original reseach report

Inhibition of Akt pathway phosphorylation as a mechanism in the pathogenesis of functional intestinal obstruction in carcinomatosis peritonei

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BACKGROUND AND OBJECTIVES: The purpose of this study was to confirm our hypothesis that the development of functional intestinal obstruction in carcinomatosis peritonei (CP) is related to cytokine-mediated inhibition of the Akt pathway and to investigate the phenomenon of relative adrenal insufficiency in CP.

METHODS: Human adrenocortical cells (NCI-H295R) were treated with serum derived from eight cancer patients who had intestinal obstruction and functional adrenal insufficiency. Serum from three normal healthy subjects and three who had CP but without intestinal obstruction or adrenal insufficiency were used as controls. The differential effects of serum on the treated cells were studied using Western blot analysis. Cortisol production of these treated cells was assayed with cortisol ELISA kits.

RESULTS: Phosphorylation of Akt at Ser473 and Ser308 in cells was significantly reduced when treated with serum from patients with intestinal obstruction but not controls. Phosphorylation of PDK1 at Ser241, mTOR downstream targets like p70S6 at Thr421/Ser424 and Thr389, and lastly 4EBP-1 at Ser70 a downstream target of p70S6 was reduced by approximately 50%, 40%, and 70%, respectively. There was enhanced phosphorylation of eIF4E an initiating factor in protein translation in cells treated with patient serum compared to controls. Cortisol synthesis was stimulated upon treatment with patient serum but not with control serum.

CONCLUSION: Inhibition of Akt phosphorylation is a mechanism that could play a major role in the development of intestinal obstruction in carcinomatosis peritonei. The identification of the mediating cytokines will lead to the development of cogent targeted therapeutic strategies.

Use of corticosteroids in the treatment of intestinal obstruction from carcinomatosis peritonei (CP) is an established practice.[1,2] The purported mechanism of action of steroids in relieving intestinal obstruction is the reduction in inflammation of the intestinal wall and surrounding soft tissue, thereby facilitating peristalsis with improved motility. We recently published our observation of an incidence of 45% of functional adrenal insufficiency in 29 consecutive patients with intestinal obstruction secondary to CP admitted to our oncology inpatient service. Short corticotrophin stimulation testing was performed in this prospective cohort of patients.[3,4] Patients with pre-existing causes for adrenal suppression such as steroid/ketoconazole usage, radiotherapy to the abdominal region encompassing the adrenals within the field, and known pituitary hypofunction were excluded in the comparative analysis of this prospective observational study. The group of patients with functional adrenal insufficiency was found to have a significantly longer duration of hospitalization for management of intestinal obstruction (mean duration 7.9 versus 4.0 days per month of survival) and a shorter overall survival compared to the group with normal adrenal function (190 days versus 111 days; hazard ratio was 1.7; 95% confidence interval, 1.3-2.2). Based on the results of this observational study, we hypothesized that there may be increased production of a single cytokine or mul-

tiple cytokines leading to direct inhibition of intestinal smooth muscle activity and that corticosteroids are able to reverse the smooth muscle function inhibition. Based on our observation of 45% relative adrenal insufficiency in such a situation, we further hypothesized that adrenal response represented by compensatory endogenous cortisol production may be insufficient to surmount this inhibition, therefore requiring the use of exogenous corticosteroids in maintaining normal intestinal motility. The activation of the Akt pathway and its downstream target the 70-kDa ribosomal S6 kinase (p70S6 kinase) are implicated in the hyperplasia of human intestinal smooth muscle cells and maintenance of their physiological mass.[5] Corticosteroids regulate the phosphatidylinositol 3-kinase (PI3K) signaling pathway via serum and glucocorticoid-regulated protein kinases (SGKs) with Akt as one of the effectors.[6] Furthermore, recent evidence suggests an important role for non-transcriptional effects of glucocorticoid receptor in the vascular system. The non-transcriptional actions of glucocorticoid receptor involve the rapid activation of protein kinases, such as PI3K and Akt, leading to the activation of endothelial nitric oxide synthase.[7,8] This novel pathway of steroid hormone action protects against ischemic injury by augmenting blood flow and decreasing vascular inflammation. Therefore, the Akt pathway was identified as a potential key mechanism in the development of functional intestinal obstruction in CP. We sought to confirm our postulation with this follow-up study. We treated cortisol-producing human adrenocortical cells (NCI-H295R) with serum derived from patients with intestinal obstruction from CP with biochemically confirmed adrenal insufficiency. Serum from both healthy subjects and patients with CP but without intestinal obstruction were used as controls. The aim was to study the differential effects of patient serum on the Akt pathway compared to controls and to determine the effect of the patients' serum on cortisol production.

