Elevated Akt Phosphorylation as an Indicator of Renal Tubular Epithelial Cell Stress

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Characterization of the phosphoinositide 3-kinase-signaling pathway in a human renal tubular epithelial cell (TEC) line HKC-8 revealed high levels of Akt phosphorylation in serum-starved cultures. In contrast to Erk1/2, little additional phosphorylation of Akt was observed after cytokine or serum stimulation. Replacement of the conditioned medium attenuated Akt phosphorylation such that 90 min after the addition of warmed serum-free media, Akt phosphorylation had fallen sufficiently to allow an epidermal growth factor-stimulated increase to be detected readily. Although the mechanism by which the phosphoinositide 3-kinase/Akt pathway is activated in serum-starved TEC is unknown, the mediator responsible is secreted from these cells. Thus, conditioned media removed from a dish of quiescent TECs stimulated Akt phosphorylation in washed TEC cultures within 10 min. Biochemical characterization of the bioactive agent identified a heat labile factor of small apparent molecular mass. The basal level of Akt phosphorylation observed in serum-starved cultures was inhibited by wortmannin at concentrations that demonstrated its dependence on 3-phosphoinositide synthesis (IC50 = 8 nM). Regular removal of conditioned media from TEC cultures and its replacement with serum-free media resulted in a sustained attenuation of Akt phosphorylation. Interestingly, after 5 days of this treatment, washed TEC cultures contained a greater number of viable cells than cultures maintained in conditioned media throughout. This observation was not explained by a difference in the rate of DNA synthesis. Instead, the number of cells undergoing apoptosis increased markedly in the unwashed cultures. Consequently, we propose that in HKC-8 cells Akt phosphorylation is up-regulated in an effort to minimize cell death. This stress-activated response is initiated by a factor secreted into the conditioned medium that stimulates the phosphoinositide 3-kinase signaling pathway.

Loss of renal tubular epithelial cell (TEC) function and atrophy of tubular architecture is a common feature of acute and chronic renal disease. Ischemia and cytotoxic agents that permeate the glomerular filtration mechanism damage these cells directly. In many forms of chronic renal disease, perturbation of the glomerular architecture results in potentially toxic agents entering the interstitium and mediating the injury. In both forms of disease, a series of common pathogenic processes are initiated, resulting in epithelial cell death and the loss of renal function. Consequently, damage to the interstitium is widely regarded as the most accurate prognostic indicator of renal failure (1, 2).

Serum deprivation and exposure to noxious agents such as oxidants, ionizing radiation, and alkylating agents have direct effects upon mammalian gene expression and cell proliferation (3). The resultant cellular response depends upon the duration and intensity of the exposure. Cells can enter a quiescent state to limit metabolic activity, mechanisms to initiate DNA repair and mitogenesis may be stimulated, or cells can undergo cell death by necrosis or apoptosis. Under such circumstances, decisions regarding cell cycle progression and initiation of transcriptional activity need to be made that require activation of intracellular signaling pathways. Until recently, the identity of these signaling events remained unclear. However, it is now accepted that exposure to cytotoxic agents evokes ligand-independent activation of many receptors including G-protein-coupled receptors for lysophosphatidic acid (LPA) (4) and angiotensin II (5), cytokines such as transforming growth factor β1 (6) and tumor necrosis factor α (7), and growth factors such as EGF and platelet-derived growth factor (8, 9). In this way, a spectrum of intracellular effectors are activated in a manner analogous to that observed after ligand addition.

