to a variety of injury stimuli and toxicants, including $HgCl_2$, t-butylhydroperoxide, bromohydroquinone, tetrafluoroethyl-L-cysteine, and hypoxia.[341,342] The mechanism by

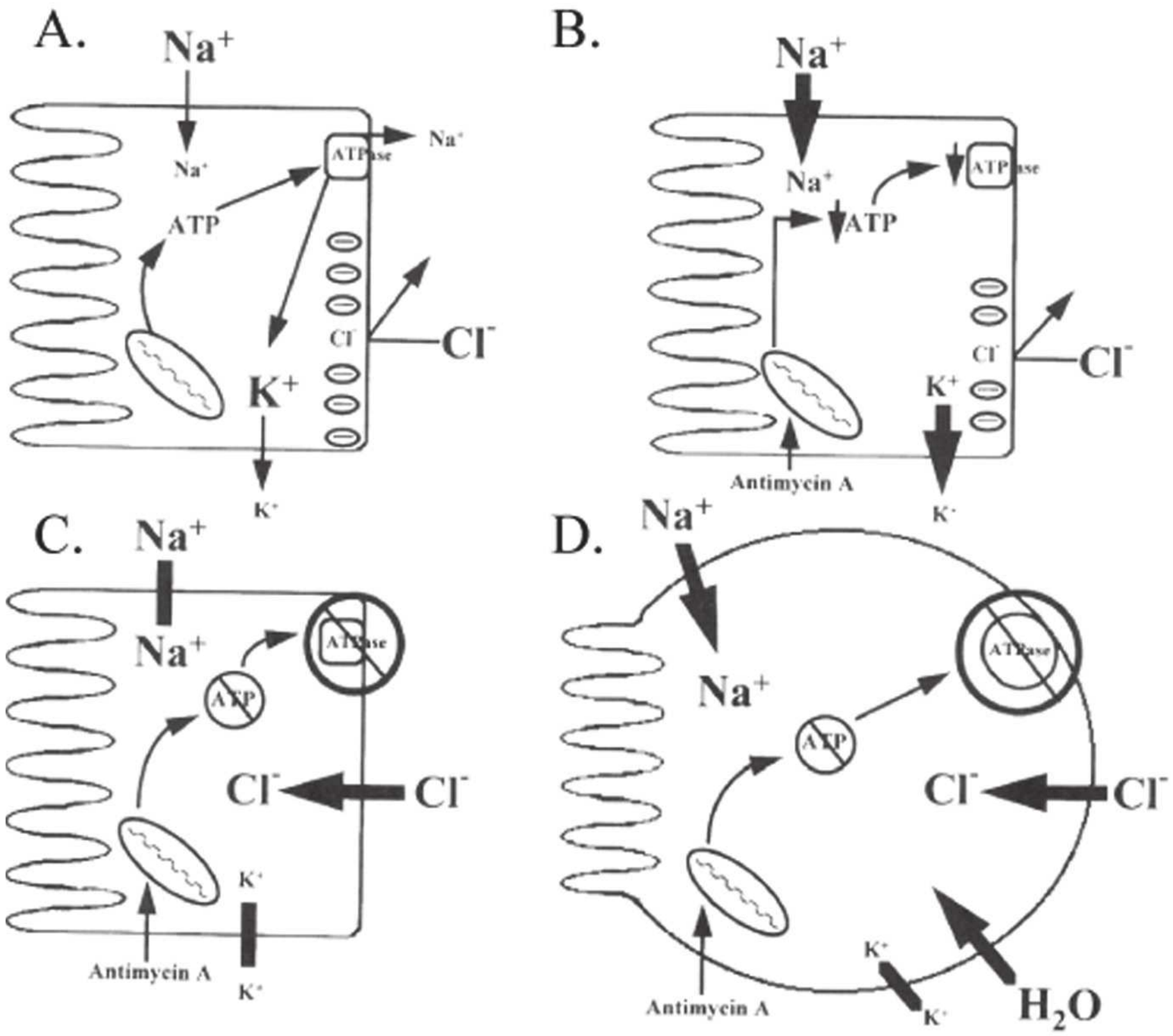

FIGURE 30.8 **A:** A schematic representation of a normally functioning renal cell. Note that the inside of the cell is negative with respect to the outside, which decreases the ability of Cl^- to enter the cell. **B:** The addition of a mitochondrial inhibitor such as antimycin A blocks cellular respiration, decreases ATP levels and Na^+-K^+-ATPase activity, increases Na^+ influx and K^+ efflux, and decreases the membrane potential. **C:** Subsequently, there is an increase in Cl^- influx (down the concentration gradient) by an unidentified pathway. **D:** The increase in Cl^- influx results in water influx, increased Na^+ influx, and cellular swelling. These processes provide the osmotic force that ultimately leads to cellular lysis.

