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Cytoprotection by darbepoetin/epoetin alfa in pig tubular and mouse mesangial cells[1]

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Background. Erythropoietin has recently been found to have cytoprotective effects in the central nervous system (CNS) and retina. The purpose of this study was to determine if darbepoetin alfa (DA) has cytoprotective properties in renal tissues.

Methods. DA was studied in LLC/PK1 and mesangial cells. Renal cellular injury was induced in different experiments by prostaglandin D_2 synthase (PGDS), camptothecin, hydrogen peroxide, and hypoxia. Cellular proliferation and apoptosis were measured [apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay or by caspase-3 activity]. In a separate experiment, an inactive form of erythropoietin alfa was used to study receptor effects.

Results. DA protected against the antiproliferative effects of PGDS. In both LLC/PK1 (TUNEL and caspase-3) and mesangial cells (TUNEL), DA reduced the apoptotic stimulus of PGDS. Epoetin alfa was also found to reduce apoptosis. In LLC/PK1 cells, DA reduced apoptosis induced by camptothecin, but not hydrogen peroxide. DA reduced LLC/PK1 apoptosis induced by hypoxia when added 24 hours before hypoxia, but not when given concurrent with the hypoxic stimulus. Erythropoietin inactive did not protect against PGDS-induced apoptosis.

Conclusion. DA has renal antiapoptotic effects for both toxic and hypoxic stimuli. The effect may be mediated via the Erythropoietin receptor.

Erythropoietin is a glycoprotein hormone produced in adults primarily in the kidneys. It was originally understood to be an erythropoietic stimulator, promoting survival of committed erythroid progenitor cells [1, 2]. The hormone's effects are signaled through the erythropoietin receptor and increasingly understood transduction intermediates [3]. The recombinant forms of the hormone have been used to effectively treat anemia in large numbers of patients with renal insufficiency, cancer, and other disorders. Darbepoetin alfa (DA), an erythropoetic agent with extended duration of action, has a serum half-life approximately three times as long as epoetin alfa [4].

Recently, it has been suggested that erythropoietin may have effects outside of the hematopoietic system. Stimulation of the erythropoietin receptor reportedly leads to biologic actions in vascular endothelial and smooth muscle cells [5, 6], and the uterus [7]. Moreover, recent studies suggest that erythropoietin may have important cytoprotective effects in neurons in the brain and retina [8–11]. Hypoxia leads to increased erythropoietin production by neurons via sensing by hypoxia inducible factor-1 [12]. It is reported that erythropoietin, via signal transduction intermediates, greatly reduces neuronal apoptosis [13]. Generation of erythropoietin would seem to be a normal protective mechanism used by endangered neurons. Moreover, with ischemic and traumatic brain insults in animal models, exogenously administered erythropoietin may reduce functional deficits and greatly reduce areas of necrosis [8, 10]. The protective effect purportedly occurs with administration prior to or concurrent with the insult, or up to 3 hours after the insult.

Renal cellular injury occurs in a wide variety of acute and chronic clinical settings. One route of renal cell death, apoptosis, or programmed cell death, has been demonstrated in diseases such as glomerulonephritis, acute and chronic renal failure, diabetic nephropathy, and polycystic kidney disease [14, 15]. Promotion of cellular survival by reducing programmed cell death has the potential to benefit many renal disease states. Recently, it has been found that functional erythropoietin receptors are produced in renal tubular and mesangial cells [16]. In addition, Rosenberger et al [17] recently reported finding hypoxia inducible factor-1 activity in renal tubular cells. These intriguing findings suggest the possibility that erythropoietin could have cytoprotective effects in the kidneys, as it does in the brain. The purpose of these

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studies was to evaluate the ability of DA to positively affect the balance between renal cellular proliferation and programmed cell death.