Of these signaling events, the activation of phosphoinositide 3-kinase (PI3K) activity has attracted particular attention. In mammals, PI3K is a large enzyme family containing eight human catalytic subunits (10). PI3K activity is increased after stimulation by a wide variety of chemokines, cytokines, and growth factors. Largely through the use of the pharmacological inhibitors wortmannin and LY294002, PI3K has been shown to play a critical role in cell proliferation and survival (11). The major 3-phosphoinositide products are PtdIns 3-P, PtdIns (3,4)P2, and PtdIns (3,4,5)P3 (12). Synthesis of PtdIns 3-P is constitutive, but PtdIns (3,4)P2 and PtdIns (3,4,5)P3 are dramatically elevated after ligand stimulation. This reflects their greater significance in receptor-mediated signal transduction. Identity of downstream targets for these 3-phosphoinositides remained unclear for many years until the identification of the serine threonine protein kinases Akt and phosphoinositide-dependent kinase-1 (13). The protooncogene c-Akt (also termed protein kinase B) was originally implicated in the PI3K-signaling pathway downstream of the platelet-derived growth factor receptor (14). After cell stimulation, the pleckstrin homology domain within Akt binds PtdIns (3,4,5)P3, allowing its translocation to the plasma membrane, where it becomes phospho-
rylated on residues Thr-308 and Ser-473. Phosphorylation of Thr-308 is mediated by the 3-phosphoinositide-dependent protein kinase (PKD-1), but the identity of the kinase that phosphorylates residue Ser-473 remains controversial. Phosphorylation of Akt is now widely used to identify activation of the PI3K-signaling pathway. Akt promotes cell survival in part by phosphorylating and inhibiting several proteins involved in promoting apoptosis (15).

Although the anti-apoptotic role of the PI3K/Akt pathway is widely acknowledged, few studies demonstrate increased levels of Akt phosphorylation under conditions where cell viability has been compromised. This is somewhat surprising given that activation of this signaling pathway might be expected in response to stress and cell damage. Indeed, inactivation and dephosphorylation of Akt has been described after hyperosmotic stress (16). In this study we investigate the significance of markedly elevated levels of Akt phosphorylation found in serum-starved cultures of the renal TEC line HKC-8. We investigate whether this increased Akt phosphorylation correlates with alterations in either cell viability or proliferation.

MATERIALS AND METHODS

Cell Culture—Stock cultures of the TEC line HKC-8 cells were generous gifts from Dr. L. Racusen, Dept. of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD. They were derived from human proximal tubule, and they have been extensively characterized (17). Cells were passaged every 3–4 days in 90-mm dishes (Nunc) using DMEM-F-12 supplemented with 10% FBS, insulin-transferrin-sodium selenite media supplement (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cultures were incubated in a humidified atmosphere of 5% CO₂, 95% air at 37°C. For experimental use, cells were switched to serum-free DMEM-F-12. After 16 h, cells were confluent and quiescent. Cell viability was assessed by trypan blue staining and quantifying dye exclusion.

Preparation of Cell Lysates—Confluent HKC-8 cell cultures, treated in the absence or presence of various cytokines at 37°C were lysed at 4°C using 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, 1% Triton-X100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 2 μg/ml aprotnin, 5 μg/ml leupeptin, and 5 μg/ml antipain (lysis buffer). Lysates were clarified by centrifugation (13,000 × g, 20 min), and the supernatants were transferred to a fresh tube, extracted with 2× sample buffer (200 mM Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol (pH 6.8)), and fractionated by SDS-PAGE.

In Vitro Kinase Assay—Lysates were incubated with agarose-linked anti-Akt antibody (Akt1G1, Cell Signaling Technology, Inc.) for 4 h at 4°C. The immune complexes were isolated by centrifugation (13,000 × g, 20 min), and the supernatants were transferred to a fresh tube, extracted with 2× sample buffer (200 mM Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol (pH 6.8)), and fractionated by SDS-PAGE.

RESULTS

Serum Starved HKC-8 Cells Have High Basal Akt Phosphorylation—Serum deprivation after attainment of confluence is a widely used treatment to obtain cultures in a quiescent state (18). Under these conditions, cells exit the cell cycle and enter a state of G₀. The addition of serum, cytokines, or growth factors allows synchronous entry into the cell cycle, and a unified response is obtained. In contrast to numerous cell types previously examined, quiescent HKC-8 cells demonstrated a high level of Akt phosphorylation (Fig. 1A). Consequently, after treatment with FBS or a variety of cytokines, no additional Akt phosphorylation was observed. This was not due to a failure of the treatment regimen because quiescent cultures had low levels of phosphorylated Erk1/2 (Fig. 1B), and phosphorylation of Erk1/2 increased dramatically after stimulation with serum, bradykinin, LPA, or EGF. In contrast, vasopressin and angiotensin II evoked a more modest stimulation.