METHODS

Patient selection and controls

Patients who were admitted to our oncology inpatient service for management of intestinal obstruction from CP with adrenal insufficiency were eligible for this study. Written informed consent was obtained from all subjects prior to participation in this study. All patients had histological confirmation of primary gastrointestinal cancers. The diagnosis of peritoneal carcinomatosis was based on the computed tomography scan of the abdomen, or positive peritoneal fluid cytology, or intraoperative finding. Exclusion criteria were mainly prior diagnosis of adrenal insufficiency, known hypothalamic or pituitary dysfunction, the presence of brain metastases, previous cranial irradiation, and use of corticosteroids and ketoconazole. The control serum was derived from healthy volunteers among the investigators who were without any present illness and from patients with CP but without intestinal obstruction.

Corticotropin stimulation test and definition of adrenal insufficiency

Adrenal function was assessed using the short cosyntropin stimulation test. This test involved intravenous administration of 250 μg of cosyntropin with plasma cortisol levels measured at 0, 30, and 60 minutes after administration. The timing of sampling for the baseline cortisol level was random. All blood specimens were drawn from a pre-inserted heparinized venous cannula with the aid of a tourniquet. Functional adrenal insufficiency was defined as having a baseline random cortisol level of less than 400 nmol/L and/or a best incremental response to cosyntropin of less than 250 nmol/L from baseline level. This definition was similar to our previous study based on current clinical guidelines.[3,4]

A 5 mL blood sample was collected from each healthy volunteer and patient. Blood was centrifuged at 3000*g* for 10 minutes at room temperature to separate the plasma and collected plasma was stored at -800°C until utilisation.

Cell culture and treatment

NCI-H295R pluripotent adrenocortical carcinoma cells were obtained from the American Type Culture Collection, Manassas, Virginia. They were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (growth medium). To study the effects of serum derived from patients and controls, NCI-H295R cells were plated at 2.5×10^6 cells per 100 mm tissue culture dish. Cells were allowed to grow in the growth medium for 24 hours. They were washed once with serum-free DMEM/F12 medium and then treated with DMEM/F12 medium containing either 5% serum from healthy individuals as controls or 5% serum derived from patients for 24 hours. At the end of the experiment, conditioned medium (CM) and cells were collected. CM was centrifuged at 3000*g* for 10 minutes at room temperature to remove the cell debris and stored at -800°C until analysis. Experiments were repeated at least three times, and the data were expressed as the mean±SD of the quadruplicate of each group.

Cortisol production assay

The amount of cortisol secreted into CM was measured using cortisol ELISA kits (IBL-Hamburg GmbH, Hamburg, Germany) according to the protocol of the manufacturer. The sensitivity for cortisol ELISA kit was 2.5 ng/mL. Intra-assay variation and inter-assay variation were 5.6% and 6.5%, respectively. The absorbance at 450 nm was measured using an ELISA plate reader (Benchmark Plus microplate spectrophotometer, Bio-Rad, Hercules, California, USA).