METHODS

Cell culture

The pig kidney epithelial cell line LLC-PK1, provided by Dr. Julia Lever, University of Texas, Houston, was maintained in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal calf serum (FCS), 7.5% sodium bicarbonate, 15 mol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 200 mmol/L L-glutamate, 50 U/mL penicillin, and 50 μg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA) at 37°C in 5% CO_2. The mouse mesangial cell line was provided by Dr. Pravin Singhal, North Shore-Long Island Jewish Hospital, Manhasset, New York, and maintained in DMEM supplemented with 10% heat-inactivated FCS, 3.7% sodium bicarbonate, 1% HEPES, and 50 U/mL penicillin, and 50 μg/mL streptomycin. For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay, the cells were plated at a density of 4.0×10^4 cells/cm^2 onto Nunc Permanox 8-well chamber slides (Rochester, NY, USA), otherwise cells were plated in 35 mm plates. In experiments in which DA was used, cells were pretreated with DA for 1 hour prior to adding apoptotic stimulators for 16-hour incubations. Hypoxic simulation was produced using a controlled chamber with oxygen monitoring and carbon dioxide/nitrogen gas.

Detection of apoptosis

Nuclear fragmentation consistent with apoptosis was determined by the TUNEL assay as previously described in Maesaka et al [14] using the ApopDetek Cell Death Assay Kit (Enzo, Farmingdale, NY, USA). Confluent cells were cultured as described above and then incubated with prostaglandin D_2 synthase (L-PGDS) (50 μg/mL) and DA (50 ng/mL) individually and in combination for 16 hours. Cells were scored as TUNEL positive if they appeared as condensed cells with dark nuclei. In all assays the apoptotic index (AI) is calculated as the percentage of TUNEL positive cells and represents at least 1000 cells counted and is expressed as the mean ± SEM. In addition to the TUNEL assay described above, apoptosis was confirmed by caspase-3 activity as previously described [15].

Cell proliferation by [^{3}H]-thymidine incorporation. Cells were grown as described above until confluency. Cells were then incubated with L-PGDS (50 μg/mL) and DA (50 ng/mL) individually and in combination for 16 hours, followed by a pulse-label with [^{3}H]-thymidine (1 μCi/mL) for 3 hours. At the end of the incubation period, the medium was removed and the cell monolayers washed with ice-cold phosphate-buffered saline (PBS) three times followed by one wash with ice-cold 10% trichloroacetic acid (TCA) and incubated for 10 minutes at 37°C. The cells were solubilized by the addition of 200 μL of a 0.1% sodium dodecyl sulfate (SDS)/0.1 N NaOH solution. After a 10-minute incubation, 100 μL of cell lysate was added to 5 mL of scintillation fluid (Instagel, Packard, Wellesley, MA, USA) and the incorporation of [^{3}H]-thymidine into DNA was determined by liquid scintillation spectroscopy. Incorporation was expressed as dpm/mg of protein/hour.

Protein assay. Protein content of cell extracts were determined with bicinchoninic acid [18].

Statistics

Results are expressed as mean ± SEM of at least three independent experiments, each performed in duplicate at different times. A paired Student *t* test was used to compare the basal versus treated preparations and an analysis of variance (ANOVA) was used to compare the mean values between treatments. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of DA and L-PGDS challenge on LLC-PK1 proliferative activity

To assess the effects of L-PGDS and DA on proliferation, cells were incubated individually and in combination with each compound(s) for 16 hours. DA by itself had no significant effect on [^{3}H]-thymidine incorporation. In contrast, L-PGDS incubation by itself led to a 33.1% reduction in proliferation ($P < 0.05$). When DA was added to LLC-PK1 cells prior to L-PGDS incubation, cellular proliferation was not significantly different than in control cells (Fig. 1).

Effects of DA, epoetin alfa, and L-PGDS challenge on LLC-PK1 and mouse mesangial cell apoptotic activity

Incubation with DA by itself had no significant effect on basal LLC-PK1 apoptotic activity. Incubation for 16 hours with L-PGDS led to a 3.6-fold increase in apoptosis ($P < 0.001$). Pretreatment with DA significantly reduced the proapoptotic effect of L-PGDS (Fig. 2).

To determine whether the antiapoptotic effect was specific for darbepoetin alfa, or more generically an erythropoietin class effect, we repeated this experiment using epoetin alfa at a concentration of 10 u/mL. Epoetin alfa demonstrated essentially the same antiapoptotic effect, reducing LLC-PK1 apoptosis after exposure to L-PGDS.