We also examined if elevated Akt phosphorylation in quiescent cultures of HKC-8 cells corresponded to constitutively increased Akt enzyme activity. Lysates were prepared from control and serum-stimulated cultures of murine Swiss 3T3 fibroblasts and HKC-8 cells. Akt isolated from lysates of FBS-stimulated Swiss 3T3 cells produced greater phosphorylation of the glycogen synthase kinase-3/β cross-tide substrate than did the enzyme obtained from control lysates (Fig. 1C). A phosphorylated doublet was produced due to the presence of partially degraded paramyosin that occurs during large scale bacterial expression of the fusion protein.² A. McIlwrath (New England Biolabs), personal communication.

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Swiss 3T3 lysates (Fig. 1D). In contrast, Akt isolated from lysates of HKC-8 cells incubated in the absence or presence of serum revealed equivalent protein kinase activity (Fig. 1C). Western blotting demonstrated that Akt isolated from control and serum stimulated lysates were phosphorylated on Ser-473 similarly (Fig. 1D). Finally, Western blotting of total Akt confirmed an equivalent enzyme expression in stimulated and control lysates (Fig. 1E). The HKC-8 cells used throughout this study were mycoplasma-negative (data not shown), thereby eliminating contamination with this bacterium as a possible explanation for our findings.

**A Soluble Factor Mediates Akt Phosphorylation**—To establish if HKC-8 cells were producing a factor to stimulate Akt phosphorylation in an autocrine manner, cells were washed, and the conditioned media was replaced with fresh, serum-free media. After media replacement, Akt phosphorylation decreased in a time-dependent manner (Fig. 2). Within 3 h, Akt phosphorylation became markedly attenuated (<20% control). This effect was maintained over 12 h, after which time phosphorylation increased once again. Basal levels of Akt phosphorylation were eventually restored after 48 h.

To exclude the possibility that media replacement blocks Akt phosphorylation in HKC-8 cells, quiescent cultures were washed with DMEM, and 90 min later, treated with EGF for various times. In contrast to quiescent cultures that were unwashed (control), Akt phosphorylation in washed cells increased dramatically after EGF addition (Fig. 3, upper panels). This increase became maximal by 3 min and was maintained for more than 1 h. In contrast, the magnitude and kinetics of Erk1/2 phosphorylation were identical in washed and control cultures after EGF stimulation (Fig. 3, middle panels). Phos-
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FIG. 4. Characterization of the factor produced by HKC-8 cells that stimulates Akt phosphorylation. Confluent cultures of HKC-8 cells were washed and incubated with DMEM-F-12 for 3 days. The resultant conditioned medium was removed, and aliquots were heated for 15 min at 60 °C. Heated and non-heated media were then examined for their ability to stimulate Akt phosphorylation in confluent cultures of HKC-8 cells that were washed with warmed DMEM-F-12 2 h earlier (upper panel). Aliquots of conditioned media were also fractionated by gel filtration chromatography to determine the apparent molecular mass of the bioactive agent (lower panel). The first peak eluted in the void volume, and the second eluted with an apparent molecular mass smaller than the 25-kDa chymotrypsinogen A (Ct) standard. It is currently unclear if the activity present in the void volume represents a high molecular mass protein or a complex containing the smaller molecular mass protein. Further purification was precluded due to loss of biological activity upon prolonged handling.

3-Phosphoinositide Production Is Responsible for Akt Phosphorylation in Serum-starved Cultures—Treatment of quiescent cultures with the PI3K inhibitor wortmannin revealed a dose-dependent inhibition of Akt phosphorylation with an IC50 of 8 nM and complete attenuation by 30 nM (Fig. 5). The role of the PI3K/Akt pathway in mediating the viability of HKC-8 cells was demonstrated by their treatment with the PI3K inhibitor LY294002 (20 μM). Cell number was quantified by trypsin blue exclusion, whereas cell viability was assessed by MTT assay (Fig. 6). With both assays a significant difference was revealed after one day of LY294002 treatment, and this difference became greater with time. Inhibition of PI3K activity markedly attenuated the proliferation of HKC-8 cells, resulting in their death upon prolonged exposure (>3 days).