Western blot analysis

To examine the effects of serum derived from patients on the Akt/mTOR pathways, cells were treated as described above. To inhibit the PI-3 kinase/Akt pathway, NCI-H295R cells were treated with serum derived from a normal individual (N2) in the absence and presence of 10 μM of PI-3 kinase inhibitor, LY294002. Treated cells were lysed in a lysis buffer and 100 μg of total cell lysate was subjected to Western blot analysis as described in reference 9. Blots were incubated with anti-mTOR, Akt-1 and anti-4E-BP1 antibodies and phosphorylation-specific antibodies against mTOR (Ser2448), p70S6 kinase (Thr421/Ser424), p70S6 kinase (Thr389), and 4E-BP1 (Thr70) (Cell Signaling Technology, Beverly, MA, USA and 1:7500 horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibody. All the primary antibodies were used at a final concentration of 1 μg/mL. Blots were then visualized with a chemiluminescent detection system (Amersham, Pharmacia Biotech, Arlington Heights, IL, USA) as described by the manufacturer. Blots were blotted with anti-α-tubulin (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) to serve as control.

Statistical analysis

The Fischer exact test was used to determine significance of differences in results between controls and study subjects. Data analysis was performed with the aid of statistical software SPSS version 14.0 for Windows.

RESULTS

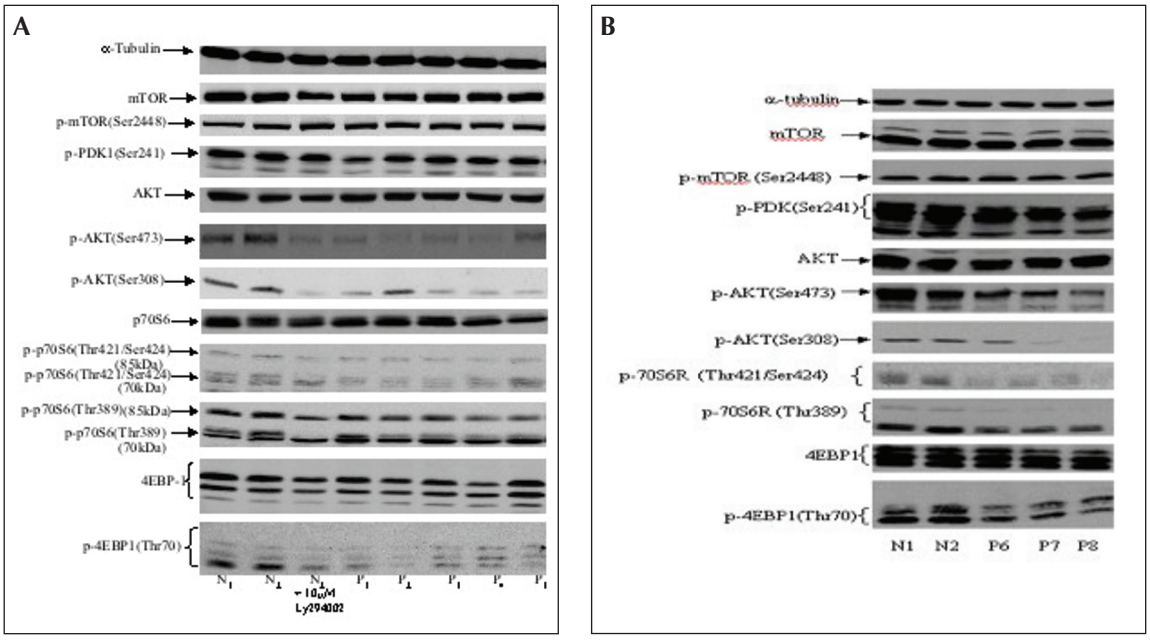
Serum was obtained from eight patients with intestinal obstruction from CP with adrenal insufficiency. Of these eight patients, two had gastric carcinoma, four had colon carcinoma and one had pancreatic carcinoma. Control serum was from three patients with colon carcinoma with CP but without intestinal obstruction or adrenal insufficiency, and also from three healthy subjects among the investigators. Phosphorylation of Akt at Ser473 and Ser308 in cells treated with study serum derived from the eight patients was significantly reduced (Figure 1), while control serum demonstrated no inhibition. Inhibition of the Akt pathway was not homogenous; serum from two patients (P2 and P6 in Figures 1a and 1b) did not significantly inhibit phosporylation at Ser308. Neither serum from patients nor controls affected mTOR and its phospho-form. Phosphorylated PDK1 (upstream positive regulator of mTOR) at Ser241 was reduced by approximately 50%. Phosphorylation of p70S6 at Thr421/Ser424 and Thr389 (downstream targets of mTOR) were reduced by approximately 40%. Phosphorylated 4EBP-1 at Ser70 (downstream target of p70S6) was decreased by approximately 70%, while eIF4E phosphorylation was enhanced. Study serum derived from five patients with intestinal obstruction enhanced cortisol production by NCI-H295R cells. Control serum did not enhance cortisol production in the latter (Figure 2). Serum from patients who had CP but not intestinal obstruction was similar to control serum from healthy subjects, and did not inhibit phosphorylation of Akt at Ser473 and Ser308, nor inhibit phosphorylation of p70S6 kinase (Thr389) and 4E-BP1 (Thr70) (Figure 3). The results of the statistical analysis showing significant inhibition of phosphorylation of Ser473, Ser308, Thr421, and Thr70 by patient serum compared with control serum as summarized in Table 1.