Mouse mesangial cells had a basal apoptotic index (TUNEL) of 5.3%. Incubation with DA did not

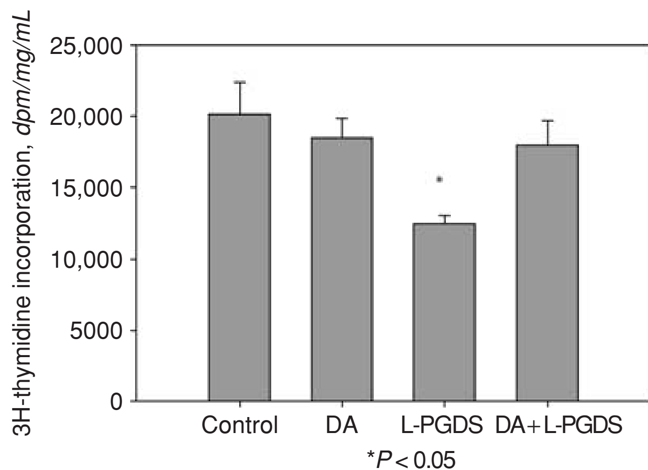

Fig. 1. The effect of darbepoetin alfa (DA) on LLC-PK1 cell proliferation. Cells were either untreated (control), incubated separately with DA (50 ng/mL) or prostaglandin D_2 synthase (L-PGDS) (50 µg/mL) for 16 hours, or pretreated with DA 1 hour prior to a 16-hour L-PGDS exposure.

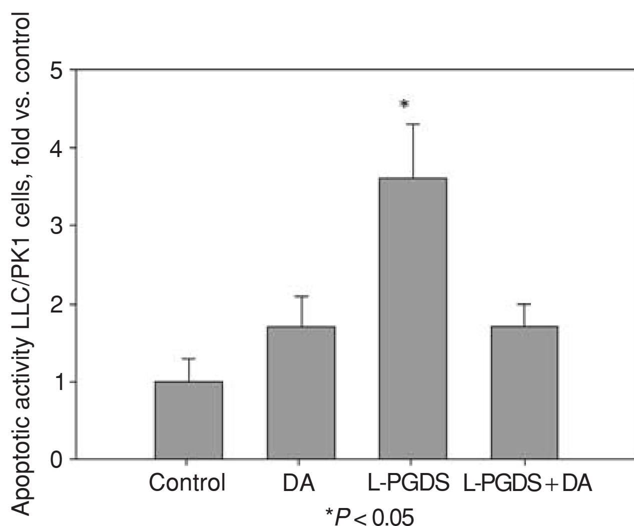

Fig. 2. The effect of darbepoetin alfa (DA) on apoptosis induced by prostaglandin D_2 synthase (L-PGDS) in LLC-PK1 cells. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay. Cells were either untreated (control), incubated separately with DA (50 ng/mL) or L-PGDS (50 µg/mL) for 16 hours, or pretreated with DA 1 hour prior to a 16-hour L-PGDS exposure. Results are reported as fold-difference vs. controls (assigned value of 1).

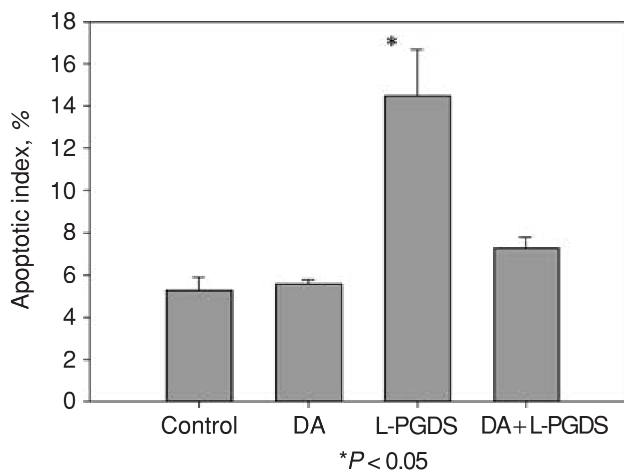

Fig. 3. The effect of darbepoetin alfa (DA) on apoptosis induced by prostaglandin D_2 synthase (L-PGDS) in mouse mesangial cells. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay. Cells were either untreated (control), incubated separately with DA (50 ng/mL) or L-PGDS (50 µg/mL) for 16 hours, or pretreated with DA 1 hour prior to a 160-hour L-PGDS exposure.