which Cl^- influx occurs under these conditions is still under study, but was inhibited by blockers of Ca^{2+}-activated Cl^- channels (e.g., niflumic acid, indanyloxyacetic acid [IAA-94], 5-nitro-2-[3]-phenylpropylamino-benzoate [NPPB], and diphenylamine-2-carboxylate [DPC]).[150–153] The Cl^- influx was insensitive to the Cl^- channel blockers 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) or to the Cl^- transport inhibitors bumetanide and hydrochlorothiazide.[341] Therefore, the Cl^- influx that occurs during the late phase of cell death may be through a Ca^{2+}-activated Cl^- channel.

The plasma membrane can also mediate apoptosis due to the presence of death receptors. These include receptors for TNF-α, Fas, and LPS, all of which mediate renal cell apoptosis.[343,344] The exact receptors involved depend on the toxicant and cell type, but typically activate the extrinsic caspase-mediated pathway (see previous text). Additionally, and as mentioned previously, plasma membrane Ca^{2+} receptors can directly mediate apoptosis[213]; however, this pathway has not received as much attention with regard to nephrotoxicant-induced renal cell injury.

Lysosomes

Lysosomes are membrane-bound vesicles that pinch off from the Golgi-apparatus and contain a variety of hydrolytic enzymes.[345] Lysosomes contain hydrolytic enzymes that function as intracellular digestive enzymes under normal conditions. Lysosomes play an important role in the nephrotoxicity of several compounds such as aminoglycoside antibiotics. Lysosomes can also mediate a specific pathology called α_{2u} nephropathy. α_{2u}-Nephropathy occurs in male rats when compounds such as unleaded gasoline, d-limonene, 1,4-dichlorobenzene, tetrachloroethylene, decalin, 2,2,4-trimethylpentane, and lindane bind to α_{2u}-globulin, which prevents its normal degradation in renal proximal tubular cells.[346,347] α_{2u}-Globulin is synthesized in the liver of male rats under androgen control. Serum α_{2u}-globulin (18.7 kDa) is freely filtered by the glomerulus with approximately half being reabsorbed via endocytosis in the S_2 segment of the proximal tubule. The binding of these agents to α_{2u}-globulin inhibits its normal degradation and results in the accumulation of α_{2u}-globulin in the proximal tubule. Over time, the size and number of lysosomes increase, and characteristic protein-droplet morphology is observed. Ultimately, this leads to single-cell necrosis, the formation of granular casts at the junction of the proximal tubule and the thin loop of Henle, and cellular regeneration.

Recent data show that a 73 kDa heat shock cognate protein mediates the binding of α_{2u}-globulin to a 96 kDa membrane glycoprotein in male rat kidney lysosomes.[348] This HSP also is involved in the degradation of other cellular proteins. Treatment of rats with 2,2,4-trimethylpentane increases the rate of transport of not only α_{2u}-globulin into the lysosome, but also increases the rate of lysosomal

transport of many proteins. Increased transport is a result of α_{2u}-globulin-mediated increases in the level of the receptor proteins in the lysosomal membrane. Thus, α_{2u}-globulin may induce lysosomal overload by increasing the rate of transport of cellular proteins to the lysosome. In this manner, chronic exposure to the preceding compounds may lead to a chronic nephropathy, and may increase the incidence of renal adenomas/carcinomas by nongenotoxic mechanisms.

α_{2u}-Globulin nephropathy is sex- and species-specific, occurring in particular strains of male rats but not in female rats, male or female mice, rabbits, or guinea pigs. This raises the question if humans are at risk for α_{2u}-globulin-induced nephropathy and renal tumors. The current evidence suggest no because: (1) humans do not synthesize α_{2u}-globulin; (2) humans secrete fewer proteins in general and, in particular, fewer low-molecular-weight proteins in the urine than the rat; (3) the low-molecular-weight proteins in human urine are either not related structurally to α_{2u}-globulin, do not bind to compounds that bind to α_{2u}-globulin, or are similar to proteins in female rats, male Black Reiter rats, rabbits, or guinea pigs that do not exhibit α_{2u}-globulin nephropathy; and (4) mice excrete a low-molecular-weight urinary protein that is 90% homologous to α_{2u}-globulin but do not exhibit α_{2u}-globulin nephropathy and renal tumors after exposure to α_{2u}-globulin nephropathy-inducing agents.[349]