DISCUSSION

Intestinal obstruction secondary to peritoneal carcinomatosis is frequently not relieved by surgery. The

Table 1. Statistical analysis.

	t	df	*P* value (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
SER473	-3.031	10	.013	-22.2727	-38.6464	-5.8991
SER308	-2.849	10	.017	-24.1818	-43.0925	-5.2711
THR421	-2.904	10	.016	-18.2727	-32.2944	-4.2511
THR70	-3.586	10	.005	-19.4545	-31.5410	-7.3681



Figures 1ab. Effects of serum derived from patients with peritoneal carcinomatosis and intestinal obstruction on the phosphorylation of mTOR, p70S6 kinase, and 4E-BP1 in NCI-H295R adrenal carcinoma cells. Cells were grown in the DMEM/F12 medium containing 10% FBS for 24 h. They were washed once with serum free DMEM/F12 medium and then treated with DMEM/F12 medium containing either 5% serum from healthy individuals (N1, N2), N2 in the presence of 10 μM of PI-3 kinase inhibitor, LY294002 or 5% serum derived from patients (P1-P5) for 24 h. Lysates from cells were subjected to Western blot analysis described. The blots were incubated with the indicated antibodies. Representative blots and changes in the expression of indicated proteins are shown. Experiments were repeated at least three times with similar results (N denotes controls, P denotes patients).

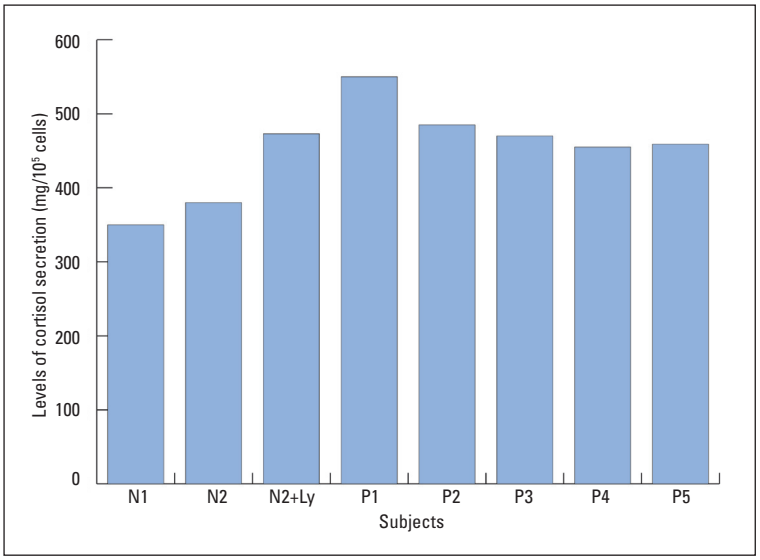


Figure 2. Effects of serum derived from patients with peritoneal carcinomatosis and intestinal obstruction on cortisol production by NCI-H295R adrenal carcinoma cells. Cells were grown in the DMEM/F12 medium containing 10% FBS for 24 h. They were washed once with serum free DMEM/F12 medium and then treated with DMEM/F12 medium containing either 5% serum from healthy individuals (N1, N2), N2 in the presence of 10 μM of PI-3 kinase inhibitor, LY294002 or 5% serum derived from patients (P1-P5) for 24 h. Conditioned medium was assayed for cortisol production using cortisol ELISA kits as described. The amount of cortisol secretion was expressed in ng/mL per 10^6 cells. Standard deviation is 62.2 ng/mL per 10^6 cells.