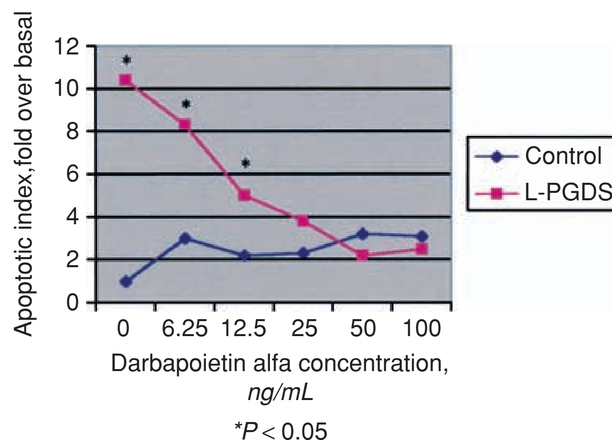

Fig. 4. The dose-effect of darbepoetin alfa (DA) on apoptosis induced by prostaglandin D_2 synthase (L-PGDS) in LLC-PK1 cells. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay. Cells were either untreated (control), incubated separately with DA (varying concentrations from 0 to 100 ng/mL), or pretreated with DA 1 hour prior to a 16-hour L-PGDS (50 µg/mL) exposure.

significantly change the apoptotic index (P = NS). Incubation with L-PGDS caused the apoptotic index to increase to 14.4%. Pretreatment with DA significantly reduced the apoptotic effect of L-PGDS (Fig. 3).

Dose response of DA to protect against L-PGDS–induced LLC-PK1 cell apoptosis measured by TUNEL assay

In a separate incubation of LLC-PK1 cells with L-PGDS, apoptotic activity was increased by 10.4-fold compared to control cells ($P < 0.05$). When cells were pretreated with DA at concentrations (doses) from 6.25 to 100 ng/mL, the apoptotic effect of L-PGDS was significantly reduced. The protective effect increased progressively from doses of 6.25 ng/mL up to 25 ng/mL. At a 25 ng/mL dose of darbepoetin alfa the protective effect was essentially complete. The dose of 100 ng/mL had a similar complete protective effect compared to the 25 to 50 ng/mL doses (Fig. 4).

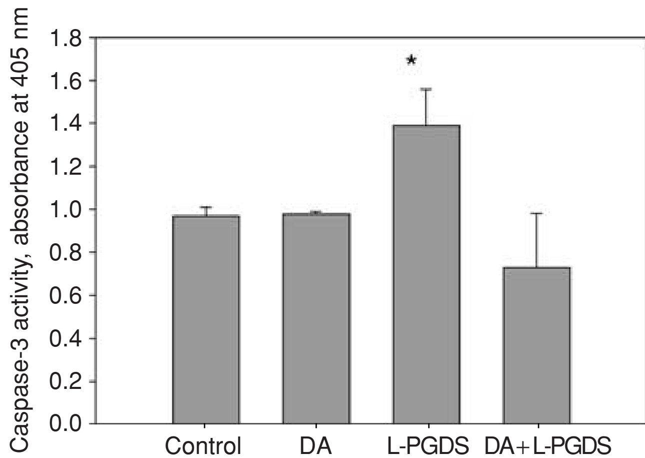

Fig. 5. The effect of darbepoetin alfa (DA) on apoptosis induced by prostaglandin D_2 synthase (L-PGDS) in LLC-PK1 cells. Apoptosis was measured by caspase-3 assay. Cells were either untreated (control), incubated separately with DA (50 ng/mL) or L-PGDS (50 μg/mL) for 16 hours, or pretreated with DA 1 hour prior to a 16-hour L-PGDS exposure.