Aminoglycoside antibiotics also induce lysosomal dysfunction and cause acute renal failure (ARF) (see Chapter 31).[120,350,351] In this case, the aminoglycosides are filtered, bound to anionic phospholipids in the brush border, reabsorbed by endocytosis in the S_1 and S_2 segments of the proximal tubule, and accumulated in the lysosomes. Over time, the size and number of lysosomes increase and electron-dense lamellar structures called myeloid bodies appear. The myeloid bodies contain undegraded phospholipids and are thought to occur through aminoglycoside-induced inhibition of lysosomal hydrolases such as sphingomyelinase and phospholipases. However, the steps between lysosomal phospholipid overload and tubule cell death are less clear.

Lysosomes play a central role in autophagy.[56,112,113,237] Basically, autophagosomes formed during autophagy fuse with the lysosomes, which leads to the degradation of cellular proteins.[112] Studies in renal cells and tissues demonstrate that autophagy occurs after nephrotoxicant exposure including that induced by cyclosporine, cisplatin, thapsigargin, and after ischemia reperfusion.[56,115,116,237,352] Because it is not clear if nephrotoxicant-induced renal cell autophagy mediates cell death or survival, it is not known if the role of the lysosome in this type of pathology is protective or damaging. Further studies are needed to address this gap in knowledge.

CELLULAR DEFENSES

Renal epithelial cells have numerous defenses against both reactive intermediates and ROS (Fig. 30.6). GSH is the primary cellular protectant and the most abundant cellular nonprotein thiol in cells. It is found in high concentrations in at least three subcellular compartments (cytosol, mitochondria, nucleus).[353] Normally, GSH detoxifies electrophiles by forming a glutathione conjugate either directly or with the aid of GST. This includes compounds containing a quinone nucleus such as bromohydroquinone, which results in formation of mono- and di-substituted glutathione conjugates in renal cells.[321]

GSH also acts in conjunction with glutathione peroxidase and glutathione reductase to neutralize ROS. This produces organic peroxide that is reduced to water and alcohol by glutathione peroxidase, forming glutathione disulfide (Fig. 30.6). Glutathione disulfides are reduced to glutathione by glutathione reductase in an NADPH-dependent reaction. Catalase and superoxide dismutase are two other enzymes that detoxify ROS. Superoxide dismutase converts the superoxide anion to hydrogen peroxide, and catalase converts the hydrogen peroxide to water.

Several studies show that the activity of glutathione-dependent enzymes differs among the nephron. Differences in the activity of these enzymes may account for differences in the susceptibility of different kidney regions to oxidative stress. Cummings and associates[354] reported that the levels of glutathione peroxidase and γ-glutamylcysteinyl synthetase are higher in rat proximal tubular cells than distal tubule cells. The activity of glutathione reductase and GST appeared to be equal between the two cell populations; however, the proximal tubular cells had a much higher concentration of glutathione than distal tubular cells (27 nmol per mg for proximal tubular cells versus 13 nmol per mg for distal tubular cells).[355]

In order to protect against nephrotoxicant-induced cellular injury, GSH must be able to cross plasma and organelle membranes. The dicarboxylate carrier is one protein responsible for transport of GSH into mitochondria, and overexpression of this protein protected normal rat kidney-52E cell lines from both oxidant (t-butylhydroperoxide) and DCVC-induced apoptosis.[356] Protection against injury correlated to increases in mitochondrial GSH concentration, as well as decreased mitochondrial dysfunction, the release of cytochrome c, and caspase activation.

Vitamin C (ascorbic acid) is a very effective reducing agent and free radical scavenger and functions in the recycling of the vitamin E radical back to vitamin E.[357] Like GSH, vitamin C can detoxify compounds containing a quinone nucleus such as bromohydroquinone, but in this case vitamin C reduces the bromoquinone and the bromoquinone radical back to bromohydroquinone.[321]

Vitamin C can also promote repair and regeneration after nephrotoxicant-induced injury. For example, pharmacologic levels of vitamin C improved recovery of rabbit RPTC after exposure to t-butylhydroperoxide and DCVC.[358–360] Increased recovery correlated to increased cell number and mitochondrial function.[358] Vitamin C may also improve recovery by promoting collagen deposition in the extracellular matrix.[358] The effect of vitamin C was not the result of its antioxidant function, because both t-butylhydroperoxide and

DCVC caused the same amount of damage in treated and untreated cultures, and vitamin C was added after injury and removal of the nephrotoxicants.