development of intestinal obstruction invariably leads to frequent and prolonged hospitalizations that herald irreversible deterioration in the quality of life of the patient with gastrointestinal cancer. While a venting gastrostomy, nasogastric tube suction, or bypass may palliate symptoms from intestinal obstruction, the treatment of intestinal obstruction in such a context is frequently medical management. In most instances, the outcome of medical management in achieving durable palliation is far from satisfactory. Aggressive use of narcotics, anticholinergics, antiemetics, phenothiazines, butyrophenones, tricyclic antidepressants, corticosteroids, and somatostatin analogs may palliate symptoms associated with intestinal obstruction.[10,11] The disparity of actions of these drugs reflects the empiricism of current medical management of intestinal obstruction in CP. It is imperative that our understanding be enhanced by a rational and systematic research into the mechanisms leading to the development of this condition. Mammalian target of rapamycin (mTOR) functions as a serine/threonine kinase to regulate protein translation, cell cycle progression, and cellular function.[12,13] mTOR is phosphorylated in response to stimuli that activate the PI 3K/Akt pathway and regulates protein transla-

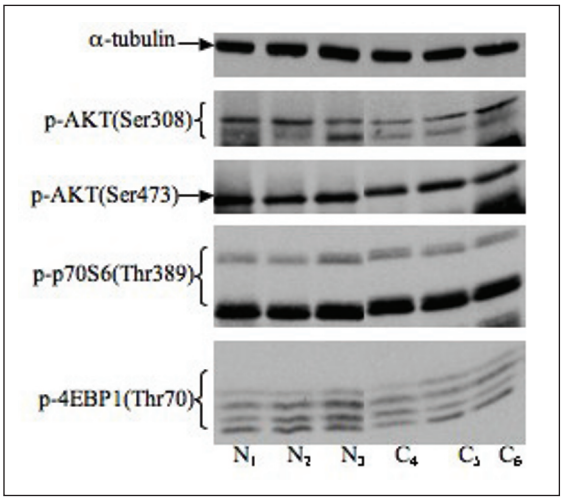


Figure 3. Effects of serum derived from patients with peritoneal carcinomatosis but without intestinal obstruction on the phosphorylation of mTOR, p70S6 kinase, and 4E-BP1 in NCI-H295R adrenal carcinoma cells. Cells were grown in the DMEM/F12 medium containing 10% FBS for 24 h. They were washed once with serum free DMEM/F12 medium and then treated with DMEM/F12 medium containing either 5% serum from healthy individuals (N1, N2, N3), LY294002 or 5% serum derived from patients (C4-C6) for 24 h. Lysates from cells were subjected to Western blot analysis described. Blots were incubated with indicated antibodies. Representative blots and changes in the expression of indicated proteins are shown. Experiments were repeated at least three times with similar results.