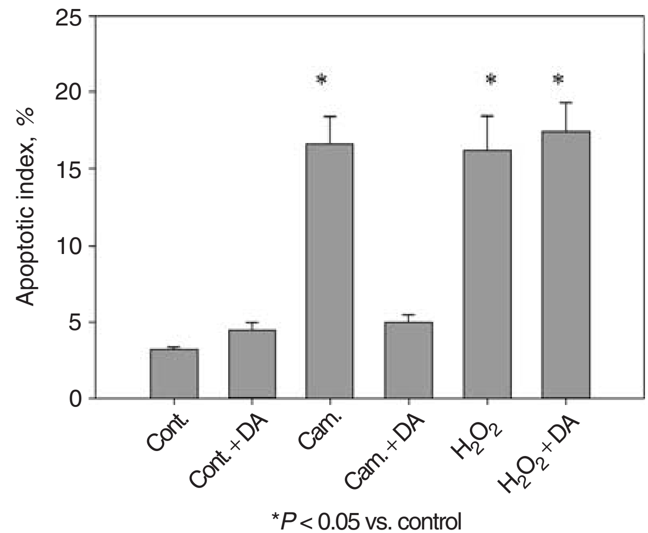

Fig. 6. The effect of darbepoetin alfa (DA) on apoptosis induced by camptothecin (5 μmol/L) and hydrogen peroxide (H_2O_2) (0.65 mmol/L) in LLC-PK1 cells. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay. Cells were either untreated (control), incubated separately with camptothecin or H_2O_2, or pretreated with DA 1 hour prior to a 16-hour exposure to camptothecin or H_2O_2.

Effect of DA and L-PGDS challenge on LLC-PK1 apoptotic activity measured by caspase-3 assay

In the previous experiments, TUNEL assay was used to measure apoptosis. To confirm these results, experiments were performed with another apoptosis measure, caspase-3 activity. Incubation of LLC-PK1 cells with DA led to no significant change in caspase-3 activity. In contrast, incubation with L-PGDS by itself led to a significant increase in caspase-3 activity ($P < 0.05$). When LLC-PK1 cells were pretreated with DA and then incubated with L-PGDS, caspase-3 activity was not significantly different than in control cells (Fig. 5).

Effects of DA on protection against apoptosis induced by camptothecin and hydrogen peroxide

There was no significant difference between basal apoptotic activity in control LLC-PK1 cells and those treated with DA 10 ng/mL (P = NS). Incubation of cells with camptothecin led to an increase in apoptotic activity compared to control cells from 2.4% to 16.1% ($P < 0.001$). When cells were pretreated with DA prior to incubation with camptothecin, apoptotic index was reduced to near baseline levels. Incubation of cells with hydrogen peroxide resulted in an increase in apoptotic index from 2.4% to 15.9% ($P < 0.001$). Pretreatment with DA had no protective effect on hydrogen peroxide induced apoptosis (Fig. 6).

Effects of DA on protection against apoptosis induced by hypoxia in LLC-PK1 cells

When grown in hypoxic conditions (3.5% oxygen) for 15 hours, apoptotic activity of LLC-PK1 cells measured by caspase-3 increased significantly. When cells were pretreated 24 hours before hypoxia with 10 ng/mL DA there was a significant attenuation of apoptosis. In contrast, when cells were treated with DA at the time of initiation of hypoxia, there was no significant protection against apoptosis (Fig. 7).

Study of inactive recombinant human erythropoietin in LLC-PK1 cells

To evaluate the necessity of erythropoietin receptor binding for protection against apoptosis, an experiment was performed with inactive recombinant human erythropoietin (kindly provided by Ken Aoki, Tony Lorenzini, and Steven Elliott, Amgen, Inc., Thousand Oaks, CA, USA). This substance is very similar to recombinant human erythropoietin in physicochemical properties, but does not activate the erythropoietin receptor. By itself, inactive erythropoietin did not significantly alter the basal apoptotic activity. Incubation of the cells with L-PGDS led to a sharp increase in apoptotic activity. Treatment with inactive recombinant human erythropoietin did not significantly reduce the apoptotic activity induced by L-PGDS (Fig. 8).

DISCUSSION

We studied the ability of DA to protect against apoptosis induced by renal toxins (L-PGDS, camptothecin, and hydrogen peroxide) and hypoxia in porcine tubular