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant found in cell membranes.[361] Vitamin E is a chain-breaking antioxidant because it contributes an electron to a peroxyl radical formed during lipid peroxidation and thereby prevents further lipid peroxidation. The vitamin E radical produced is unreactive and is recycled back to vitamin E. Vitamin E suppresses cyclosporin A-mediated toxicity in vivo in rat renal kidneys by inhibiting lipid peroxidation.[362] Vitamin E also protects against cephaloridine-induced toxicity in freshly isolated rat proximal tubule cells.[363] The protective effect of vitamin E on proximal tubule cell death correlated to decreases in lipid peroxidation.

Glycine

Glycine is cytoprotective in a number of models of renal injury.[364] In addition, several other small amino acids with similar structure to glycine, including D- and L-alanine, β-alanine, and 1-aminocylopropane-1-carboxylic acid, also protect against nephrotoxicity. This suggests that there is a structural requirement for cytoprotection with these compounds.

Glycine is cytoprotective against a diverse group of chemical insults such as anoxia, metabolic inhibitors, bromohydroquinone, halogenated alkene, and alkane cysteine conjugates and, to a lesser extent, t-butylhydroperoxide and $HgCl_2$.[215,365] The mechanism of glycine cytoprotection has remained elusive, but studies suggest that glycine acts during the terminal phase of cell injury.[365] Further, the mechanism of protection may be receptor-based as the neuronal glycine receptor antagonist strychnine protected against renal cell injury under a variety of conditions.[341,366] Strychnine binds to a low-affinity binding site on the basolateral membrane of the rabbit RPT cell in a saturable and reversible manner at the same concentrations that are cytoprotective.[245] Proteins corresponding to two of the three subunits of the neuronal strychnine-sensitive glycine receptor are expressed at the basolateral membrane as well, which may represent the glycine receptor β subunit.[366,367] The signal transduction pathway for the neuronal glycine receptor involves Cl^-. Collectively, these studies suggest that glycine and strychnine are cytoprotective by directly or indirectly altering Cl^- influx. These compounds may inhibit the ability of Cl^- to increase the osmotic force, which drives increases in cell swelling during injury.

Alternatively, Nichols and associates[368] proposed that glycine is cytoprotective in hepatocytes through its ability to inhibit calpains. However, Edelstein and coworkers[246] reported that glycine did not inhibit calpain activity in rat RPT exposed to hypoxia. Studies in rabbit RPT demonstrated that glycine and strychnine did not inhibit basal calpain activity, but did inhibit calpain activity observed during the late phase of cellular injury.[369] Later studies confirmed that glycine does not directly affect calpain activity, but rather inhibited toxicant-mediated extracellular Ca^{2+} influx, calpain translocation, and Cl^- influx.[369]

Acidosis

Acidosis is not a normal cellular defense mechanism per se; however, decreasing extracellular pH is cytoprotective in a variety of in vitro models of renal cell injury.[370,371] For example, reducing the extracellular pH to 6.8 to 7.0 protected against anoxia-induced cell death in isolated renal tubules.[371–373] In addition, Rodeheaver and Schnellmann[370] demonstrated that extracellular acidosis (pH 6.4) ameliorated renal proximal tubular cell death produced by a series of mitochondrial inhibitors (antimycin A, rotenone, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, oligomycin) and ion exchangers (nigericin, monensin, valinomycin), but potentiated cell death produced by the oxidants t-butylhydroperoxide, hydrogen peroxide, and ochratoxin A. Thus, the effect of extracellular acidosis on renal cell injury is toxicant-specific. Increased cell death in the presence of oxidants correlated to increases in oxidized GSH (GSSG), lipid peroxidation, and mitochondrial dysfunction. This suggests that extracellular acidosis during oxidant exposure decreases free radical detoxification.

It is unlikely that extracellular acidosis protects against nephrotoxic-induced renal cell injury by preserving mitochondrial function or ATP levels.[370–372] This hypothesis is supported by studies demonstrating that extracellular acidosis initiated at various times after toxicant exposure was still cytoprotective.[238,371] For example, extracellular acidosis initiated 15 minutes after antimycin A or carbonyl cyanide-p-trifluoromethoxy phenylhydrazone addition—a time point after the cessation of respiration, depletion of ATP, and increases in intracellular sodium and decreases in intracellular potassium—was completely cytoprotective at 45 and 105 minutes, respectively; however, cytoprotection did not prevent increases in Cl^- influx that occurs in the late stages of cell injury. Extracellular acidosis initiated 2 hours after tetrafluoroethyl-L-cysteine or t-butylhydroperoxide addition also was cytoprotective 2 hours later. These results demonstrate that the cytoprotective effect of extracellular acidosis occurs very late in the cell injury process distal to Cl^- influx.