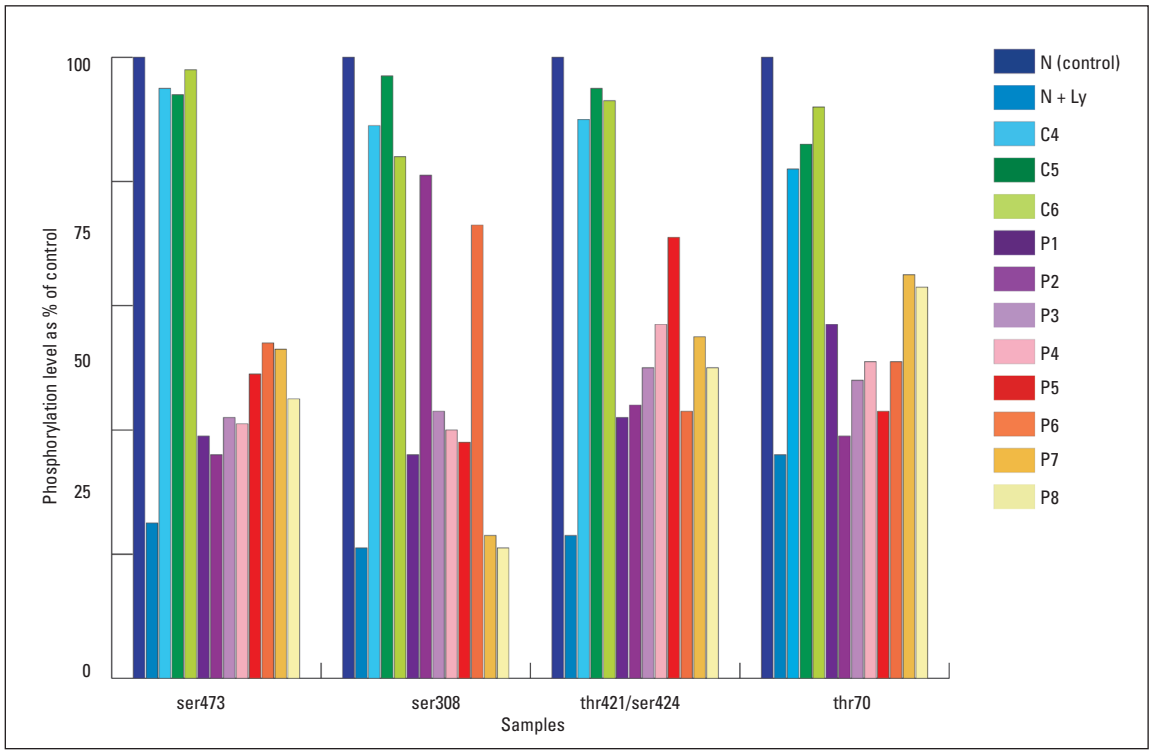


Figure 4. Effects of serum derived from patients with peritoneal carcinomatosis and intestinal obstruction on the levels of phospho-Akt (Ser473) (SD=27%), phospho-Akt (Ser308) (SD=30%), phospho-p70S6 (Thr421/Ser424) (SD=25%), and phospho-4EBP-1 (Thr70) (SD=21%) expressed as percentage of controls.

tion through phosphorylation of ribosomal S6 kinase (S6K), eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K). p70S6K directly phosphorylates the 40S ribosomal protein S6, which correlated with enhanced translation of transcripts with 5'-terminal oligopyrimidine (5'-TOP) sequences that encode components of translational machinery such as ribosomal proteins and elongation factors.[14,15] Multi-site phosphorylation of the translational repressor 4E-BP1 by mTOR resulted in its dissociation from eIF4E, facilitating the recruitment of other translation initiation factors to form the eIF4E complex and initiate cap-dependent translation.[15] The activation of Akt pathway and its downstream target the 70-kDa ri-