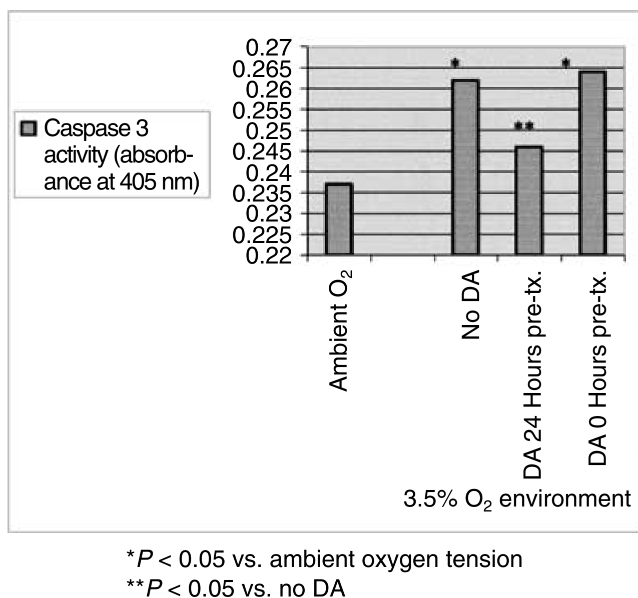

Fig. 7. The effect of darbepoetin alfa (DA) on apoptosis induced by hypoxia in LLC-PK1 cells. Apoptosis was measured by caspase-3 assay. The first vertical bar represents basal apoptotic activity with cells grown in ambient oxygen. The second vertical bar represents cells grown in 3.5% oxygen, with no DA added. The third vertical bar represents cells grown in 3.5% oxygen, with DA (50 ng/mL) added 24 hours prior to exposure to the hypoxic environment. The fourth vertical bar represents cells grown in 3.5% oxygen, with DA (50 ng/mL) added concurrently with initiation of exposure to the hypoxic environment.

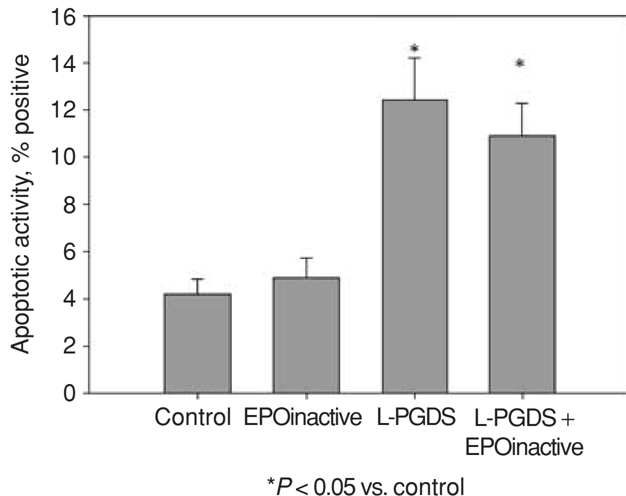

Fig. 8. The effect of inactivated erythropoietin inactive on apoptosis induced by prostaglandin D_2 synthase (L-PGDS) in LLC-PK1 cells. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay. Cells were either untreated (control), incubated separately with erythropoietin inactive (50 ng/mL) or L-PGDS (50 µg/mL) for 16 hours, or pretreated with erythropoietin inactive 1 hour prior to a 16-hour L-PGDS exposure.

cells, and a toxin (L-PGDS) in mouse mesangial cells. We found that DA was highly protective against all challenges except hydrogen peroxide. Moreover, the dose response relationship was studied for protection against L-PGDS–induced apoptosis. Indeed, there was a robust dose response effect demonstrated. To determine whether the cytoprotection was mediated via the erythropoietin receptor, we studied an altered form of erythropoietin that does not interact with the receptor. We found that this agent did not confer protection against apoptosis. Taken together, these findings suggest that DA has important protective effects in renal mesangial and tubular tissues, probably mediated via the erythropoietin receptor. In addition, we studied epoetin alfa, and found that this agent offered similar antiapoptotic effects in LLC-PK1 cells compared to darbepoetin alfa. This suggests that the protective actions of darbepoetin alfa may represent a class effect.

Erythropoietin is best understood as the body's major promoter of erythropoiesis [19]. It accomplishes this by promoting survival of committed erythroid precursors, primarily by inhibiting cellular apoptosis [20]. DA has five amino acid differences from erythropoietin allowing for two additional N-linked carbohydrates increasing the carbohydrate content from 40% to 51% [21, 22]. The structural change results in a longer serum half-life that allows for an extended dosing interval [23]. We chose to study this molecule, rather than recombinant human erythropoietin, because the extended serum half-life might be advantageous for future anticipated clinical use in states such as acute renal failure.