Replacement of Conditioned Media Improves Cell Viability—Although we had shown that removal of conditioned media transiently decreased the level of Akt phosphorylation in HKC-8 cells, we wondered if a regimen of repeated media change would produce a sustained decrease in Akt phosphorylation. To this end, conditioned media was removed and replaced with warmed serum-free DMEM every 12 h. Conditioned media remained on control cultures throughout. Fig. 7 shows that regular media exchange produced a sustained attenuation of Akt phosphorylation that became maximal after 4 days of treatment compared with the control cultures. To exclude the possibility that this procedure reduced expression of the Akt enzyme, total Akt levels were determined by Western blotting. These were unaltered in washed and control cultures over the entire duration of the experiment.

Having established that regular replacement of conditioned media suppressed the high basal level of Akt phosphorylation in quiescent cultures, we examined its implications for cell viability. Fig. 8, upper panel, shows that initially no effect on cell number was observed. However, after day 4 the number of cells maintained in conditioned media reached a plateau and later decreased. In contrast, cells that were washed regularly continued to increase in number, reaching a higher plateau at day 6. The results of the MTT assay used to assess cell viability revealed a difference by day 3 (Fig. 8, lower panel). Cultures
where media was replaced were more viable than those that remained in conditioned media. The decrease in cell viability exhibited by control cultures after day 3 correlated with the plateau in their cell number observed at day 4 (Fig. 8, upper panel). DNA synthesis was also quantified by BrdUrd incorporation (Fig. 9). Although the proportion of BrdUrd-labeled cells decreased slightly over the study period, there was no significant difference between those cultures that were washed and those left in conditioned media. In an attempt to explain the difference in cell number between the two treatments, we assessed the degree of apoptotic cell death in each culture. Fig. 10 shows that during the first 3 days, the number of terminal dUTP nick-end labeling (TUNEL)-positive cells was low (<5%) in both washed cultures and those maintained in conditioned media. However, after day 4, the number of apoptotic cells in cultures maintained in conditioned media increased dramati-
pressed as a percentage of the total cell number ($n$).

Cells were counterstained with methyl green and visualized. Fields were fixed in paraformaldehyde, permeabilized, and labeled with TdT.

Regular removal of conditioned media was also able to maintain a markedly attenuated phosphorylation of the Akt enzyme (Fig. 7). However, in contrast to the use of LY294002, Akt phosphorylation was not completely abolished. Consequently, if the autocrine factor were mitogenic, then cells remaining in conditioned media would remain more viable than those cultures where the conditioned media was continuously replaced and Akt phosphorylation suppressed. However, if this factor were cytotoxic, phosphorylation of Akt might be an attempt to preserve cell viability. Data presented in Figs. 7 and 9 demonstrated markedly increased apoptotic cell death in those cultures that remained in conditioned media. We cannot exclude the possibility that HKC-8 cells also produce growth factors to support their viability during periods of serum deprivation, although increased Erk1/2 phosphorylation was not observed (Figs. 1 and 3). We conclude that if the conditioned media remains on these cultures, an accumulation of cytotoxic factors or metabolites occurs, and the cells respond by activating the PI3K/Akt pathway. Consequently, the high level of Akt phosphorylation observed in serum-starved cultures of HKC-8 cells is indicative of cell stress and suggests that it is an attempt to minimize the cell damage and death that will later ensue. A role of Akt phosphorylation in cell proliferation cannot be supported in this model since the rate of DNA synthesis in both sets of cultures remained unaltered.

Other examples of stress-mediated activation of the PI3K pathway include mechanical stress (28, 29), UV irradiation (30), and treatment with cytotoxic agents such as H$_2$O$_2$ (31, 32). It is becoming clear that under such conditions, proliferative signaling mechanisms become activated in an attempt to sustain cell number. H$_2$O$_2$ treatment leads to ligand-independent phosphorylation and activation of the ErbB receptor family, thereby activating the PI3K and Erk/mitogen-activated protein kinase pathways (33). Induction of cell death by use of PI3K antagonists has demonstrated that 3-phosphoinositide synthesis must be maintained to ensure cell viability (Ref. 26 and Fig. 5). This is supported by the role of the PI3K pathway in regulating the activity of the Bcl-2 protein family. Akt phosphorylates BAD to relieve its inhibitory effect on Bcl-2 enzyme activity (27). Akt also inhibits the stress-activated kinase SEK1 and its substrate, JNK1 (34). Cells in which constitutively active Akt is overexpressed and cells heterozygous for the 3-phosphatase PTEN show reduced levels of cell death (35, 36).