bosomal S6 kinase (p70S6 kinase) are implicated in the hyperplasia of human intestinal smooth muscle cells and maintenance of their physiological mass through activation of translation initiators.[5] Our study finding of inhibition of phosphorylation of PDK1 at Ser241, p70S6 at Thr421/Ser424 and Thr389, and 4EBP-1 at Ser70 mediated by factors in the serum from patients with intestinal obstruction in CP confirms our hypothesis that reduced myogenicity of intestinal smooth muscle through cytokine-mediated Akt pathway inhibition is one of the underlying mechanisms leading to intestinal obstruction (Figure 4). It is not known whether these cytokines are produced by the tumors or are part of an immune response mounted against the cancer. Identification of the cytokines that mediate these inhibitions will allow the development of a rational therapeutic strategy. It is improbable that these effects are mediated by a single cytokine in view of heterogeneous inhibition of the Akt pathway as evident by the fact that serum from two patients (P2 and P6 in Figure 1a and 1b) did not significantly inhibit phosporylation at Ser308. This strategy may entail reducing the production of these inhibitory cytokines, antagonizing the effect of these cytokines at the sites of pathway inhibition, and lastly, reversing the inhibition that may be already present. Candidate cytokines include IL-22 and myostatin, a transforming growth factor β super family member.[16,17] This is the subject of our current ongoing research effort. The reason for enhanced phosphorylation of initiation factor eIF4E is complex and probably related to decreased protein intake in intestinal obstruction.[18,19] Phosphorylation of eIF4E is likely independent of upstream events in the Akt pathway. Reduced protein intake cannot, however, entirely account for the selective inhibition of phosphorylation of PDK1, p70S6 and 4EBP-1 found in our study. Our finding of increased cortisol production by the NCI-H295R pluripotent adrenocortical carcinoma cells when treated with patient serum was unexpected. Whether this increase in cortisol production is mediated by the same cytokines inhibiting the Akt pathway remains unknown. Corticosteroids activate the PI3K/Akt pathway and acutely increase the endothelial NO synthase (eNOS) activity through a rapid non-nuclear non-transcriptional process.[8] Increased NO will result in vasodilatation and counterintuitively also anti-inflammatory effects.[15] Akt may in turn regulate corticosteroid synthesis via eNOS production through a negative feedback loop. Phosphorylation of eNOS at Ser1177 by Akt directly stimulates eNOS activity and NO production.[21] NO is a soluble gas and has been shown, like molecular oxygen and CO, to bind directly to the heme region of a number of P450s and inhibit their activity. The key steps regulating the initiation of steroidogenesis (P450scc) and two important committing steps to aldosterone (P450aldo) or cortisol/C19 steroids (P450c17) are all enzymes that use multiple rounds of attack of the heme-oxygen complex on the steroid substrate. P450c17 is a target of NO inhibition.[22] Further studies that confirm NO inhibition of P450scc also show that NO donors can still inhibit the conversion of progesterone to aldosterone, suggesting another site of NO action, possibly P450aldo.[23] To further complicate matters, the effects of VEGF on permeability[24] and vascular tone[25] are coupled to nitric oxide (NO) production. VEGF has been shown to induce the release of NO from vascular endothelial cells by increasing phosphorylation of endothelial nitric-oxide synthase (eNOS).[26] Further studies of Akt, NO functions in adrenocortical steroidogenesis will be helpful in clarifying our findings. But certainly, increased cortisol production in our clinical context does not equal adequate adrenal response in relation to the physiological stress experienced. Unfortunately, in vitro tests will never allow a dynamic functional response assessment. Even if we identify the optimal test to determine functional adrenal insufficiency in cancer, several factors complicate the diagnosis of functional adrenal insufficiency based on laboratory tests. Since the highest levels of cortisol are found in patients with the most severe illness, both high and low cortisol levels have been reported to be associated with poor prognosis. This was seen in our study in that all of those patients with cortisol levels of more than 1000 nmol/L and a sub-optimal response to corticotropin stimulation test survived less than a week.[3] Therefore, a solitary random cortisol level without a corticotropin stimulation test is inadequate in the diagnosis of functional adrenal insufficiency in an ill patient. Changes in levels of cortisol-binding globulin further complicate matters. Fluctuations in tissue sensitivity to the physiological action of cortisol certainly render a standard diagnostic range of test results that is reproducible in different patients with varying degrees of illness implausible.

In conclusion, our data suggest that cytokine-mediated inhibition of the Akt pathway leading to reduced myogenicity of the intestinal smooth muscles is a mechanism that could play a major role in the development of intestinal obstruction in patients with CP. Identification of the mediating cytokines will help to determine opportune strategies to adopt in prevention and treatment of intestinal obstruction in CP. Clarification of the roles that Akt, NO, VEGF and corticosteroids play in the complex web linking adrenal

steroidogenesis, relative adrenal insufficiency, vascular permeability and vascular tone in future studies will be of great interest.

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