Recently, erythropoietin has been suggested to have profound cytoprotective effects in neurons of the brain and retina. For example, Brines et al [8] reported that experimental ischemic or concussive brain injury in rats could be reduced by 50% to 75% by treatment with erythropoietin. The data suggest that erythropoietin gene expression in many tissues, including the brain, is regulated by hypoxia via hypoxia-inducible factor-1 [24]. Indeed, in the brain, hypoxia appears to induce production of both erythropoietin and the erythropoietin receptor [9, 10, 25]. Subsequent local binding of erythropoietin to its receptor may lead to activation of antiapoptotic signal pathways [13] Indeed, Siren et al [10] reported that treatment with erythropoietin led to nearly complete protection against apoptosis after ischemic stroke in rats. The antiapoptotic effect of erythropoietin are reported to greatly reduce functional neurological deficits in animal studies [26–32].

The possibility that erythropoietin may play a cytoprotective role in the kidney is suggested by clinical evidence associating erythropoietin treatment with improved renal outcomes [33, 34]. Furthermore, Westenfelder, Biddle, and Baranowski [16] report that erythropoietin receptors are expressed in human, rat and mouse renal cells, and that erythropoietin stimulated cell proliferation. More recently, Rosenberger et al [17] reports expression of hypoxia-inducible factor-1 in rat renal tubular cells. While the kidney is critically linked with erythropoietin production as part of regulated erythropoiesis, it is

possible that erythropoietin could also have an important effect in renal cytoprotection. In fact, other growth factors, such as epidermal growth factor, hepatocyte growth factor and insulin-like growth factor-1 may have protective effects in experimental models of renal failure [35].

We tested the ability of DA to protect against toxins, L-PGDS, camptothecin and hydrogen peroxide, and against hypoxia. Of these, L-PGDS has special properties of interest. This lipocalin-type prostaglandin, also known as beta-trace protein, has effects on sleep, platelet function, vasomotor tone, and is an allergic and inflammatory mediator [36]. Depending on its relative glycosylation, its size ranges from 20 to 29 kD. With renal insufficiency, L-PGDS accumulates in serum, and may act as a mediator of renal disease progression [14]. Moreover, we have previously found L-PGDS to be a potent renal tubular toxin [14].

We found that DA protected against L-PGDS induced apoptosis in both renal tubular and mesangial cells. The effect in tubular cells was dose dependent, with a peak effect at a concentration that approximates serum levels of DA achieved in anemia treatment. It appears that the protective effect might be mediated via the erythropoietin receptor, since inactive erythropoietin, which does not interact with the receptor, did not provide protection. We found that DA reduced the antiproliferative effect of PGDS in LLC/PK1 cells, but did not itself have a proproliferative effect. One possible explanation for this finding is that DA (or erythropoietin generally) does not function as a basal growth factor for renal tubular cells. Rather, DA may promote cellular survival by preventing apoptosis during toxic challenge.

DA also provided protection against apoptosis induced by camptothecin. This alkaloid, an inhibitor of DNA topoisomerase I, is a potent stimulus of apoptosis in a variety of tissue types including renal carcinoma cells [37]. In contrast, we did not find DA to protect against apoptosis induced by hydrogen peroxide. The mechanistic implications of this finding are a subject of further study. Hypoxia is a potent stimulus for cellular apoptosis, and we did find this to be true in LLC-PK1 cells. DA was not protective when administered concurrently with the initiation of hypoxia, but was protective when incubated for 24 hours prior to hypoxia. This timing is in some contrast to studies of neurologic injury in rats, where epoetin alfa administration up to 6 hours after an ischemic insult has been found to be protective [8].

DA's antiapoptotic effects against toxins and hypoxia suggest that the drug may eventually be found to be beneficial for patients with acute renal failure. In normal homeostasis, cellular survival depends on an interplay between apoptotic and antiapoptotic stimuli that usually balances strongly in favor of survival. In cellular injury, such as in acute renal failure, the balance shifts, and apoptosis occurs [38–40]. Indeed, apoptosis plays an important role on the pathogenesis of acute renal failure. However, it is not yet entirely possible to separate out the relative roles of apoptosis/necrosis in cell death in acute renal failure. Our findings suggest that DA may help prevent toxic and ischemic apoptosis and promote cellular survival. Moreover, DA might help in the process of postinjury recovery and regeneration. We would suggest that animal studies and clinical trials of DA in acute renal failure and other renal diseases may be warranted.

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