Peroxisomes and Peroxisomal Proliferating Activated Receptors

Peroxisomes are membrane-bound vesicles that contain degradative enzymes for fatty acids and amino acids.[345] Peroxisomes also contain catalase, which converts H_2O_2 to oxygen and water. Thus, peroxisomes are a major site of antioxidant defense. In addition, peroxisomal proliferation has been linked to the preservation of mitochondrial function[374] and the reduction in renal cell death following injury induced by gallic acid,[375] cisplatin,[259,376] and ischemia/reperfusion-induced injury.

Peroxisomes also may protect against renal cell death via mechanisms linked to the activation of peroxisomal proliferator-linked receptors (PPAR). PPAR are members of a nuclear hormone-activated receptor and transactivation protein family.[377] At this time three different PPAR have been identified and cloned (PPAR-α, PAR-β/δ, and PPAR-γ,).[377] PPAR-β/δ are

detected in almost all tissues including the kidney cortex.[377–379] PPAR-γ is present in distal medullary collecting ducts, glomeruli, and the renal microvasculature.[379,380] PPAR-α is expressed in the proximal tubule, medullary thick ascending limbs, and the glomerular mesangial cells. It is hypothesized that differences in the distribution of PPAR isoforms may result in different mechanisms of protection between different cells.

Toxicants that activate PPAR are structurally diverse and include plasticizers (di(2-ethyhexl)phthalate),[381] herbicides,[382] hypolipidemic drugs (fenofibrate, clofibrate, and clofibric acid),[377] and antidiabetic drugs[377,383] (e.g., troglitazone and rosiglitazone).[383–385] Activators of PPAR increase the number of peroxisomes within the cell and increase the expression of enzymes for fatty acid β-oxidation including fatty acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme, and 3-ketoacyl-CoA thiolase.[381,382,386] Activators of PPAR isoforms also increase mitochondrial enzymes including carnitine palmitoyltransferase, medium chain acyl-CoA dehydrogenase, and pyruvate dehydrogenase complex.[376,379] The increase in these proteins is believed to be key in the protection against nephrotoxicants.[377,379,387]

Activation of PPAR, at least activation of PPAR-α, appears to protect against renal cell death. This hypothesis is supported by studies showing that the PPAR-α agonists clofibrate and WY14643 protect against ischemia/reperfusion-induced renal cell dysfunction in rat kidneys.[379,388] In addition, knocking out PPAR-α increased cisplatin-induced renal cell apoptosis in mouse kidneys, in correlation with the release of Endo G from the mitochondria.[259]

The mechanism of protection afforded by a PPAR-α agonist against nephrotoxicant-induced renal cell injury correlates to increases in mitochondrial function. This is demonstrated by in vivo studies demonstrating that PPAR-α induction correlated to increased mitochondrial medium-chain acyl-CoA dehydrogenase and pyruvate dehydrogenase complex activity, which correlated to decreases in cisplatin-induced proximal tubular necrosis.[376]

Schnellmann and colleagues demonstrated that overexpression of peroxisomal proliferator-activated receptor γ coactivator-1α (PGC-1α), the master regulator of mitochondrial biogenesis, induced mitochondrial biogenesis in renal proximal tubular cells.[252,389] Further, PGC-1α expression was increased following sublethal injury induced by t-butylhydroperoxide, and only returned to base values after mitochondrial function was restored. Interestingly, overexpression of PGC-1α following oxidant injury stimulated the recovery of mitochondrial and cellular function. These data suggest that PGC-1α is a therapeutic target for stimulating renal cell recovery and regeneration after nephrotoxicity.

SPECIFIC TOXICANTS

It is critical to identify the ultimate toxic species and the cell type targeted in order to understand the mechanism by which a chemical produces nephrotoxicity. For example, is the glomerulus, proximal convoluted tubule, proximal straight tubule, the thick ascending limb of Henle, or the distal convoluted tubule the target of the parent compound, a primary, or secondary metabolite? Thus, biotransformation, toxicokinetic, and morphologic studies are paramount in determining the sites of biotransformation, which metabolites reach the kidney, the quantity of metabolites in the kidney, the target cell type in the kidney, and ultimately the mechanism of nephrotoxicity. Other chapters in this book focus on specific toxicants such as analgesics (Chapter 32), antibiotics (Chapter 31), antineoplastics (Chapter 31), heavy metals (Chapter 34), immunosuppressives (Chapter 31), and radiocontrast media (Chapter 33).

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