Because epithelial cells line the renal tubules in situ they are continually exposed to shear stress and alterations in the ionic content of the extracellular medium at their apical surface. The marked up-regulation of Akt phosphorylation observed under conditions of low serum may reflect the mechanism of their sustained viability in such an environment. Examination of another human epithelial cell line, HK2, also revealed increased Akt phosphorylation in quiescent cultures. However, in

![Fig. 10. Conditioned media increases HKC-8 apoptotic cell death.](Image)  

**Fig. 10.** Conditioned media increases HKC-8 apoptotic cell death. HKC-8 cells were grown on 13-mm glass coverslips in 24-well plates. Once confluent and quiescent, cultures were washed with DMEM-F-12, and incubation was continued at 37 °C. Every 12 h, cultures were washed with fresh media (open symbols). Control cultures remained unwashed (closed symbols). Daily, representative cultures remained unaltered. HKC-8 cells in washed cultures remained negligible during this time.

**DISCUSSION**

The results of this study reveal an inverse correlation between the basal level of Akt phosphorylation and long term viability of HKC-8 cells under serum-free conditions. Originally, the degree of Akt phosphorylation observed in serum-starved cultures precluded further increase after serum or cytokine stimulation (19, 11). This effect was clearly selective for Akt since phosphorylation of Erk1/2 was negligible in quiescent cultures and increased dramatically after ligand addition (Fig. 1).

It soon became clear that the factor responsible for Akt phosphorylation was secreted into the conditioned media since its replacement with fresh media markedly attenuated this activity (Fig. 2). Although the identity of this factor remains elusive, we have demonstrated that it is heat-labile, and it can be isolated by biochemical fractionation (Fig. 4). Chemokines and cytokines including heparin binding epidermal growth factor-like growth factor, transforming growth factor-alpha, acidic and basic fibroblast growth factor, endothelin-1, vascular endothelial growth factor, and platelet-derived growth factor-BB have all been previously proposed to act as autocrine mediators of this cell type (20–22). Additional candidates included LPA and EGF since LPA is a major mitogenic component of serum, and EGF is very highly expressed by renal tubular cells (23, 24). However, neither is involved in this model since the ability of LPA and EGF to stimulate phosphorylation of Akt and Erk1/2 both differ from that of the conditioned media (Fig. 3). Biochemical analysis revealed the factor to be heat-labile and have an apparent molecular mass less than 25 kDa (Fig. 4). In addition, the detection of activity eluting in the void volume implies that conditioned media either contains two factors able to stimulate the PI3K pathway or that the smaller molecule may aggregate or bind larger proteins.
contrast to HKC-8 cells, 10% FBS was able to increase Akt phosphorylation further (data not shown). Possibly HKC-8 cells demonstrate a greater sensitivity to noxious stimuli and respond by an accentuated activation of the PI3K/Akt pathway. Marked Akt phosphorylation in quiescent cultures has also been shown with other cell lines, although the significance of this observation is rarely noted (37, 38). In addition to cytokine-receptor-driven anti-apoptotic mechanisms, the role of cell adhesion to extracellular matrix through integrin receptors should also be considered (39). The focal adhesion kinase FAK has recently been shown to suppress chemically induced apoptosis of TEC (40). Phosphorylation of FAK on Tyr-397 creates a consensus motif for binding of the SH2 domain of the class IA PI3K adaptor p85. This association plays a role in extracellular matrix-mediated cell survival that involves activation of Akt (41).

Although we have demonstrated that Akt phosphorylation is dependent upon 3-phosphoinositide synthesis, the enzyme responsible and the mechanism of its activation remains elusive. In contrast to the mitogen-activated protein kinase enzyme family, the identification of PI3K isoforms involved in stress-mediated responses has received little attention. The class IB family, the identification of PI3K isozymes involved in stress-sensitive and the mechanism of its activation remains elusive. Dependent upon 3-phosphoinositide synthesis, the enzyme responsible. This association plays a role in extracellular matrix-mediated cell survival that involves activation of Akt (41).

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